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Contribution of flavonoids and iridoids to health properties of edible plants of *Arbutus*, *Cornus*, and *Vaccinium* genera from Mediterranean area

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The PhD thesis entitled “Contribution of flavonoids and iridoids to health properties of edible plants of *Arbutus, Cornus,* and *Vaccinium* genera from Mediterranean area” was carried in joint supervision between University of Calabria and Université de Paris.

The PhD student dr. Maria Concetta Tenuta is enrolled at the doctoral school in Translational Medicine (University of Calabria, Italy), coordinated by Prof. Sebastiano Andò, under the supervision of Prof. Rosa Tundis, and at the doctoral school Médicament, Toxicologie, Chimie, Imageries, (MTCI - ED 563) (Université de Paris, France) within “Produits Naturels Analyses et Synthèses” team, under the supervision of Prof. Brigitte Deguin.

In joint supervision, the research period was carried out for 18 months at the University of Calabria, Laboratory of Phytopharmacy and Food Science and Technology of the Department of Pharmacy, Health and Nutritional Sciences, and for 18 months at the Université de Paris, within the "Natural Products Analysis and Synthesis" team of the CiTCoM laboratory (Cibles Thérapeutiques et Conception de Médicaments**)**, UMR 8038 (CNRS, Université de Paris) of the UFR de Pharmacie de Paris.

Abstract

***Aim of the study*:** Natural compounds produced by the "nature laboratory" continue to play a leading role in the process of discovering and developing of new drugs, food supplements and/or functional foods. Today, in fact, we are witnessing a renewed interest in these products for the prevention and treatment of numerous diseases, including those associated with oxidative stress such as diabetes and inflammatory diseases. In this context, the present research project aimed at identifying the best techniques that allow the extraction of two classes of active compounds, flavonoids and iridoids, from the fruits and leaves of *Arbutus unedo* L., *Vaccinium corymbosum* L., *Cornus mas* L., and *Cornus sanguinea* L. Their edible fruits but also their leaves (considered byproducts) represent rich sources of bioactive compounds. The work continued with the evaluation of *in vitro* biological activity and the fractionation of the most active extracts in order to envisage their potential use as a source of bioactive compounds for the development of functional foods, nutraceuticals and/or food supplements .

***Materials and methods*:** The chemical profile of extracts was evaluated by using LC/ESI/QTOF/MS. Four different *in vitro* tests (DPPH, ABTS, β-carotene bleaching test and FRAP), were performed to investigate the antioxidant activity. The potential hypoglycaemic activity was investigated by the inhibition of α-amylase and α-glucosidase enzymes. The inhibitory effects of the extracts on the production of nitric oxide have been studied by Griess assay. The most active extracts of *C. mas* and *C. sanguinea* were subjected to fractionation in order to obtain a separation of flavonoids and iridoids using XAD-16 resin. Obtained fractions were tested to evaluate their ability to reduce the activation of the NF-kB factor.

***Results*:** LC/ESI/QTOF/MS analyses detected for the first time in the A. unedo the presence of ellagic acid 4-*O*-β-D-glucopiranoside, kaempferol 3-*O*-glucoside, myricetin, myricetin 3-*O*-rhamnopiranoside, naringenin 7-*O*-glucoside, isovitexin 7-*O*-glucoside, and norbergenine and *V. corymbosum* the presence of the iridoids scandoside, vaccinoside, geniposide, and dihydromonotropein. The complete chemical characterization of *C. sanguinea* fruits and leaves was conducted herein for the first time, reporting the presence of flavonoids and iridoids. Worthy of note are the results obtained from the hypoglycaemic activity. The extracts of both *Cornus* species showed a high inhibition of α-glucosidase and a moderate inhibition of α-amylase. Of particular note were the results obtained with the hydroalcoholic maceration of leaves and dried fruits of *C. mas* (TDB and MDB), the ethanol maceration of fresh leaves (PF1) and the hydroalcoholic maceration of dry leaves (SD2) of *C. sanguinea*, which for this reason were subjected to fractionation using XAD-16 resin. *C. sanguinea* PF1 (II) and SD2 (II) fractions showed the best antioxidant and NF-KB inhibition activity.

***Conclusion*:** All the investigated extracts showed a promising antioxidant, hypoglycaemic and anti-inflammatory potential, confirming the positive contribution of the two classes of compounds under study, flavonoids and iridoids, suggesting their potential use as a rich source of useful bioactive compounds for the formulation of new products to prevent diseases associated with oxidative stress such as inflammatory diseases and diabetes. In particular, results obtained with the *C. sanguinea* fractions PF1 (II) and SD2 (II) encourage researchers to continue the study with further *in vivo* studies.

Abstract

Scopo dello studio: I composti naturali prodotti dal "laboratorio natura" continuano ad avere un ruolo di primo piano nel processo di scoperta e sviluppo di nuovi farmaci, integratori alimentari e/o alimenti funzionali. Oggi, infatti, si assiste a un rinnovato interesse nei riguardi di tali prodotti nella prevenzione e nel trattamento di numerose patologie, tra cui quelle associate allo stress ossidativo come diabete e malattie infiammatorie. In questo ambito si inserisce il presente lavoro di ricerca, volto all'identificazione delle migliori tecniche che consentano l'estrazione di due classi di principi attivi, i flavonoidi e gli iridoidi, dai frutti e dalle foglie di *Arbutus unedo* L., *Vaccinium corymbosum* L., *Cornus mas* L. e *Cornus sanguinea* L. I loro frutti commestibili ma anche le foglie (considerate prodotti di scarto) rappresentano ricche fonti di composti bioattivi. Il lavoro ha quindi riguardato la valutazione dell'attività biologica *in vitro* e il frazionamento bio-guidato degli estratti risultati più attivi al fine di prospettare un loro potenziale impiego come fonte di composti bioattivi per lo sviluppo di alimenti funzionali, nutraceutici e/o integratori alimentari.

Materiali e metodi: Il profilo fitochimico degli estratti è stato valutato mediante LC/ESI/QTOF/MS. Per la determinazione *in vitro* della capacità antiossidante sono stati eseguiti quattro diversi test (DPPH, ABTS, FRAP e β-carotene bleaching test). La potenziale attività ipoglicemizzante è stata investigata mediante l'inibizione degli enzimi α-amilasi e α-glucosidasi. Gli effetti inibitori degli estratti sulla produzione di ossido nitrico sono stati studiati mediante saggio di Griess. Gli estratti maggiormente attivi di *C. mas* e *C. sanguinea* sono stati sottoposti a frazionamento per la separazione di flavonoidi e iridoidi mediante l'impiego della resina XAD-16. Le frazioni ottenute sono state saggiate per valutare la capacità di ridurre l'attivazione del fattore NF-kB.

Risultati: L'analisi LC/ESI/QTOF/MS ha rilevato per la prima volta nell'*A. unedo* la presenza di acido ellagico 4-*O*-β-D-glucopiranoside, kaemferolo 3-*O*-glucoside, miricetina, miricetina 3-*O*-ramnopiranoside, naringenina 7-*O*-glucoside, isovitexina 7-*O*-glucoside, e norbergenina e nel *V. corymbosum* la presenza degli iridoidi scandoside, vaccinoside, geniposide e diidromonotropeina. La caratterizzazione chimica completa di frutti e foglie del *C. sanguinea* è stata condotta per la prima volta, riportando la presenza flavonoidi ed iridoidi. Degni di nota sono i risultati ottenuti dall'attività ipoglicemizzante. Gli estratti di entrambe le specie di *Cornus* hanno mostrato un'alta inibizione dell'α-glucosidasi e una moderata inibizione dell'α-amilasi. Di particolare rilievo sono stati i risultati ottenuti con la macerazione idroalcolica di foglie e frutti secchi di *C. mas* (TDB e MDB), la macerazione in etanolo delle foglie fresche (PF1) e la macerazione idroalcolica delle foglie secche (SD2) di *C. sanguinea*, che per tale motivo sono stati sottoposti a bio-frazionamento mediante l'impiego della resina XAD-16. Le frazioni PF1 (II) e SD2 (II) del *C. sanguinea* hanno mostrato la migliore attività antiossidante e di inibizione dell'NF-$_K$B.

Conclusioni: Tutti gli estratti investigati hanno mostrato un promettente potenziale antiossidante, ipoglicemizzante ed antinfiammatorio, confermando il positivo contributo delle due classi di composti oggetto di studio, i flavonoidi e iridoidi prospettando il loro potenziale impiego come ricca fonte di composti bioattivi utili per la formulazione di nuovi prodotti per prevenire le malattie associate allo stress ossidativo come malattie infiammatorie e diabete. In particolare, i risultati ottenuti con le frazioni PF1 (II) e SD2 (II) del *C. sanguinea* incoraggiano i ricercatori nel proseguire lo studio con eventuali studi *in vivo.*

Résumé

Objet de l'étude : Les substances naturelles produites par le laboratoire « Nature » continuent de jouer un rôle de premier plan dans le processus de découverte et de développement de nouveaux médicaments, compléments alimentaires et/ou aliments fonctionnels. Aujourd'hui, en effet, nous assistons à un regain d'intérêt pour ces produits pour la prévention et le traitement de nombreuses maladies, notamment celles associées au stress oxydatif, telles que le diabète et les maladies inflammatoires. Le présent travail de recherche vise à identifier les meilleures techniques permettant d'extraire deux classes de principes actifs, les flavonoïdes et les iridoïdes, des fruits et des feuilles d'*Arbutus unedo* L., *Vaccinium corymbosum* L., *Cornus mas* L. et *Cornus sanguinea* L. Leurs fruits comestibles, comme leurs feuilles pourtant actuellement considérées comme des déchets représentent de riches sources de composés bioactifs. Les travaux ont donc porté sur l'évaluation de l'activité biologique *in vitro* et le fractionnement bio-guidé des extraits les plus actifs afin d'envisager leur utilisation potentielle comme source de composés bioactifs pour le développement d'aliments fonctionnels, et nutraceutiques.

Matériels et méthodes : Le profil phytochimique des extraits a été évalué par LC/ESI/QTOF/MS. Pour la détermination in vitro de la capacité antioxydante, quatre tests différents ont été réalisés (DPPH, ABTS, FRAP et β-carotene bleaching test). L'activité hypoglycémique potentielle a été étudiée par inhibition des enzymes α-amylase et α-glucosidase. Les effets inhibiteurs des extraits sur la production d'oxyde nitrique ont été étudiés par le test de Griess. Les extraits les plus actifs de *C. mas* et *C. sanguinea* ont été soumis à un bio-fractionnement pour la séparation des flavonoïdes et des iridoïdes au moyen de la résine XAD-16. Les fractions obtenues ont été testées pour évaluer l'aptitude à réduire l'activation du facteur NF-kB.

Résultats : L'analyse par LC/ESI/QTOF/MS détectée pour la première fois dans l'*A. unedo* la présence d'acide ellagique 4-*O*-β-D-glucopiranoside, de kaempférol 3-*O*-glucoside, de myricétine, de myricétine 3-O-rhamnopyranoside, de naringénine 7-*O*-glucoside, d'isovitexine 7-*O*-glucoside et de norbergénine, et *V. corymbosum* la présence d'iridoïdes scandoside, vaccinoside, géniposide et dihydromonotropeine. La caractérisation chimique complète des fruits et des feuilles de *C. sanguinea* a été réalisée pour la première fois, en indiquant la présence de flavonoïdes et d'iridoïdes. Il convient de noter les résultats obtenus à partir de l'activité hypoglycémique. Les extraits des deux espèces de *Cornus* ont montré une forte inhibition de l'α-glucosidase et une inhibition modérée de l'α-amylase. Résultats intéressants ont été obtenus avec la macération hydro-alcoolique de feuilles et de fruits séchés de *C. mas* (TDB et MDB), la macération en éthanol des feuilles fraîches (PF1) et la macération hydro-alcoolique de feuilles sèches (SD2) de *C. sanguinea* qui, pour cette raison, ont été soumis à un fractionnement avec la résine XAD-16. Les fractions PF1 (II) et SD2 (II) de *C. sanguinea* présentaient la meilleure activité inhibitrice des antioxydants et du NF-kB.

Conclusion: Tous les extraits étudiés ont montrés un potentiel antioxydants, hypoglycémiants et anti-inflammatoire, confirmant l'apport positif des deux classes de composés étudiés, les flavonoïdes et les iridoïdes, suggérant leur utilisation potentielle comme source riche de composés bioactifs utiles pour la formulation de nouveaux produits pour prévenir les maladies associées au stress oxydatif, telles que les maladies inflammatoires et le diabète. En particulier, les résultats obtenus avec les fractions PF1 (II) et SD2 (II) de *C. sanguinea* incitent les chercheurs à poursuivre l'étude en proposant éventuellement des études *in vivo*.

Abbreviations

5-ASA	5-Aminosalicylic Acid
AAE	Ascorbic Acid Equivalent
ABTS	2,2'-Azino-Bis(3-Ethylbenzothiazoline-6-Sulphonic Acid)
AChE	Acetylcholinesterase
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
AST	Aspartate Transaminase
AU	Aucubin
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
BW	Body Weight
CA	Chlorogenic Acid
CAT	Catalase
CoA	Coenzyme A
COX-2	Cyclooxygenase-2
CREB	cAMP Response Element-Binding Protein
DAD	Diode Array Detector
DIAN	*o*-Dianisidine
DM	Diabetes Mellitus
DMSO	Dimethyl Sulfoxide
DNA	DeoxyriboNucleic Acid
DPP4	Dipeptidyl Peptidase-4
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dried Weight
ESI	Electrospray Ionization
FRAP	Ferric Reducing Antioxidant Power
FW	Fresh Weight
GAE	Gallic Acid Equivalent
GAS	Global Antioxidant Score
GLP-1	Glucagon-Like Peptide 1
GLUT4	Glucose Transporter 4
HDL	High Density Lypoproteins
HFF1	Human Foreskin Fibroblast
HPLC	High Performance Liquid Chromatography
ICAM	Intracellular Adhesion Molecule
IC_{50}	Half Maximal Inhibitory Concentration
IGF1	Insulin-like Growth Factor 1
IL	Interleukin
*i*NOS	Inducible Nitric Oxide Synthase
IVGTT	Intravenous Glucose Tolerance Test

LC	Liquid Chromatography
LDL	Low Density Lypoproteins
L-NAME	L-NG-Nitroarginine Methyl Ester
LPS	Lipopolysaccharide
MAO-A	Monoamine Oxidase A
MIC	Minimum Inhibitory Concentration
MMP	Matrix Metalloproteinase
MPO	Myeloperoxidase
MS	Mass Spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NF-kB	Nuclear Factor kB
NO	Nitric Oxide
OD_{550}	Optical Density at 550 nm
OGTT	Oral Glucose Tolerance Test
PDW	Platelet Distribution Width
PGE2	Prostaglandin E_2
PGO	Peroxidase-Glucose Oxidase
PPARγ	Peroxisome Proliferator-Activated Receptor γ
QE	Quercetin
QTOF	Quadrupole Time Of Flight
RACI	Relative Antioxidant Capacity Index
RBCs	Red Blood Cells
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SD	Standard Deviation
STAT3	Signal Transducer and Activator of Transcription 3
STZ	Streptozotocin
TC	Total Cholesterol
TFC	Total Flavonoids Content
TG	Triglycerides
TIC	Total Iridoids Content
TNBS	2,4,6-Trinitrobenzenesulfonic acid
TNF-α	Tumor Necrosis Factor-α
TPC	Total Phenols Content
TPTZ	2,4,6-Tri(2-pyridyl)-s-triazine
TYR	Tyrosinase
UV	Ultraviolet
VCAM-1	Vascular Cell Adhesion Molecule-1
VLDL	Very Low Density Lipoprotein

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Introduction

General introduction

For millennia, plants have been a valuable source of therapeutic agents and still many of today's drugs are plant-derived natural products or their secondary metabolites.

An unprecedented revitalization has been taking place in the interest for natural products with impact on several fields of technical advancements, scientific knowledge, and economy (Koehn et al., 2005). The revived scientific interest in plant-derived natural product-based drug discovery is related with major scientific and technological advances in the relevant research fields. These include a better understanding of diseases and their underlying mechanisms, advances in screening methods and analytical equipment, an increasing number of targets available for testing and improved possibilities for optimization of natural leads using synthetic modification strategies. In addition, natural products provide important clues for identifying and developing synergistic drugs that, so far, research has neglected.

Berry fruits from edible plants of Ericaceae and Cornaceae families are known as natural sources of food, beverage and nutraceutical ingredients due to their richness in nutritional and bioactive compounds. Increased attention is given to the potential health benefits of berries. A diet rich in antioxidants has shown its efficiency in the prevention of several degenerative diseases such as cardiovascular diseases, cancer, obesity, and diabetes (Dai et al., 2010).

Interest in the berry constituents of these families has increased in recent years, mainly because of their high phenol content, known for their excellent antioxidant effect.

Considering their important health properties, the choice of efficient extraction methods is drawing great attention. Extraction process is the most important first step for the isolation of natural compounds from raw materials. According to the extraction principle, extraction methods include solvent extraction, pressing, sublimation, and distillation methods. Solvent extraction is the most used method. The extraction of natural compounds proceeds through the following steps: 1) the solvent penetrates into the solid matrix; 2) the solute dissolves in the solvents; 3) the solute is diffused out of the solid matrix; 4) the extracted solutes are collected. Any factor enhancing the diffusivity and solubility in the above steps will facilitate the extraction. The extraction efficiency is affected by several factors such as the properties of the solvent used for the extraction, the particle size of the raw materials, the solvent/solid *ratio*, the extraction duration, and the extraction temperature.

Crucial for the extraction is the selection of the solvent. Usually, the choice of the solvent is made according to the nature of the metabolites to solubilize, it must be selective of classes of phytochemicals having similar polarity, safe and inexpensive.

Alcohols (ethanol and methanol) are universal solvents used for a phytochemical investigation of polar metabolites. The use of binary solvents was better compared with mono-solvents for the extraction of phenolic compounds (Thoo et al., 2010).

Jayaprakasha et al. (2001) have indicated a low yield of phenolic compounds when alone solvent as methanol was used. Moreover, aqueous solution with acetone, methanol, and ethanol demonstrated to be more efficient compared with mono solvent solution with the *Vitis rotundifolia* seeds (Yilmaz et al., 2006). The possible explanation could be that majority of phenolic compounds are glycosides and sugar portion is more soluble in water (Ignat et al., 2011). The choice of the solvents must take into account the legislation (directive 2009/32/EC), the effectiveness towards the extraction of bioactive compounds, their toxicity, and environmental impact (Socaci et al. 2018).

High temperatures increase both diffusion and solubility, consequently reducing the extraction times. Hence, high temperatures increase the concentration of poorly soluble compounds such as certain flavonoids and iridoids, but also determine the breakdown of cellular components that would retain the bioactive compounds present (Lim et al., 2007; Náthia-Neves et al., 2017). As reported by Mokrani et al. (2016), majority flavonols are glycosides and their yields are increased through heating. Heat could be soften the plant tissue with weaken phenol-polysaccharides and phenols interactions, with consequently migration of flavonols in the solvent. However, too high temperatures may cause the extraction of undesirable impurities due to the decomposition of thermolabile components. The extraction efficiency increases with the duration in a certain time range.

Extraction time influences both solubility and transfer of bioactive compounds in the solvent; these processes are correlated with their molecular weight and structure (Belwal et al., 2016; Vuong et al., 2011). Increasing time will not affect the extraction after the equilibrium of the solute is reached inside and outside the solid material. Prolonged extraction time could trigger degradative processes of bioactive compounds, such as catechin (Vuong et al., 2011). In general, a higher extraction yield corresponds to a greater solvent/solid ratio. However, a solvent/solid ratio too high could determine excessive extraction solvent and require a long time for the concentration of the obtained extractive solutions. The conventional extraction methods, including maceration, percolation and reflux extraction, usually use organic solvents and require a large volume of solvents and long extraction time. Some modern or greener extraction methods such as super critical fluid extraction, pressurized liquid extraction, and microwave-assisted extraction, have also been applied in natural products extraction, and they offer some advantages such as lower organic solvent consumption and shorter extraction time.

The aim of the study

Extracts from selected plants from Ericaceae and Cornaceae families, namely *Arbutus unedo* L. (strawberry tree), *Vaccinium corymbosum* L. (highbush blueberry), *Cornus mas* L. (cornelian cherry), and *Cornus sanguinea* L. (blood twig dogwood,

European dogwood or common dogwood), are a rich source of health-promoting compounds, and are suitable for use as natural food additives and as functional foods.

Until now, several previous works have focused on the study of a single morphological part of these plant species and especially of fruits and anthocyanins-rich extracts. To the best of our knowledge, there are not present in the literature studies that investigated the potential contribution of flavonoids and in particular iridoids to their biological activities. These two phytochemical classes characterize these botanical families and are present in different plant organs. Flavonoids are a class of phenolic compounds commonly distributed in fruits and vegetables and are considered as health promoting dietary supplements. *In vitro, in vivo,* epidemiological, and clinical studies reveal that flavonoids may exert protective effects against several degenerative diseases including metabolic disease, cardiovascular disease, and cancer (Gonzales, 2017).

Flavonoids have been reported to exert antioxidant, antibacterial, hypolipidemic, antithrombotic, antiviral, and anti-inflammatory effects. Therefore, recently flavonoids are the focus of current considerable therapeutic and nutritional interest.

Iridoids are monoterpenes widely distributed in nature, characterized by a cyclopentantetrahydropyran ring type, also known as iridane. Typically, they are present as glycosides and are known for some interesting biological activities, including hepatoprotective, antimicrobial, sedative, antihypertensive, antioxidant, antiviral, neuroprotective, lipid-lowering, anti-cancer, immunomodulatory, and anti-inflammatory (Deng et al., 2013).

Moreover, berry fruits are worldwide recognized as “superfoods” due to the high content of bioactive molecules and the health benefits deriving from their consumption.

A body of scientific research studies proved the contribution of berry consumption to the main targets of functional foods such as health maintenance and reduced risk of some chronic diseases. However, not only the fruits, but also the leaves of these plants have been used in traditional remedies. Leaves are considered byproducts of berries cultivation. Their traditional use against several diseases, such as inflammation, diabetes, and ocular dysfunction, has been almost forgotten nowadays. The scientific interest regarding the leaf composition and beneficial properties grows, documenting that leaves may be considered an alternative source of bioactive compounds. Analytical studies reveal that the leaves chemical composition is similar to that of the fruits or even richer and higher, indicating that they may be used as an alternative source of bioactive compounds for the development of functional foods, nutraceuticals, and/or food supplements.

In this context, the aim of this thesis is:

- ✓ to find the best extraction procedure to take advantage of the biological properties of constituents (flavonoids and iridoids) in the fruits and leaves (byproducts) of *Arbutus unedo* L., *Vaccinium corymbosum* L., *Cornus mas* L., and *Cornus sanguinea* L. The project will explore the most promising extractive techniques with a focus on their main advantages in terms of rapidity, low energy costs, low toxicity with respect to

the traditional extraction methods, thus promoting the adoption of environmentally-friendly procedures. Different extraction techniques such as maceration, ultrasound-assisted extraction, Soxhlet extractor and decoction, will be applied by using food grade ethanol/water as solvent mixture, selected as environmentally friendly solvents.

- ✓ to investigate samples for their potential *in vitro* antioxidant activity, hypoglycaemic potential, and nitric oxide production. Considering that different antioxidant compounds may act *in vivo* through different mechanisms, not a single method can fully evaluate the antioxidant capacity of a sample. Therefore, to investigate the antioxidant activity of a sample choosing an adequate assay is critical. The antioxidant activity of *Arbutus*, *Cornus*, and *Vaccinium* species will be evaluated employing four established *in vitro* systems such 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ammonium salts (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP), and β-carotene bleaching tests. Diabetes mellitus is a metabolic disorder characterized by hyperglycaemia and alterations in carbohydrate, lipid and protein metabolism, associated with absolute or relative deficiencies in insulin secretion and/or insulin action. The high prevalence of diabetes as well as its long-term complications has led to an ongoing search for hypoglycaemic agents from natural sources (Nicasio-Torres et al., 2009). One therapeutic approach to treat the early stage of diabetes is to decrease post-prandial hyperglycaemia. This is done by retarding the absorption of glucose through the inhibition of the carbohydrate-hydrolysing enzymes, α-amylase and α-glucosidase, in the digestive tract. Consequently, inhibitors of these enzymes determine a reduction in the rate of glucose absorption and consequently blunting the post-prandial plasma glucose rise. Therefore, the α-amylase and α-glucosidase inhibition assays will be performed. Nitric oxide (NO) is a potent mediator in several cellular processes including inflammation (Sharma et al., 2007). The use of NO inhibitors represent an important therapeutic approach in the management of inflammatory diseases. Herein, the inhibitory effects of extracts on NO production were investigated by using the assay based on the reaction of diazocopulation of nitrite with the Griess reagent.
- ✓ to establish the chemical profile of extracts by LC/ESI/QTOF/MS analyses.
- ✓ to correlate the founded bioactivities to the main classes of identified constituents, flavonoids and iridoids.
- ✓ to identify the most active extracts and to fractionate them through the use of resin.
- ✓ to investigate the biological properties of the obtained enriched-fractions in order to prospect their future use for the development of functional foods, nutraceuticals, and/or food supplements.

The thesis is structured in two parts. The first part presents a literature review regarding the interest in flavonoids and iridoids as health-promoting compounds from Ericaceae and Cornaceae families (Chapter 1), general aspects on the constituents and

their health benefits of species from *Arbutus*, *Cornus*, and *Vaccinium* genera (Chapter 2-4), and the scientific interest in the search of multitarget agents for the treatment of high social impact diseases related to oxidative stress (Chapter 5).

The available information was collected from some scientific databases such as SciFinder, PubMed, Science Direct, Scopus, and Web of Science, using the keywords "Ericaceae" or "Cornaceae" or "*Arbutus unedo*" or "*Vaccinium corymbosum*" or "*Cornus mas*" or "*Cornus sanguinea*" or "strawberry tree" or "cornelian cherry" or "blood twig dogwood" or "European dogwood" or "common dogwood" or "highbush blueberry" and "chemical compounds" or "iridoids" or "flavonoids" or "phenolic acids" or "tannins" or "byproducts" or "extraction" or "activity" or "antioxidant" or "hypoglycaemic" or "anti-inflammatory" or "nitric oxide" or "toxicity". Only articles written in English and published in peer-reviewed scientific journals were used. To find relevant studies, papers were primarily screened based on titles, abstracts, and keywords.

The second part concerns the experimental results and contains four chapters. Chapter 6 reports the description of materials and methods. In the chapters 7 and 8, the chemical analyses of *A. unedo* and *V. corymbosum* and the *in vitro* biological properties of their extracts are presented. Chapter 9 is focused on the evaluation of the chemical profile and bioactivity of *C. mas* and *C. sanguinea* extracts, fractions, and pure compounds.

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Bibliographic part

Chapter 1

Cornaceae and Ericaceae: families rich in health-promising molecules

1.1. Introduction

Among polyphenols and iridoids-rich plants, in depth bibliographic investigation about the edible plants from Mediterranean area allowed to select some species from *Arbutus, Cornus*, and *Vaccinium* genera.

Arbutus and *Vaccinium* genera belong to the Ericaceae family, while *Cornus* belongs to the Cornaceae family. The Asterids gather most Triporees with petals and fused carpels; they contain 91800 species divided into 99 families in order, the largest clade of Angiosperms. Genetic analysis carried out after APG IV maintains its position as the Cornales (Figure 1.1). A second order that splits from the base of the Asterids are the Ericales. The remaining orders cluster into two clades, the Lamiids and the Campanulids.

Cornales include 657 species distributed in 7 families, including Cornaceae (85 species) with single leaves, whole, opposite, 4 petal flowers, 4 free herbs, post-obdiplostemones and an inferior ovary.

Ericales include 11500 species and 22 families including Theaceae, Ericaceae, and Primulaceae. The flower typically comprises two whorls of obdiplostemones etamines, but by abortion, it can become post-obdiplostemone. Some families are meristemones.

The Ericaceae are widespread throughout the world, from mountains to tropics, but are located mainly in the temperate or cold regions. Part of the family is also adapted to the Mediterranean climate. They are shrubs, more or less lignified, rich in iridoids. Most are adapted to acid and poor soils, such as moors, maquis; the result is a particular vegetative port called ericoide characterised by: very slow-growing, hardwood-shaped, contoured stems; narrow leaves whose limb margins are bristly and fold over themselves, protecting their lower side, carrying the stomata to limit perspiration.

In addition, Ericaceae species present original characters: endomycorrhizae; the fillet of the stamens is not soldered to the corolla, and each box of the antechamber opens with a pore; the anther is often provided with two appendices erect or horn-shaped, hence the name “Bicornes” formerly given to the order; the pollen grains are usually arranged in tetrads.

The ovary is composed of five “closed” carpels; axial placentas carry many ovules; styles and stigmas are welded. The fruit is a capsule. Both Cornaceae and Ericaceae families are known for a high content in flavonoids and iridoids (Iwashina, 2000; Plouvier et al., 1971).

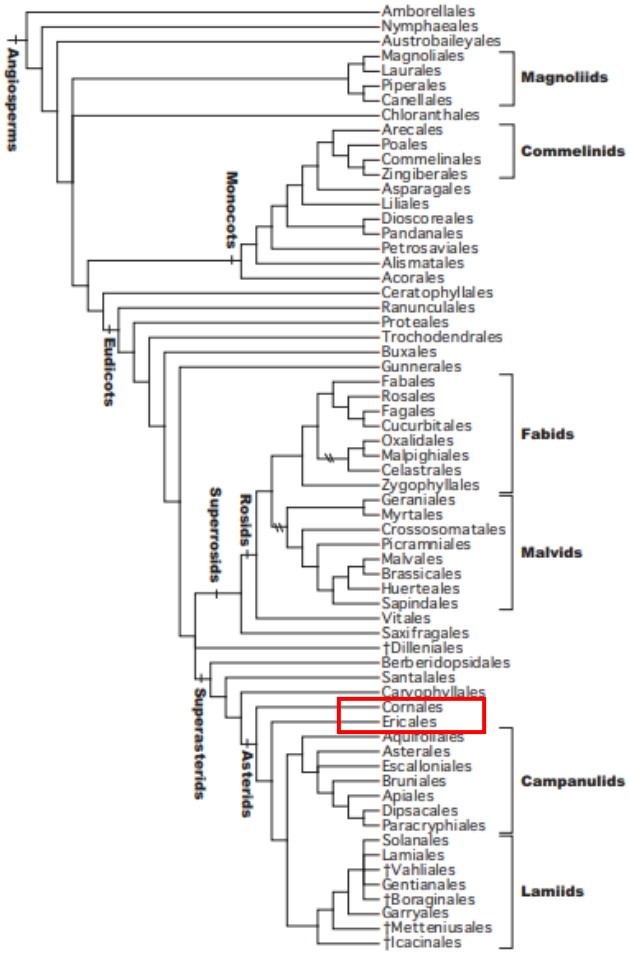

Figure 1.1. Plant classification according to APG IV

1.2. Flavonoids: biogenesis, classification, and toxicity

1.2.1. Definition

Flavonoids *lato sensu* are universal pigments of plants and constitute a class of phenols that consists of more than 6000 compounds ubiquitously present in the plant kingdom, with a wide range of biological properties, including antitumor, antiviral, anti-allergic, antioxidant and anti-inflammatory activities (Ginwala et al., 2019). These compounds have a basic structural element namely 2-phenyl chromane and can be classified into a dozen subgroups (Bruneton, 2016), according to the degree of oxidation of the pyranic ring, which can be opened and recycled in the furan cycle:

- anthocyanin (2-phenylbenzopyiliums);
- 2-phenylchromones: flavones, flavonols and dimers; flavanones and dihydroflavonols;
- 2-phenylchromanes: flavanes, flavan-3-ols, flavan-3,4-diols;
- aurones (present only in some species);
- chalcones and dihydrochalcones.

Many authors use the term flavonoids to indicate all these compounds. However, in the following dissertation, we will use "flavonoids" for indicate flavones, flavonols, flavanones, dihydroflavonols, bioflavonoids, and chacones. In this way, we will distinguish these flavonoids *stricto sensu* from proanthocyanidins and anthocyanins.

1.2.2. Biogenesis

Flavonoids are synthesized by the phenylpropanoid metabolic pathway, in which the amino acid phenylalanine is used to produce 4-coumaroyl-CoA (Figure 1.2) and with a chain extension coming from acetate pathway (Dewick, 2009).

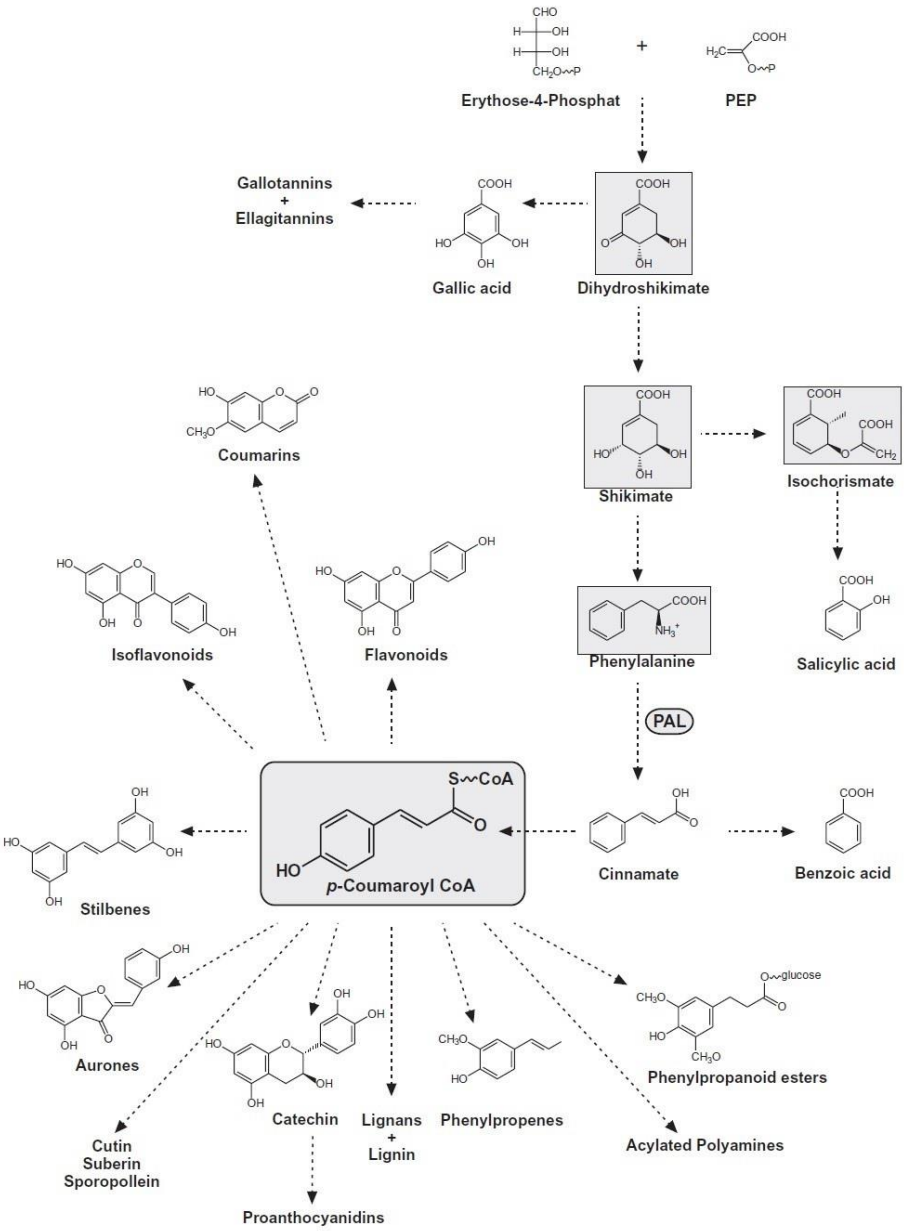

Figure 1.2. Flavonoids biosynthetic pathway (adapted by Vogt, 2010)

Shikimic acid is the basis for the formation of phenylalanine, a precursor of phenylpropanoid pathways to reach flavonoids as a final product. The enzyme phenylalanine ammonia lyase (PAL) converts phenylalanine into cinnamic acid, which in turn is used by cinnamate-4-hydroxylase (C4H) to give 4-coumaric acid. Subsequently, coumaric acid is added to a molecule of Coenzyme A (CoA) through the action of a CoA ligase obtaining the 4-coumaroyl-CoA (or 4-hydroxycinnamoyl-CoA), which represents the pivot point in the phenylpropanoid pathway. The condensation of this substrate with 3 units of malonyl-CoA by chalcone synthase (CHS) leads to the formation of narigenin chalcone that in turn lets get flavanone by a Michael-type nucleophilic attack of a phenol group on to the unsatured ketone.

Flavanones represent the basic skeleton for the formation of catechins, anthocyanidins, flavones, and flavonols (Figure 1.3).

Flavonoids are characterised by the presence of 15 carbon atoms in their basic skeleton, arranged in the form C6-C3-C6, which corresponds to two aromatic rings A and B linked by a unit of three carbon atoms, which may or may not give rise to a third ring. The rings are labeled A, B and C.

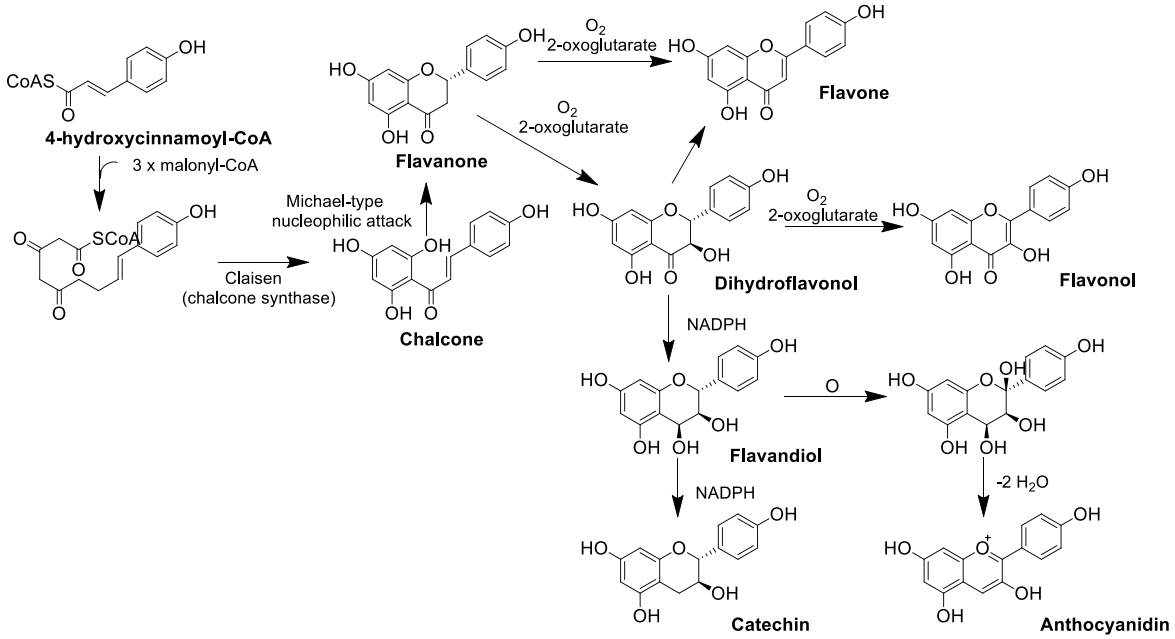

Figure 1.3. Flavonoids biogenesis

For the difference on the position of the link of ring B on ring C can be due to the classification: flavonoids, isoflavonoids, and neoflavonoids (Figure 1.4).

There are other flavonoid classes (chalcones and aurones) with an “incomplete structure”, because the ring C has not yet been formed. As previously described in the

biogenesis, chalcones (skeleton 2a) are precursor of flavonoids. In nature, it is possible to find the rearrangement of chalcone (skeleton 2b).

The term dihydrochalcones (skeleton 3) are attributed to chalcone without double bond on “pseudo” ring C. The base structure of flavonoids in restricted sense include flavans (1) as well as compounds of type 1 with one or two additional double bonds, and carbonyl or hydroxy groups or both in ring C, used to rank flavonoids into different classes, as flavones, flavonols, and anthocyanidins.

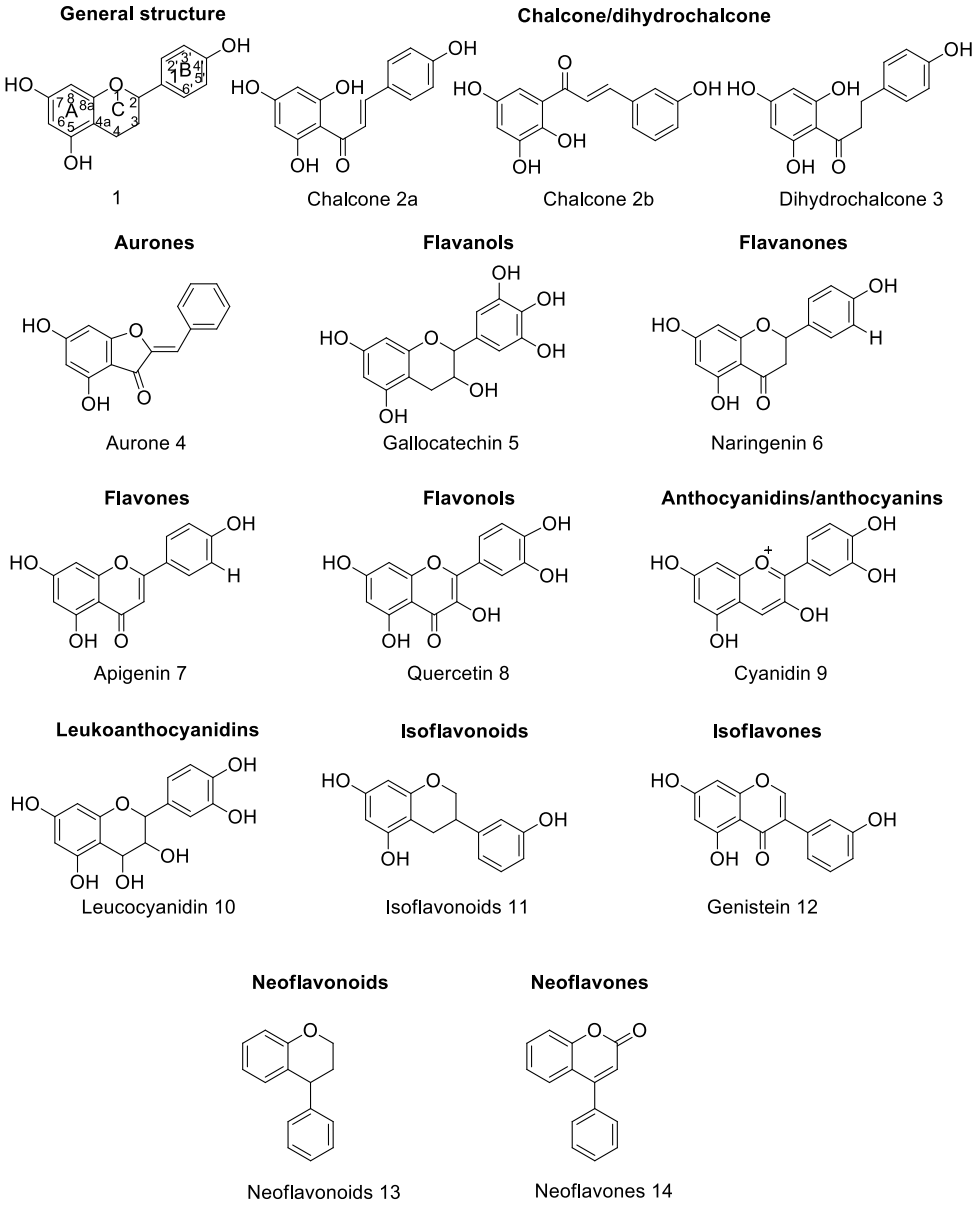

Figure 1.4. Flavonoids classification

Flavans are compounds with a skeleton 1, that may be substituted including flavan-3-ols (with -OH in position 3; skeleton 5) and flavan-4-ones (with a carbonyl function in position 4; skeleton 6), which are generically known as flavanols and flavanones, respectively.

Flavones and flavonols are derived by skeleton 6 but there is a double link between the C2 and C3 of ring C with formation of flavones (skeleton 7). The term "flavonol" (skeleton 8) is used as a class name for compounds with a skeleton 6 that present a hydroxyl substitution on C3. Anthocyanidins (skeleton 9) are responsible to colours (from red to purple) in the plant kingdom and, together with the 3-glycosides of anthocyanidin, the anthocyanins, represent a large group of plant pigments.

Compounds that present the same skeleton 5 but with other hydroxyl substitution in position 4 are designated leukoanthocyanidins (skeleton 10). Isoflavones (skeleton 12) present the same structure of flavones (7) but the ring B is connected with ring C by position 3.

1.2.3. Potential toxicity

Diet-derived flavonoids are considered as safe for humans because many foods are of course rich in these polyphenols. It is difficult to find tolerable reference daily intake, but for the quantities present in the food is not a concern. The potential toxicity of flavonoids rely on dose, type, and duration of intake. However, the toxicity found *in vitro* can be null when tested direct on human, because genotoxicity was observed at high concentrations that are impossible to achieve with diet. In fact, for genistein and quercetin genotoxicity demonstrated in *in vitro* tests, was not confirmed in *in vivo* tests (Harword et al., 2007; Jerome-Morais et al., 2011). Genotoxicity observed *in vitro* could be associated with flavonoids pro-oxidant activity that show under certain condition as a result of its autoxidation or enzymatic conversion, *in vivo* there are different protective mechanism that prevent their autoxidation.

In vitro, flavonoids anti-oxidant abilities may be due at least in part to an ability to chelate transition metal ions such as iron (Sestili et al., 1998), but metals can promoted auto-oxidation. Interesting is that, *in vivo*, transition metals are complexed with proteins and this not allowing their auto-oxidation (Galati et al., 2002).

Several toxicology studies about anthocyanins extracted by grape-skin and blackcurrant, showed the daily intake of 2.5 mg/kg per day is a dose safe acceptable (European Food Safety Authority, 2013). Previous studies *in vivo* not reported any toxic effects of anthocyanins derived by berries at quantities of 25 mg/kg *per day* in mice and 20 mg/kg *per day* in rats. Moreover, increasing the dose (9g/kg *per day*) not presented toxicity over three generation in rats, mice and rabbits (Pourrat et al., 1976).

1.3. Iridoids: biogenesis, classification and toxicity

1.3.1. Definition

Iridoids represent a large group of cyclopenta[c]pyran monoterpenoids found in a number of medicinal plants and food plants (Villaseñor et al., 2007).

Eucommiaceae, Actiniaceae, Saxifragaceae, Hamamelidaceae, Daphniphyllaceae, Hippuridaceae, Cornaceae, Garryaceae, Pyrolaceae, Ericaceae, Oleaceae, Loganiaceae, Gentianaceae, Menyanthaceae, Apocynaceae, Rubiaceae, Fouqueriaceae, Verbenaceae, Callitrichaceae, Labieae, Buddleiaceae, Scrophulariaceae, Globulariaceae, Bignoniaceae, Pedaliaceae, Orobanchaceae, Lentibulariaceae, Myoporaceae, Plantaginaceae, Caprifoliaceae, and Valerianaceae are the families known for their iridoids content.

Iridoids presented a skeleton with 15 carbon atoms. Traditional and IUPAC nomenclatures of iridoids are presented in Figure 1.5.

Traditional nomenclature IUPAC nomenclature

Figure 1.5. Iridoids nomenclature

Intensive research revealed that iridoids exhibit a wide range of activities, such as cardioprotective, antioxidant, anti-inflammatory, anticancer, neuroprotective, antimicrobial, antispasmodic, purgative, immunomodulating, hepatoprotective, and hypoglycaemic activities (Bas et al., 2007a,b; Crisan et al., 2010; Jaishree et al., 2010; Li et al., 2004; Sharma et al., 1994; Tundis et al., 2008).

1.3.2. Biogenesis

All iridoids derived from geraniol (Figure 1.6), which is transformed by hydroxylation and oxidation into iridodial (geraniol cyclised). Another oxidation gives iridotrial, in which hemiacetal formation then leads to production of the heterocyclic ring. Loganin is a key intermediate in the biosynthesis of many other iridoids. Its oxidation and phosphorylation give opening to ring and secoiridoids (secologanin). Secologanin is a precursor of other secoiridoids and alkaloid indole-monoterpenes. Iridoids present in plantkingdom were classified in various groups basing on biosynthesis.

Since of 1980, different authors have reported various classifications of natural iridoids. El-Naggar et al., (1980) have summarised only iridoid glycosides excepting iridoids contained nitrogen. Iridoids are divided in secoiridoid glucosides (not necessary glucose) and non-glycosidic compounds. Hegnauer (1986) has divided the iridoids in nine structural group, cyclopentanoid monoterpenes and secoiridoids.

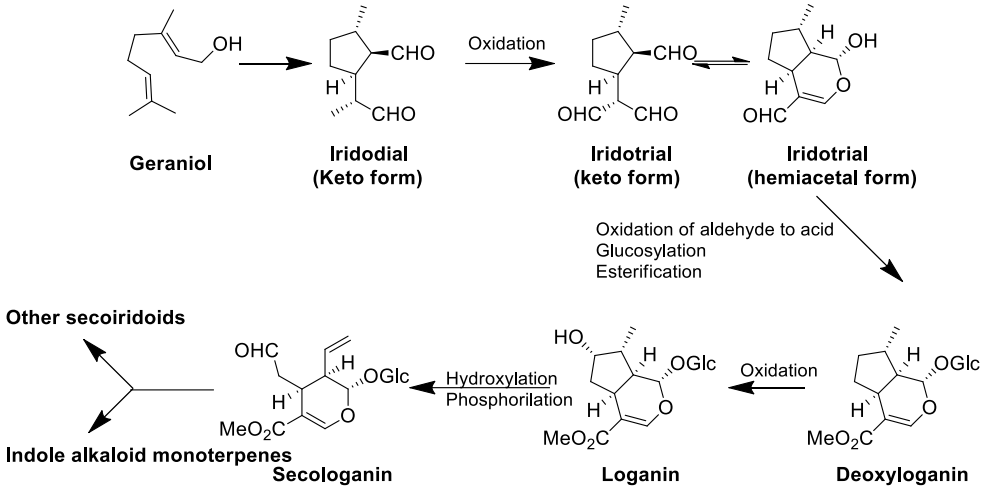

Figure 1.6. Iridoids biogenesis

This last characterised by general structure complexed with indole and isochinoline. Bianco et al. (1990) divided the cyclopentane monoterpenes in two categories: iridoids (glycosilated iridoids, simple iridoids, secoiridoids, and bisiridoids) and alkaloid monoterpenes.

A more recent classification reports three categories: simple iridoids, alkaloid monoterpenes, and glycosilated iridoids.

The term glycosilated iridoids includes carbocycles iridoids, secoiridoids, and bisiridoids. In the majority of cases, glucopyranoside unite is linked to C1.

Another classification (Figure 1.7) divides iridoids in iridoids with eight carbon (skeleton 16-17), iridoids with nine carbon and this carbon is in position C-4 (skeleton 18), iridoids with nine carbon and this carbon is in position C-8 (skeleton 19-20), and iridoids with ten carbon (skeleton 21-22).

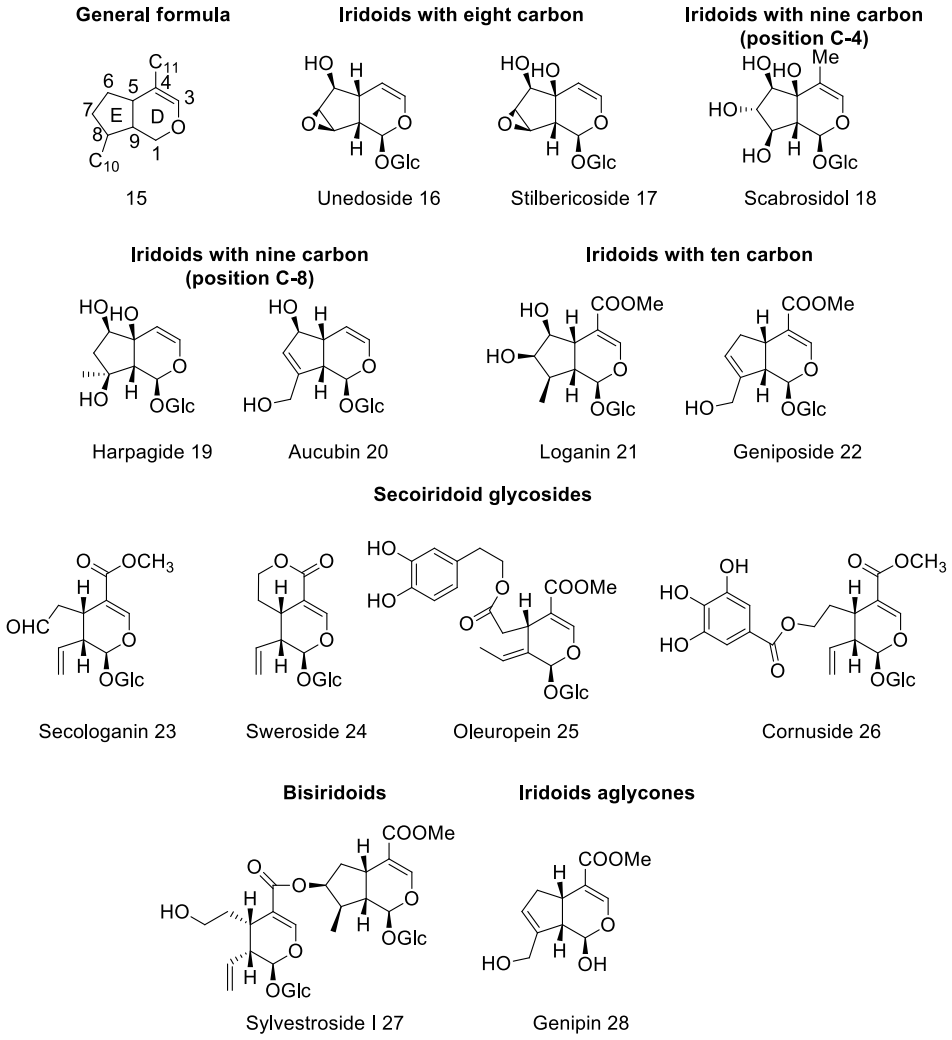

Figure 1.7. Iridoids classification

1.3.3. Toxicological aspects of iridoids

Iridoids exert many different biological activities (Dinda et al., 2007; Dinda et al., 2019; Ghisalberti, 1998). Researchers examined also their toxicological aspects.

Deng et al. (2013) demonstrated that loganin, sweroside, and cornuside did not display any toxicity and did reduce the amount of DNA damage caused by 4-nitroquinoline 1-oxide (used as induction factor DNA damage), suggesting potential anti-genotoxic activity.

The juice of *C. mas* was investigated in order to evaluate its potential toxicity on mice. No adverse symptoms or death after administration of a dose of 5 mL/kg was reported (West et al., 2012). Kidney and liver damages of normal and with jaundice rats treated with geniposide were observed (Shan et al., 2017). Hepatotoxicity associated with oxidative stress was observed in normal rats with a dose of 574 mg/kg after 24-48 h. On the other hand, in the rats with jaundice, after treatment with 1.2 g/kg of geniposide, the

liver parameters, as transaminases, bilirubin, and alkaline phosphatase were increased; showing toxicity.

Swertiamarin did not display at 2 g/kg in mouse any toxicity or mortality (Dhanavathy et al., 2017).

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Chapter 2
Arbutus species (Ericaceae)

2.1. Introduction

Arbutus L. is a genus that belongs to the Arbutoideae sub-family, which includes evergreen shrub-like woody taxa with laurel-like and sclerophyllous leaves of the Ericaceae family. This sub-family is distributed throughout the whole world from sea level to mountaintop and in latitude ranging from the Tropics to the Arctic.

The Plant List includes 122 scientific plant names of *Arbutus* species. However, twelve are the accepted *Arbutus* species growing in America and Mediterranean area.

Among American species, *A. menziesii* and *A. xalapensis* are the main investigated. *A. menziesii* bark is recognized as ingredient of "ten-barks" medicine for birth control, colds, stomach problems, and internal ailments (Turner et al., 1990). *A. xalapensis* leaves are used for the treatment of diabetes in the Mexican traditional medicine (Maiti et al., 2016). In the Mediterranean area, there are four species and two hybrids: *A. unedo*, *A. andrachne*, *A. pavarii*, *A. canariensis*, *Arbutus* × *andrachnoides* (*A. unedo* × *A. andrachne*, and *Arbutus* × *androsterilis* (*A. unedo* × *A. canariensis*) (Torres et al., 2002).

A. unedo (strawberry tree) is the most common species (Figure 2.1).

Figure 2.1. *Arbutus unedo* L. (adapted by Encyclopedia of Life)

According to "The Plant List" *A. unedo* presents various synonyms such as *A. cassinifolia* Steud., *A. crispa* Hoffmanns., *A. croomii* auct., *A. integrifolia* Sims, *A. intermedia* Heldr. ex Nyman, *A. laurifolia* L.f., *A. microphylla* auct., *A. nothocomaros* Heldr. ex Nyman, *A. procumbens* Kluk ex Besser, *A. salicifolia* (Lodd.) Cels ex Hoffmanns., *A. serratifolia* Salisb., *A. turbinata* Pers. ex Rchb., *A. unedo f. subcrenata* Maire, *A. unedo var. ellipsoidea* Aznov., *A.unedo var. salicifolia* Regel, *A. vulgaris* Bubani (The Plant List, 2013).

The literature survey revealed different traditional uses for these species based on their diuretic, antiseptic, anti-hypertensive, anti-diabetic, anti-inflammatory, and laxative properties. Anthocyanins, iridoids, phenols, triterpenes, sterols, and fatty acids are reported as main classes of constituents.

The aim of this chapter is to provide an overview of current knowledge on the chemical profile and bioactivity of *Arbutus* species, particularly *A. unedo*, *A. pavarii*, and *A. andrachne*, three edible species common in the Mediterranean area.

2.2. Traditional use of *Arbutus* species

A. unedo fruits are generally used for preparing alcoholic beverages, marmalades, jams, and jellies; less frequently are eaten fresh. They are also used in traditional medicine as antiseptic, diuretic, and laxative agent, to treat hypertension and kidney diseases (Table 2.1) (El-Hilaly et al., 2003; Fortalezas et al., 2010; Kivçak et al., 2001a; Ruiz-Rodriguez et al., 2011).

Root, bark and leaves are effective on the treatment of hypercholesterolemia, hypertension, vaginal infections, and gastrointestinal disorders, as well as to treat urological and dermatologic problems (Leonti et al., 2009; Novais et al., 2004; Ziyyat et al., 1997). Leaves are used for the treatment of skin diseases (Ait Youssef, 2006), haemorrhoidal (Cornara et al., 2009), diabetic (Ziyyat et al., 1997), rheumatic (González et al., 2010) and kidney diseases (El-Hilaly et al., 2003).

In Morocco, Italy, Turkey, and Spain, leaves and fruits of *A. unedo* are recognized for their astringent, anti-aggregant, antimicrobial, and anti-diarrheal properties (Boulanour et al., 2013; Mendes et al., 2011; Pallauf et al., 2008). In India, flowers and stems are used as anti-inflammatory agents (Govindappa et al., 2011).

A. andrachne is a small evergreen tree native to the Mediterranean region and southwestern Asia (Serçe et al., 2010) traditionally used for its astringent, anticancer, laxative, anti-diarrhoeal, urinary antiseptic and depurative properties (Said et al., 2002; Sakar et al., 1992; Şeker et al., 2010). In Jordanian traditional medicine flowers, barks, and leaves of *A. andrachne* are used for the treatment of asthma (Amro et al., 2013) and diabetes (Hamdan et al., 2008).

A. canariensis is an endemic species to the Canary Islands of Spain where its fruits are used as anti-diarrhoeal agents (Darias et al., 1989).

A. pavarii is a Mediterranean evergreen shrub endemic to Libya in Gebal Al-Akhdar (Green Mountain) escarpment (Hegazy et al., 2013), employed as food supplement in honey production, as ornament trees, and in medicine for the treatment of gastritis, renal infections, and anti-cancer agent (Hamad et al., 2011).

Table 2.1. Ethno-medicinal uses of *Arbutus* species.

Arbutus	Traditional uses	Country	Part used	References
A. andrachne	Anti-cancer	Israel	Leaves, fruits and roots	Said et al., 2002
	Anti-diabetic	Jordan	Leaves	Hamdan et al., 2008
	Anti-diabetic	Anatolian	Leaves	Sakar et al., 1992
	Antiseptic	Turkey	Leaves and fruits	Şeker et al., 2010
	Asthma	Jordan	Flowers, bark and leaves	Amro et al., 2013
	Blood tonic	Israel	Leaves, fruits and roots	Said et al., 2002
	Diuretic, laxative	Turkey	Leaves and fruits	Seker et al., 2010
	To treat wound	Israel	Leaves and fruits	Said et al., 2002
	Urinary antiseptic	Israel	Leaves, fruits and roots	Said et al., 2002
	Urinary antiseptic	Anatolian	Leaves	Sakar et al., 1992
A. canariensis	Anti-diarrheal	Canary Islands	Fruits	Darias et al., 1989
A. menziesii	Birth control, cold, to treat stomach problems	Vancouver Island	Bark	Turner et al., 1990
A. pavarii	To treat cold, tuberculosis, and stomach problems	Lybia	Bark and leaves	Hamad et al., 2011
A. unedo	Antidiabetic	Morocco	Leaves and roots	Mrabti et al., 2018 ; Ziyyat et al., 1997
	Antidiabetic	Portugal	Leaves and fruits	Fortalezas et al., 2010; Mendes et al., 2011 ; Oliveira et al., 2011a
	Antiseptic	Spain	Leaves and fruits	Pallauf et al., 2008 ; Ruiz-Rodriguez et al., 2011
	Antiseptic	Portugal	Leaves Fruits	Fortalezas et al., 2010 ; Mendes et al., 2011; Oliveira et al., 2009, 2011a
	Antiseptic	Turkey	Leaves and fruits	Pabuçcuoğlu et al., 2003; Pavlović et al., 2011; Pawlowska et al., 2006
	Diuretic	Morocco	Leaves	El Haouari et al., 2007; Ziyyat et al., 1997
	Diuretic	Turkey	Leaves et fruits	Pabuçcuoğlu et al., 2003; Pavlović et al., 2011; Pawlowska et al., 2006
	Diuretic	Portugal	Leaves and fruits	Boulanouar et al., 2013; Fortalezas et al., 2010 ; Mendes et al., 2011 Oliveira et al., 2009, 2011a
	Diuretic	Spain	Leaves and fruits	Pallauf et al., 2008; Ruiz-Rodriguez et al., 2011
	Laxative	Spain	Leaves and fruits	Pallauf et al., 2008; Ruiz-Rodriguez et al., 2011
	Laxative	Portugal	Fruits	Fortalezas et al., 2010; Mendes et al., 2011
	Laxative	Turkey	Fruits	Pawlowska et al., 2006
	Astringent	Morocco	Leaves	El Haouari et al., 2007; Ziyyat et al., 1997
	Astringent	Portugal	Leaves	Boulanour et al., 2013; Fortalezas et al., 2010 ; Mendes et al., 2011; Oliveira et al., 2009, 2011a

Astringent	Turkey	Leaves	Pabuçcuoğlu et al., 2003; Pawlowska et al., 2006
Anti-aggregant	Morocco	Leaves	El Haouari et al., 2007; Ziyyat et al., 1997
Anti-aggregant	Portugal	Leaves	Boulanour et al., 2013; Fortalezas et al., 2010 ; Mendes et al., 2011; Oliveira et al., 2009, 2011a
Anti-aggregant	Turkey	Leaves	Pabuçcuoğlu et al., 2003; Pawlowska et al., 2006
Anti-aggregant	Spain	Fruits	Pallauf et al., 2008; Ruiz-Rodriguez et al., 2011
Antimicrobial	Turkey	Leaves	Kivçak et al., 2001b
Antimicrobial	Spain	Fruits	Ruiz-Rodriguez et al., 2011
Anti-inflammatory	Morocco	Leaves	Ziyyat et al., 1997
Anti-inflammatory	Portugal	Leaves	Mendes et al., 2011; Oliveira et al., 2011a
Anti-inflammatory	India	Flowers and stem	Govindappa et al., 2011
Anti-diarrheal	Morocco	Leaves	El Haouari et al., 2007; Ziyyat et al., 1997
Anti-diarrheal	Turkey	Leaves	Pabuçcuoğlu et al., 2003 ; Pawlowska et al., 2006
Anti-diarrheal	Portugal	Leaves	Mendes et al., 2011; Oliveira et al., 2009, 2011a
Anti-hypertensive	Morocco	Leaves and roots	El Haouari et al., 2007; Ziyyat et al., 1997
Anti-hypertensive	Portugal	Leaves	Boulanour et al., 2013; Fortalezas et al., 2010
Anti-hypertensive	Turkey	Leaves	Pabuçcuoğlu et al., 2003; Pawlowska et al., 2006
Anti-hypertensive	Portugal	Leaves	Mendes et al., 2011; Oliveira et al., 2011a
Anti-hypertensive	Spain	Leaves and fruits	Ruiz-Rodriguez et al., 2011
Depurative	Morocco	Leaves	Ziyyat et al., 1997
Depurative	Portugal	Leaves	Mendes et al., 2011; Oliveira et al., 2009, 2011a
Depurative	Portugal	Roots	Novais et al., 2004
Depurative	Turkey	Leaves	Pabuçcuoğlu et al., 2003; Pawlowska et al., 2006
To treat dermatologic diseases	Italy	Leaves, fruits, bark and roots	Leonti et al., 2009
To treat dermatologic diseases	Turkey	Fruits	Pavlović et al., 2011
To treat gastrointestinal disorders	Turkey	Fruits	Pavlović et al., 2011
To treat gastrointestinal diseases	Italy	Leaves, fruits, bark and roots	Leonti et al., 2009
To treat gastrointestinal diseases	Portugal	Roots	Novais et al., 2004
To treat kidney diseases	Morocco	Fruits and leaves	El-Hilaly et al., 2003
Anti-haemorrhoidal	Italy	Leaves	Cornara et al., 2009
Anti-rheumatic	Spain	Leaves	González et al., 2010
Antihypercholesterolemic	Portugal	Roots	Novais et al., 2004

2.3. Chemical constituents

Fruits and leaves of *Arbutus* species biosynthesize phenols, anthocyanins, iridoids, carotenoids, fatty acids, and terpenoids. Some sugars and minerals also characterised both fruits and leaves. However, differences related to the extraction procedure must be taken into consideration. Previous studies showed that phenols and iridoids are well extracted by using alcoholic solutions (Karikas, 1993; Maleš et al., 2006), anthocyanins by using methanol solution of HCl (Pawlowska et al., 2006) and apolar compounds such as satured fatty acids and carotenoids by employing acetone-petroleum ether mixture. Carotenoids are not soluble in methanol and for the preservation of vitamin C and β-carotene, the extract need to be freeze-dried rapidly because the sensitivity to oxidation is known.

Nyacin was extracted only by H_2SO_4 (0.5 M) (Alarcão-E-Silva et al., 2001). Tripernoids were obtained with treatment of ethanolic extracts diluted with water until its precipitation. The precipitate was treated with petroleum ether for separated triterpene alcohols and sterol from triterpene acids (Grishkovets et al., 1979). Table 2.2 summarizes the main constituents of *A. andrachne*, *A. pavarii*, and *A. unedo*.

Table 2.2. The main chemical constituents of *A. andrachne*, *A. pavarii*, and *A. unedo*.

Arbutus species	Class	Chemical constituent	Part of plant	Reference
A. andrachne				
	Acids	Citric acid, malic acid	Fruits	Serçe et al., 2010
	Flavonoids	Catechin gallate, isoquercitrin, myricetin, myricetin 3-*O*-rhamnopyranoside, quercetin 3-*O*-arabinoside, querectin 3-*O*-rhamnopyranoside, quercitrin, rutin	Leaves	Lebreton et al., 2002; Legssyer et al., 2004; Maleš et al., 2006; Sakar et al., 1992
	Iridoids	Monotropein, monotropein methyl ester, stilbericoside, unedoside	Leaves	Sakar et al., 1991
	Phenolic glucoside	Arbutin	Leaves	Sakar et al., 1991
	Triterpenes and sterols	Lupeol, pomolic acid, ursolic acid, α- amyrin, β-amyrin, β-sitosterol,	Fruits	Čirva et al., 1980; Grishkovets et al., 1979
A. pavarii				
	Acids and phenolic acids	Caffeic acid, chlorogenic acid, ferulic acid, gallic acid, quinic acid, rosmarinic acid, salicylic acid	Leaves	El Shibani, 2017, Elmhdwi et al., 2014 Hamad et al., 2011
	Anthocyanins	Delphinidin-3-*O*-rutinoside	Leaves	El Shibani, 2017
	Flavonoids	Catechin, dihydroquercetin, isoquercitrin, isovitexin 7-*O*-glucoside, kaempferol, myricetin, naringenin-7-*O*-glucoside, naringin, neodiosmin, quercetin, rutin	Leaves	El Shibani, 2017; Hamad et al., 2011
	Phenolic glucoside	Arbutin	Leaves	Hamad et al., 2011
A. unedo				
	Anthocyanins	Cyanidin-3-*O*-arabinoside, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, delphinidin-3-*O*-galactoside	Fruits	Fortalezas et al., 2010; Pallauf et al., 2008; Pawlowska et al., 2006
	Carbohydrates	Fructose, glucose, sucrose	Fruits	Ruiz-Rodriguez et al., 2011; Şeker et al., 2010
	Carotenoids	Lutein, zeaxanthin, β-carotene, β-cryptoxanthyne	Fruits	Alarcão-E-Silva et al., 2001; Barros et al., 2010; Pallauf et al., 2008
	Fatty acids	Linoleic acid, oleic acid, palmitic acid, α-linolenic acid	Fruits	Barros et al., 2010; Fonseca et al., 2015

Flavonoids	Catechin, gallocatechin, hyperoside, isoquercitrin, kaempferol, myricetin 3-*O*-xyloside, quercetin, quercetin 3-O-rhamnoside, quercetin 3-*O*-xyloside, quercitrin, rutin	Fruits	Maleš et al., 2006, 2010; Mazza et al., 1993; Pallauf et al., 2008
Flavonoids	Afzelin, isoquercitrin, kaempferol 3-*O*-arabinoside, quercetin, quercetin 3-*O*-arabinofuranoside	Leaves	Guendouze – Bouchefa et al., 2015; Maleš et al., 2006
Iridoids	Asperuloside, gardenoside, geniposide, stilbericoside, unedide, unedoside	Fruits	Davini et al., 1981; Karikas et al., 1993, 1987;
Phenolic acids	Ellagic acid, gallic acid, gentisic acid, *m*-anisic acid, *p*-hydroxybenzoic acid, protocatechuic acid, vanillic acid	Fruits	Ayaz et al., 2000; Maleš et al., 2006; Pallauf et al., 2008
Phenolic acids	Chlorogenic acid, ellagic acid, gallic acid, *p*-hydroxybenzoic acid, vanillic acid	Leaves	Guendouze – Bouchefa et al., 2015; Maleš et al., 2006, 2013
Phenolic glucoside	Arbutin	Leaves	Guendouze-Bouchefa et al., 2015; Kivçak et al., 2001a; Pavlović et al., 2009
Proanthocyanidins	Catechin-4,8-catechin, epicatechin-4,6-catechin, epicatechin-4,8-epicatechin, epicatechin-4,8-epicatechin- 4,8-catechin, epicatechin-4,8-epicatechin-4,8-epicatechin, gallocatechin 4,8-catechin	Fruits	Pallauf et al., 2008
Triterpenes and sterols	Betulinic acid, lupenone, lupeol, olean-12-en-3β,23-diol, oleanolic acid, ursolic acid, ursolic aldehyde, uvaol, α-amyrenone, α-amyrin, β-amyrin	Fruits	Fonseca et al., 2015
Volatiles	(*E*)-2-Decen-1-ol, (*E*)-2-hexenal, (*E*)-2-nonenal, (*E*)-2-pentanal, (*E*,*E*)-2,4-nonadienal, (*Z*)-2-heptenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenyl acetate, *(Z*)-3-hexenyl butanoate, 1-hexanol, 1-penten-3-ol, caryophyllene, eucaliptol, hexanal, hexyl acetate, limonene, nonanal, α-ionone, β-ionone	Fruits	Oliveira et al., 2011a

2.3.1. Fruits

Arbutus fruits represent a good source of phytochemicals good for health, such as carotenoids, phenols, and iridoids, and sugars, minerals, and vitamins.

Sugars. Fructose, glucose, and sucrose were identified in the ethanol extract of *A. unedo* fruits collected in Turkey (Şeker et al., 2010). Fructose was the most abundant sugar also in the aqueous fruits extract collected in Portugal. Generally, the amounts of fructose and glucose depend on the ripening stage, whereas sucrose content does not change during maturation (Alarcão-E-Silva et al., 2001). Ruiz-Rodriguez et al. (2011) described a variability in fructose (12.69-3.65%) and glucose (6.50-3.24%) content in hydroalcoholic extract of *A. unedo* fruits depending on the harvest years and location (center and west of Spain). Ripe *A. andrachne* fruits (aqueous extract) contained lower amounts of fructose (4.12%) and glucose (2.73%), in comparison to *A. unedo* fruits that showed a percentage of fructose and glucose of 24.09 and 19.09%, respectively (Serçe et al., 2010).

Minerals. A wide variability in the minerals composition was found in dependence to the site of collection (Aslantas et al., 2007; Şeker et al., 2010; Salem et al., 2018). For example, fruits collected in Croatia showed potassium (118.61 mg/100 g), calcium (36.05 mg/100 g), sodium (20.63 mg/100 g), magnesium (9.66 mg/100 g), iron (1.29 mg/100 g),

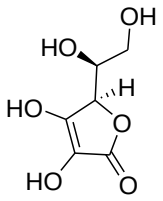
zinc (0.45 mg/100 g), and manganese (< 0.99 mg/100 g) as main minerals identified (Vidrih et al., 2013). Fruits collected in Turkey (North-eastern Anatolia region) had 2.602 mg/100 g of zinc, 12 mg/100 g of calcium, 119 mg/100 g of potassium, 9.1 mg/100 g of magnesium, 1.25 mg/100 g of iron, 0.088 mg/100 g of copper, and 0.197 mg/100 g of manganese (Aslantas et al., 2007).

A variability in the minerals content of *A. unedo* fruits was found also in the samples collected in two localities (center and west of Spain) characterised by different environmental conditions and in three years (Ruiz-Rodriguez et al., 2011). Values of 323.14-79.72 mg/100 g for potassium, 1.856-0.354 mg/100 g for iron, 104.12-40.54 mg/100 g for calcium, 9.94-4.33 mg/100 g for sodium, 45.85-9.56 mg/100 g for magnesium, 0.208-0.073 mg/100 g for copper, 0.178-0.038 mg/100 g for manganese, and 0.762-0.188 mg/100 g for zinc were found. The year of harvest mainly influenced the fruits content of potassium, magnesium, copper, iron, manganese, and zinc. The analysis of the literature revealed the presence of a less number of studies carried out on *A. andrachne* compared to *A. unedo*.

Volatiles. The analysis of volatiles of strawberry tree fruits at three different ripening stages allowed the identification of 41 compounds (10 alcohols, 10 aldehydes, 10 esters, 2 norisoprenoid derivates, 4 sesquiterpenes, and 5 monoterpenes) (Oliveira et al., 2011a).

(*Z*)-3-Hexen-1-ol and 1-hexanol are the main volatiles, followed by hexanal, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate. Generally, these volatiles decreased during ripening, maybe due to the lipoxygenase activity. Alcohols represent the major class of compounds identified in all maturation stages. Their content decreased as the maturation progresses. Aldehydes, the second major class of constituents, showed the highest content at the intermediate stage of maturation.

Vitamins. Both *A. unedo* and *A. andrachne* fruits are a rich source of ascorbic acid, (Figure 2.2; Alarcão-E-Silva et al., 2001; Oliveira et al., 2011b; Pallauf et al., 2008). fruits represent a good source of ascorbic acid (Şeker et al. 2010).



Ascorbic acid
(Vitamin C)

Figure 2.2. Ascorbic acid

***Carotenoids*.** In addition to *β*-carotene, lutein, zeaxanthin, and *β*-cryptoxanthyn are frequently reported in the ethanol and acetone-hexane extracts of *A. unedo* fruits (Figure 2.3) (Alarcão-E-Silva et al., 2001; Barros et al., 2010; Pallauf et al., 2008).

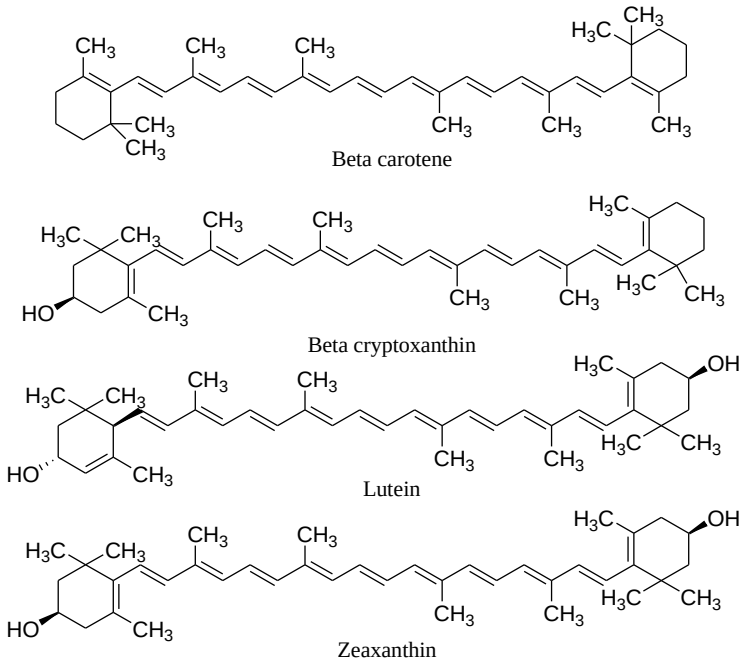


Figure 2.3. Carotenoids isolated from ethanol and acetone-hexane extracts of *A. unedo* fruits

Fatty acids. The analysis of literature data reported the fatty acids composition only of *A. unedo.* The dominant unsaturated fatty acids are *α*-linolenic, linoleic, and oleic acids. Palmitic acid is the most important saturated fatty acid identified in dichloromethane extract of strawberry tree fruits (Figure 2.4) (Barros et al., 2010; Fonseca et al., 2015). Fresh and dried fruits extracts have a high ω3/ω6 *ratio*, due to the content of *α*-linolenic as well as a good PUFA (polyunsaturated fatty acid)/SFA (saturated fatty acids) *ratio* (Barros et al., 2010; Oliveira et al., 2011b; Vidrih et al., 2013).

Differences in the content of these compounds based on the sites of collection were described. Fruits from Portugal showed *α*-linolenic acid (36.9-43.04%), linoleic acid (20.1-18.8%), and oleic acid (29.38-26.75%) as dominant compounds.

Ruiz-Rodriguez et al. (2011) investigated the petroleum ether extract of strawberry tree fruits from central and western Spain and found the same three fatty acids, namely α-linolenic acid (31.3%), linoleic acid (24.3%), and oleic acid (24.8%), as the most abundant compounds. *A. unedo* fresh fruits collected in Croatia have *α*-linolenic acid (34.8%), linoleic acid (31.3%), as main fatty acids followed by palmitic acid (19.0%) and oleic acid (14.9%) (Vidrih et al., 2013).

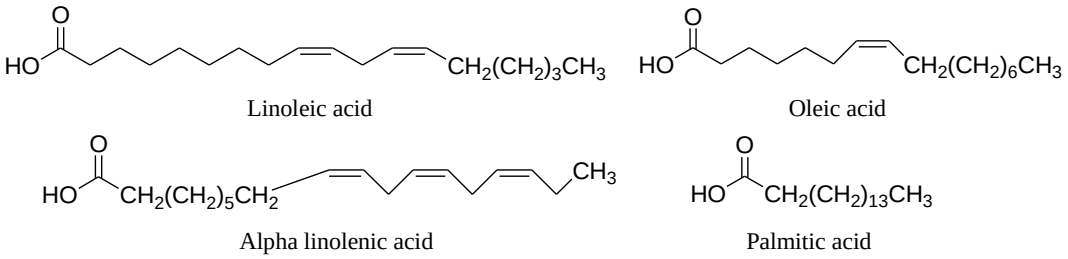
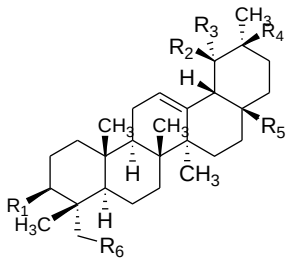
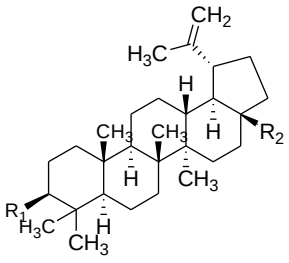


Figure 2.4. The main fatty acids identified from apolar extracts of *A. unedo*

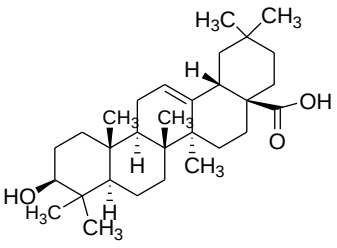
***Triterpenes and sterols*.** The triterpenes α- and β-amyrin, α-amyrenone, betulinic acid, lupeol, lupenone, ursolic aldehyde, ursolic acid, uvaol, oleanolic acid, and olean-12-en-3β,23-diol were identified in the dichloromethane extract of *A. unedo* fruits (Fonseca et al., 2015). Petroleum ether extract *of A. andrachne* fruits is characterised by β-sitosterol, and some triterpenols and triterpenoic acids such as α- and β-amyrin, lupeol, ursolic and pomolic acids (Čirva et al., 1980; Grishkovets et al., 1979). Figure 2.5 presents triterpenes and sterols isolated from *A. unedo* fruits.



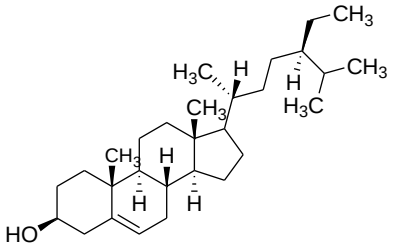


	R_1	R_2	R_3	R_4	R_5	R_6
α–Amyrenone	=O	CH_3	H	H	CH_3	H
α-Amyrin	OH	CH_3	H	H	CH_3	H
β-Amyrin	OH	H	H	CH_3	CH_3	H
Olean-12-ene-3-β,23 diol	OH	H	H	CH_3	CH_3	OH
Pomolic acid	OH	CH_3	OH	H	COOH	H
Ursolic acid	OH	CH_3	H	H	COOH	H
Ursolic aldehyde	OH	CH_3	H	H	CHO	H
Uvaol	OH	CH_3	H	H	CH_2OH	H

	R_1	R_2
Betulinic acid	OH	COOH
Lupenone	=O	CH_3
Lupeol	OH	CH_3



Oleanolic acid

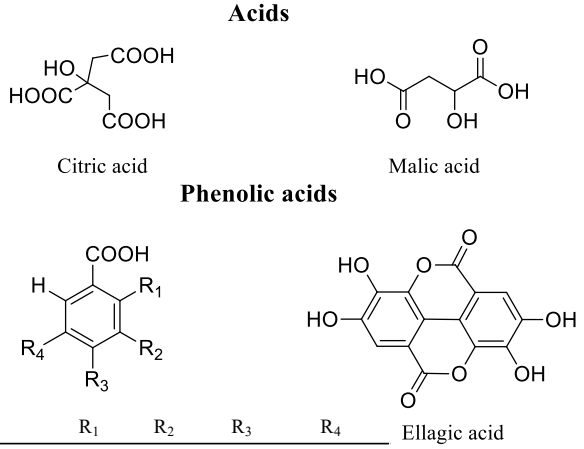


Beta-sitosterol

Figure 2.5. Triterpenes and sterols isolated from isolated from apolar extracts of *A. unedo* fruits

Acids and phenolic acids. Phytochemical studies revealed the presence of phenolic acids, ellagic acid, and gallic acid derivatives as the most abundant compounds identified in the *Arbutus* fruits (Ayaz et al., 2000; Alarcão-E-Silva et al., 2001; Barros et al., 2010; Ergun et al., 2014; Oliveira et al., 2011a; Pallauf et al., 2008; Pawlowska et al., 2006).

Some organic acids namely malic, ascorbic and citric acids (Serçe et al., 2010) characterised *A. andrachne* fruits aqueous extract. Gallic acid was the dominant phenolic compound, followed by protocatechuic acid, gentisic acid, *p*-hydroxybenzoic acid, vanillic acid, and *m*-anisic acid founded in the ethanol extract of *A. unedo* fruits (Ayaz et al., 2000). Acids and phenolic acids structures were summarised in Figure 2.6.



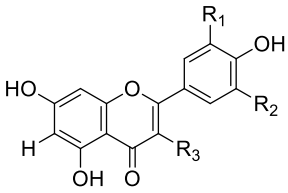
	R_1	R_2	R_3	R_4
m-Anisic acid	H	OCH_3	H	H
Gallic acid	H	OH	OH	OH
Gentisic acid	OH	H	H	OH
p-Hydroxybenzoic acid	H	H	OH	H
Protocatechuic acid	H	OH	OH	H
Vanillic acid	H	OCH_3	OH	H

Figure 2.6. Acids and phenolic acids isolated from polar extracts of *A. unedo* and *A. andrachne* fruits

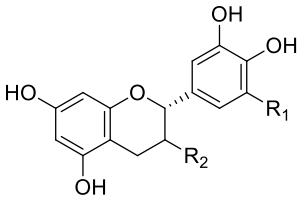
Flavonoids, proanthocyanidins, anthocyanins, and phenols. Flavonoids, proanthocyanidins, anthocyanins, and phenols are the main classes of compounds identified in *Arbutus* genus, particularly in the fruits of *A. unedo* and *A. andrachne* (Table 2.2 and Figure 2.7) (Ayaz et al., 2000; Alarcão-E-Silva et al., 2001; Barros et al., 2010; Bouzid et al., 2014; Ergun et al., 2014; Fortalezas et al., 2010; Mendes et al., 2011; Oliveira et al., 2011a; Pallauf et al., 2008; Pawlowska et al., 2006).

Analyses of the hydro-alcoholic and methanol extracts of *A. unedo* fruits lead to the identification of several flavonoids, including quercetin derivatives, kaempferol, proanthocyanidins, galactosilated and glucosilated form of delphinidin and cyanidin (Fortalezas et al., 2010; Maleš et al., 2006; Mazza et al., 1993; Pallauf et al., 2008; Pawlowska et al., 2006).

Flavonoids

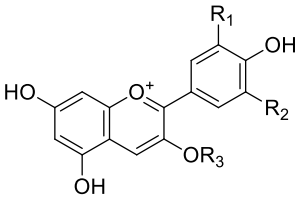


	R_1	R_2	R_3
Hyperoside	OH	H	*O*-Gal
Isoquercitrin	OH	H	*O*-Glc
Kaempferol	H	H	OH
Myricetin 3-*O*-xyloside	OH	OH	*O*-Xyl
Quercetin	OH	H	OH
Quercetin 3-*O*-rhamnopyranoside	OH	H	*O*-Rha
Quercetin 3-*O*-xyloside	OH	H	*O*-Xyl
Quercitrin	OH	H	*O*-Rha
Rutin	OH	H	*O*-Glc(6←1)Rha



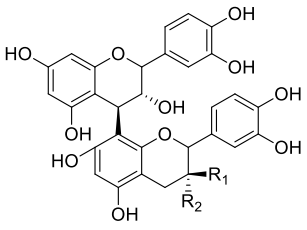
	R_1	R_2
Catechin	H	OH
Catechin gallate	H	OOC, OH, OH
Gallocatechin	OH	OH

Anthocyanins

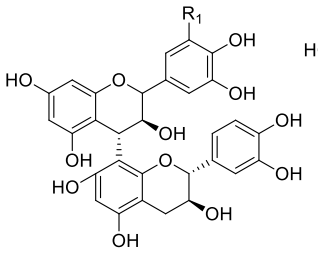


	R_1	R_2	R_3
Cyanidin 3-*O*-galactoside	OH	H	Gal
Cyanidin 3-*O*-glucoside	OH	H	Glc
Delphinidin 3-*O*-galactoside	OH	OH	Gal
Delphinidin 3-*O*-glucoside	OH	OH	Glc

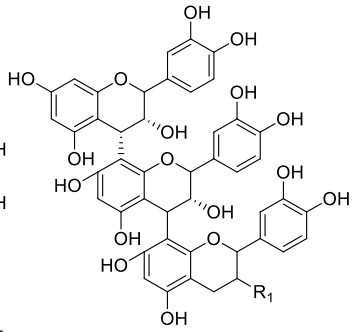
Proanthocyanidin



	R_1	R_2
Epicatechin-4,8-epicatechin	H	OH
Epicatechin-4,6-catechin	OH	H



	R_1
Catechin-4,8-catechin	H
Gallocatechin-4,8-catechin	OH

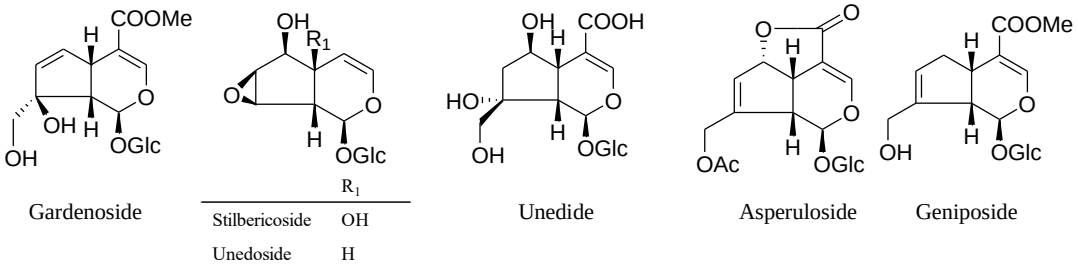


	R_1
Epicatechin-4,8-epicatechin-4,8-epicatechin	OH
Epicatechin-4,8-epicatechin-4,8-catechin	OH

Figure 2.7. Flavonoids, proanthocyanidins, anthocyanins, and phenols from polar extracts of *A. unedo* fruits.

Iridoids. The presence of iridoids are studied in the alcoholic fruits extract of *A. unedo* (Figure 2.8). Geniposide, asperuloside, gardenoside unedoside, unedide, and stilbericoside were the identified iridoids (Davini et al., 1981; Karikas et al., 1987, 1993).

Unedide and unedoside are rarely available in the plant kingdom. Nevertheless, unedide is the chemical taxonomic marker of the genus.



Gardenoside | Unedide | Asperuloside | Geniposide

Figure 2.8. Iridoids from alcoholic extracts of *A. unedo* fruits

2.3.2. Leaves

Phenols, triterpenes, and essential oils are the main classes of phytochemicals identified in the leaves of *Arbutus* species (Carcache-Blanco et al., 2006; Legssyer et al., 2004; Maleš et al., 2006, 2013; Orak et al., 2011; Tavares et al., 2010).

Essential oils. Several studies showed the main classes of phytochemicals identified in leaves essential oils of *Arbutus* species (Carcache-Blanco et al., 2006; Legssyer et al., 2004; Maleš et al., 2006, 2013; Orak et al., 2011; Tavares et al., 2010). The main volatile compounds that characterised *A. unedo* leaves collected in Italy and Spain are monoterpene hydrocarbons, oxygenated monoterpenes, and some aldehydes, particularly nonanal and decanal (Peñuelas et al., 2001). Linoleic acid, palmitic acid, and 2,6-di-tert-butyl-*p*-cresol were the main constituents of the essential oil of *A. unedo* leaves collected in Algeria (Bessah et al., 2012). A different chemical composition was reported for the essential oil of *A. unedo* leaves collected in West Anatolia (Turkey) (Kivçak et al., 2001c).

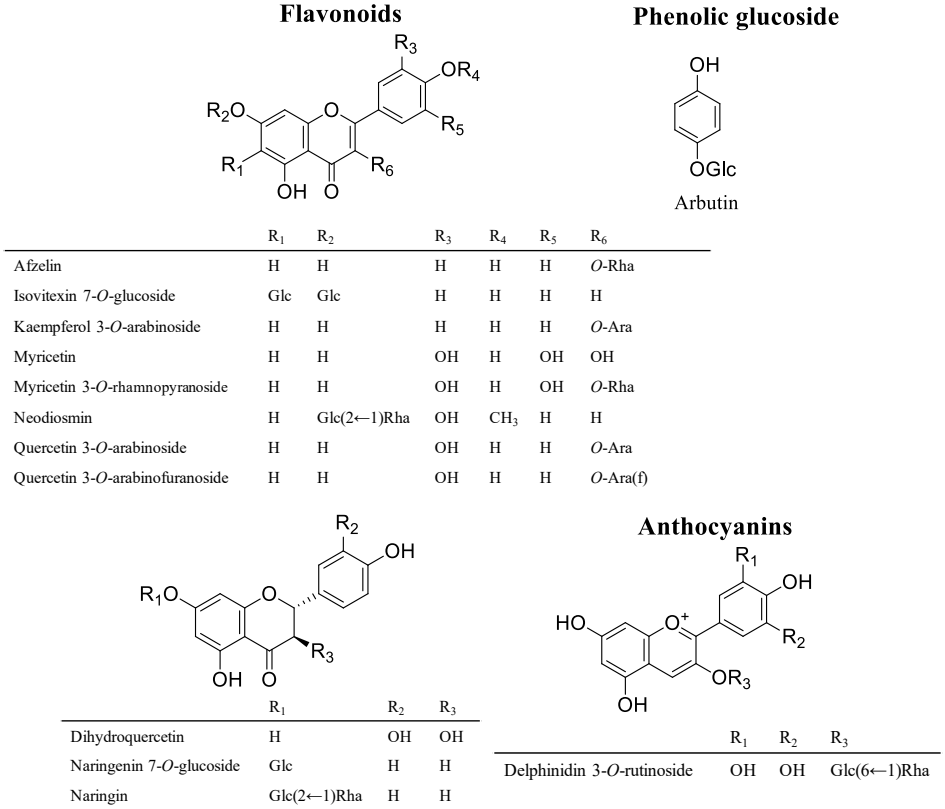
This oil showed (*E*)-2-decenal, (*E*)-2-undecenal, α-terpineol, and hexadecanoic acid as dominant compounds.

Flavonoids, anthocyanins and phenols. Data from literature revealed that generally the number of identified phenolic compounds in leaves is higher than that in fruits (Mendes et al., 2011). The decoction of *A. unedo* leaves revealed the presence of flavanols, flavonols and several galloyl and ellagic derivatives (arabinoside, xyloside, and rhamnoside). According to Maleš et al. (2006), methanol extract of *A. unedo* leaves contain tannins, and several flavonoids, including afzelin, juglanin (kaempferol-3-*O*-arabinoside), avicularin (quercetin-3-*O*-arabinofuranoside), quercetin, and isoquercetin (Ayaz et al., 2000; Maleš et al., 2006).

The phenolic glycoside arbutin was also reported in the ethanol extract of *A. unedo* leaves (Kivçak et al., 2001b; Pavlović et al., 2009), methanol extracts of *A. andrachne* and *A. pavarii* leaves (Hamad et al., 2011; Sakar et al., 1991).

Quercetin-3-*O*-rhamnopyranoside, myricetin-3-*O*-rhamnopyranoside, isoquercitrin, and quercetin-3-*O*-arabinoside were detected as the main flavonoids in the methanol extract of *A. andrachne* leaves (Sakar et al., 1992).

Some available studies reported the presence of major flavonoids as catechin, kaempferol, and naringin, followed by neodiosmin, isoquercitrin, myricetin, isovitexin 7-*O*-glucoside, naringenin 7-*O*-glucoside, quercetin, rutin, and dihydroquercetinin in the methanol extract of *A. pavarii* leaves (Elmhdwi et al., 2014; Hamad et al., 2011). One anthocyanin, delphinidin-3-*O*-rutinoside, was also identified in the methanol extract of *A. pavarii* leaves (El Shibani, 2017). Flavonoids, anthocyanins and phenols found in Arbutus genus leaves extracts were reported follow (Figure 2.9).



	R_1	R_2	R_3	R_4	R_5	R_6
Afzelin	H	H	H	H	H	*O*-Rha
Isovitexin 7-*O*-glucoside	Glc	Glc	H	H	H	H
Kaempferol 3-*O*-arabinoside	H	H	H	H	H	*O*-Ara
Myricetin	H	H	OH	H	OH	OH
Myricetin 3-*O*-rhamnopyranoside	H	H	OH	H	OH	*O*-Rha
Neodiosmin	H	Glc(2←1)Rha	OH	CH_3	H	H
Quercetin 3-*O*-arabinoside	H	H	OH	H	H	*O*-Ara
Quercetin 3-*O*-arabinofuranoside	H	H	OH	H	H	*O*-Ara(f)

	R_1	R_2	R_3
Dihydroquercetin	H	OH	OH
Naringenin 7-*O*-glucoside	Glc	H	H
Naringin	Glc(2←1)Rha	H	H

	R_1	R_2	R_3
Delphinidin 3-*O*-rutinoside	OH	OH	Glc(6←1)Rha

Figure 2.9. Flavonoids, anthocyanins and phenols from alcoholic extracts of *A. unedo* leaves

Acids and phenolics acids. The decoction of *A. unedo* leaves revealed also the presence of several galloyl and ellagic derivatives (arabinoside, xyloside, and rhamnoside). According to Maleš et al. (2006), *A. unedo* leaves also contain vanillic, syringic and chlorogenic acids (Figure 2.10). The methanol extract of *A. unedo* leaves was characterised by the presence of gallic, ellagic, and *p*-hydroxybenzoic acids

(Guendouze-Bouchefa et al. 2015; Pallauf et al., 2008). Some available studies reported the presence of ferulic acid, and gallic acid in the methanol extract of *A. pavarii* leaves (Elmhdwi et al., 2014; Hamad et al., 2011). Moreover, five phenolic acids such as rosmarinic, caffeic, gallic, salycilic, and chlorogenic acids and one carboxylic acid (quinic acid), were also identified in the methanol extract of *A. pavarii* leaves (El Shibani, 2017).

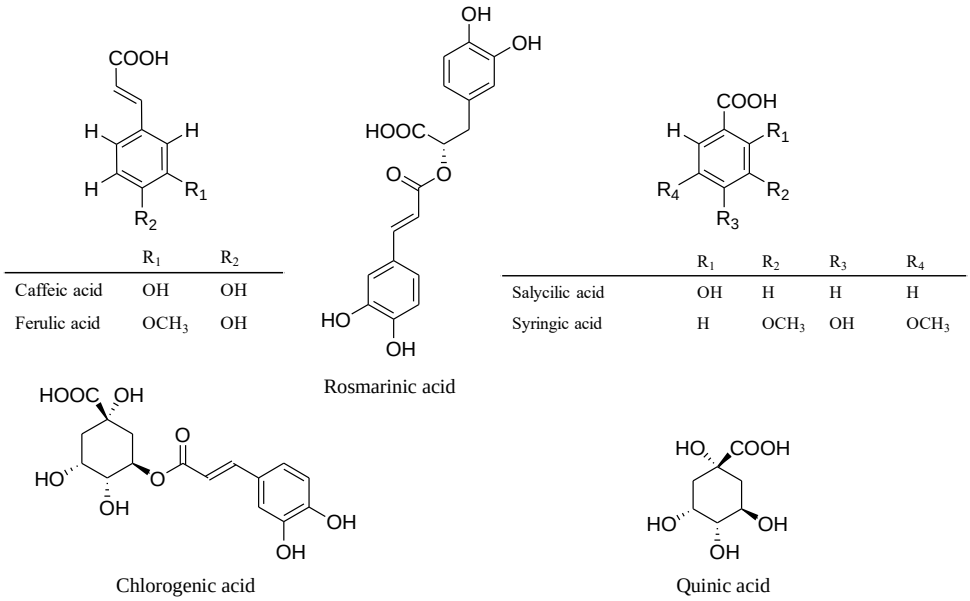
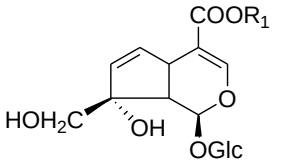


Figure 2.10. Phenolic acids from methanol extracts of *Arbutus* leaves

Iridoids. Sakar et al. (1991) identified monotropein, monotropein methyl ester, gardenoside, stilbericoside, and unedoside from methanol extracts of *A. andrachne* leaves (Sakar et al., 1991; Figure 2.11). From the chemotaxonomic point of view, all classes of compounds identified in *Arbutus* genus are present in the Ericaceae family. Iridoids with an aglycone with a C-10 skeleton are characteristic of this family of plants.

In the sub-family Arbutoideae (*Arbutus*, *Arctous*, *Arctostaphylos*, *Ornithostaphylos*, *Comarostaphylis*, and *Xylococcus* genera) only *Arctostaphylos* and *Arbutus* genera were investigated. Iridoids with a C-8 carbon skeleton are biosynthesized in both genera. This capacity is very rarely in the plant kingdom (Frederiksen et al., 1999). Unedoside is a very particular iridoid that characterises the subfamily of the Arbutoideae and it is found in the *Arbutus* genus (Kurkin et al., 2018).



	R_1
Monotropein	H
Monotropein methyl ester	CH_3

Figure 2.11. Iridoids from methanol extracts of *A. unedo* leaves

2.4. Biological properties

Arbutus species have been previously investigated for their biological properties mainly antitumor, anti-hypertensive, antimicrobial, hypoglycaemic, hypocholesterolemic, cardiovascular, anti-inflammatory, anti-diarrhoeal, antioxidant, and antidiabetic activities (Boulanouar et al., 2013; El Haouari et al., 2007; Fortalezas et al., 2010; Mendes et al., 2011; Oliveira et al., 2009; Oliveira et al., 2011a; Pabuçcuoğlu et al., 2003; Pallauf et al., 2008; Pavlović et al., 2011; Pawlowska et al., 2006; Ruiz-Rodriguez et al., 2011; Ziyyat et al., 1997).

2.4.1. Antioxidant activity

The antoxidant activity of extracts of *A. unedo* obtained from fruits, leaves and roots has been frequently studied in relation to *A. andrachne* and so far, few studies on extracts of *A. pavarii* have present.

A. unedo. The fruits of *A. unedo* demonstrated interesting antioxidant effects. This activity is influenced by various factors, including fruits ripening, geographical origin, extraction process, and processing (Akay et al., 2011; Barros et al., 2010; Bouyahya et al., 2016; Fortalezas et al., 2010; Isbilir et al., 2012; Oliveira et al., 2009, 2011a; Orak et al., 2011, 2012; Pabuçcuoğlu et al., 2003; Şeker et al., 2010).

Supercritical fluid extraction (SFE) of *A. unedo* fruits showed 99.9% DPPH radical scavenging activity at concentration of 250 μg/mL and total phenols content of 25.72 mg gallic acid equivalent (GAE)/g extract. These values resulted higher than those obtained by ethanol (15.12 mg/g; 95.8%) and conventional water (24.89 mg/g; 83.8%) extractions. In contrast, in the β-carotene bleaching test, the lipid peroxidation prevention was better by using *A. unedo* fruits water extract compared with ethanol and SFE fruits extracts. The phytochemical analysis of SFE extract revealed the presence of gallic acid as dominant phenolic acid, followed by gentisic acid, protocatechuic acid, *p*-hydroxybenzoic, vanillic, and *m*-anisic acids (Akay et al., 2011).

Isbilir et al. (2012) investigated the antioxidant potential of water, ethanol and methanol extracts of *A. unedo* fruits at different ripening stage (green, yellow, and red).

Methanol extract of red fruits exhibited the higher antioxidant capacity in all used assays (DPPH, β-carotene bleaching, FRAP, metal chelating capacity, superoxide anion scavenging) except in H_2O_2 scavenging activity test in which aqueous extract of green fruits were more active.

The antioxidant activity of ethanol extract of *A. unedo* fruits was influenced also by drying processes. Freeze-dried fruits showed higher DPPH radical scavenging capacity (IC_{50} value of 2.1 mg/mL) and prevented lipid peroxidation evaluated by β-carotene bleaching test (IC_{50} value of 0.3 mg/mL) than hot air-dried fruits (Orak et al., 2012). Probably, the high temperature used in hot air drying may be responsible to the degradation and/or loss of antioxidant compounds such as phenols and ascorbic acid (Demirsoy et al., 2007). Both Oliveira et al. (2009) and Orak et al. (2011) demonstrated that leaves methanol extract showed a more potent antioxidant activity than the ethanol and water extracts. On the other hand, Bouyahya et al. (2016) revealed that the leaves *n*-hexane extract showed a higher DPPH radicals scavenging activity (IC_{50} of 73.73 μg/mL) than ethyl acetate (IC_{50} of 276.15 μg/mL) and ethanol (IC_{50} of 280.50 μg/mL) extracts.

More recently, Erkekoglou et al. (2017) assessed the antioxidant effects of *A. unedo* leaves hot/cold infusion and decoction by different methods including ABTS, DPPH, crocin-bleaching, copper-reducing, and liposome accelerated oxidation assays. The protection of H_2O_2 induced oxidative stress in *S. cerevisiae* cells was also studied.

A higher phenolic content was obtained by decoction in comparison to hot and cold infusion. The same trend was observed for the total flavonoids content. Flavonols were the main abundant compounds in all extracts, with quercitrin accounting for ~20% of the total phenol amount. Decoction resulted the most active in DPPH radical scavenging activity. Alghazeer et al. (2016) studied the correlation between active compounds of *A. unedo* leaves and flowers and their biological properties. Flavonoids-rich extracts obtained by microwave assisted extraction by using methanol as solvent are characterised by a promising antioxidant activity. Leaves extracts were more active than flower extracts. A synergism of action was observed when the leaves flavonoids-rich extract was used in combination with the flowers extract (2:1).

The antioxidant effect of *A. unedo* leaves and fruits aqueous extracts was investigated by using different *in vitro* test including DPPH radical scavening ability, reducing power, and inhibitory effect on α,α'-azodiisobutyramidine dihydrochloride-induced hemolysis and lipid peroxidation in human erythrocytes (Mendes et al., 2011). *A. unedo* leaves extracts, characterised by the highest phenolic content (170.3 mg/g), showed a better antioxidant activity in all used assays than fruits extracts. Both extracts are able to protect the erythrocyte membrane from haemolysis with IC_{50} values of 0.430 and 0.062 mg/mL, for leaves and fruits extracts, respectively. Moreover, a reduction in malondialdehyde level, a breakdown product of lipid peroxidation, was observed (IC_{50} of 0.732 and 0.075 mg/mL, respectively) (Mendes et al., 2011).

The antioxidant effects of Morocco *A. unedo* roots and leaves aqueous extracts were investigated by using both *in vitro* (DPPH assay) and *in vivo* test (estimation of

malondialdehyde, MDA, and superoxide dismutase, SOD, levels in diabetic mice). Roots showed higher radical scavenging potential than leaves (IC_{50} values of of 4.52 and 7.24 µg/mL, respectively). No significant difference between *A. unedo* group and metformin group in MDA and SOD levels were recorded in liver and kidney tissues.

Generally, roots are characterised by a high presence of tannins, anthraquinones, terpenoids, and flavonoids.

The anti-radicals activity of several phenolic acids, including gallic, *p*-hydroxybenzoic, gentisic, protocatechuic, and vanillic acids, were determined by Karamać et al. (2005). Gallic and gentisic acids exhibited the highest DPPH radical scavenging activities with EC_{50} values of 0.0237 and 0.0292 μmol/assay, respectively, followed by protocatechuic and vanillic acids (EC_{50} values of 0.0574 and 14.37 µmol/assay, respectively). More recently, protocatechuic acid exhibited a concentration-dependently antioxidant activity. Comparing to Trolox, the relative antioxidant activity of protocatechuic acid (i.e. the ratio of IC_{50} (Trolox)/IC_{50} (protocatechuic acid) was calculated as 2.3, 2.7, 1.5, 2.8, 1.0, 3.7, 6.1, and 4.2 respectively, for ABTS, chelating ability (Fe^{2+}) and chelating ability (Cu^{2+}), DPPH, hydroxyl radical-scavenging, reducing power (Fe^{3+}), reducing power (Cu^{2+}), superoxide anion radical-scavenging activity. These data demonstrated that protocatechuic acid is more effective than Trolox in both aqueous and lipid media assays with a mechanism of action that involves scavenging free radicals *via* donating electron or hydrogen atom or chelating metal transition ions (Li et al., 2011).

The different radical-scavenging activities of phenolic acids depend on the number of hydroxyl moieties linked to the aromatic ring. Gallic acid, with three hydroxyl groups, resulted one of the most active followed by gentisic and protocatechuic acids characterised by the dihydroxylation of the aromatic ring (Brand-Williams et al., 1995).

Joshi et al. (2012), using isolated rat liver mitochondria and the human erythrocytes models, recently confirmed the antioxidant activity of gentisic acid. This acid efficiently scavenged both hydroxyl and organohaloperoxyl radicals in with a mechanism of action in which its phenoxyl group is involved.

The strawberry tree honey showed higher DPPH activity (3.34 mmol Trolox equiv/kg) compared to other unifloral honeys, as mint, thyme, sunflower, rapeseed, sage, acacia. Phenols are the main contributor to the antioxidant properties of honey, as indicated by high significant correlations between the antioxidant activity and total phenol content (Tariba Lovaković et al., 2018). One of the most important *A. unedo* phenols is homogentisic acid that showed interesting antioxidant and antiradical activities by different mechanism of action (Rosa et al., 2011).

A. andrachne. Abidi et al. (2016a) investigated the antioxidant potential of *A. andrachne* bark roots extracts. A promising DPPH radical scavenging potential was obtained with ethyl-acetate extract. A lower metal chelating activity in comparison to EDTA was observed with investigated samples. Recently, the antioxidant activity (evaluated by DPPH and FRAP tests) of methanol extract of *A. andrachne* fruits and flowers were investigated (Saral et al., 2017).

Flowers, characterised by higher contents of both phenols and flavonoids, exhibited a better ferric reducing ability power (104.81 μmol $FeSO_4 \cdot 7H_2O$/g dry sample) than fruits. Similarly, Okmen (2015) showed a higher ABTS radical scavenging activity of methanol extract of *A. andrachne* flowers than leaves extract. Analysing the effect of ripening stages on the antioxidant activity of *A. andrachne* fruits (Özgen et al., 2009), results evidenced that at fully ripeness stage fruits are characterised by the highest total phenolic content (3.90 mg GA/kg fresh weight) and antioxidant activity (FRAP value of 21.8 μmol TE/g fresh weight).

2.4.2. Anti-inflammatory activity

Only *A. unedo* has been evaluated for its anti-inflammatory action. The aqueous extract of *A. unedo* leaves exerted effects on nitric oxide synthase and intracellular adhesion molecule-(ICAM)-1 with additional effect on IFN-γ-elicited STAT1 activation and interleukin (IL)-6-elicited signal transducer and activator of transcription 3 (STAT3) activation (Mariotto et al., 2008). The extract down-regulated the activation of STAT3 with concomitant attenuation of parameters associated with inflammation such as TNF-α, IL-1β and IL-6 production, iNOS expression, cyclooxygenase-2 (COX-2), ICAM-1 expression, neutrophil infiltration, and prostaglandin E2 and nitrite/nitrate level.

Moualek et al. (2016) evidenced a good protective effect of human red blood cells membrane against haemolysis process. The anti-inflammatory activity of *A. unedo* is related to the ability of the phytocomplex to edit the calcium influx in erythrocytes, demonstrating cytoprotective properties on these last. It is known that intracellular content of calcium is closely linked to the deformability and cell volumes of erythrocytes. (Chopade et al., 2012). The inhibition of release of lysosomal content at the site of inflammation was also demonstrated by using *A. unedo* leaves aqueous extract (Govindappa et al., 2011). Carcache-Blanco et al. (2006) reported the COX-2 inhibitory activity of the *A. unedo* methanol extract (from entire plant). Bioactive fractionation process lead to the isolation of α-amyrin acetate, lupeol, and betulinic acid that showed IC_{50} values of 10.2, 11.1 and 11.4 μg/mL, respectively. The anti-inflammatory activity of betulinic acid could be ascribed to the reduction in TNF-α production induced by LPS without any effect on the IL-6 production (Costa et al., 2014). Moreover, betulinic acid inhibited the production of pro-inflammatory mediators by LPS-stimulated macrophages and promoted a significant increase in IL-6 and IL-10 production through modulation of nuclear factor kB (NF-kB) in human peripheral blood mononuclear cells (hPBMCs) (Viji et al., 2010). Successively, the same research group demonstrated that betulinic acid inhibited prostaglandin E2 production and LPS-induced COX-2 protein expression. This triterpene was able also to attenuate LPS-induced ERK and Akt phosphorylation without influencing p38. Other influenced pathway related to inflammation are the blockage LPS-induced IkBa phosphorylation and the LPS-induced

reactive oxygen species formation with consequent lactate dehydrogenase release. Among identified compounds, α-amyrin showed anti-inflammatory activity.

Vitor et al., (2009) reported that this triterpene had a comparable activity to the largely prescribed drug dexamethasone in reversing the macroscopic and microscopic outcomes of trinitrobenzene sulphonic acid-induced colitis, used as model of inflammation. The restoration of cytokine balance with reduction of interleukin IL-1β level and restore IL-10 level were detected.

Moreover, this triterpene reduced the expression of vascular endothelial growth factor, COX-2, phospho-NF-κB and phospho-CREB. Additionally, α-amyrin inhibited 40% paw oedema after 5 hours. Moreover, it inhibited heat-induced haemolysis better than the largely prescribed drug diclofenac and at the dose of 100 mg/kg (p.o.) evoked a reduction of 60.3% in total leucocyte count and suppression (47.9%) of neutrophil infiltration (Okoye et al., 2014).

2.4.3. Hypoglycaemic activity

Diabetes mellitus (DM), a group of metabolic diseases characterised by hyperglycaemia associated with long-term damage and dysfunction of various organs, is one of the most common chronic diseases worldwide. Traditional plants remedies continue to be a potential adjunct therapy for the control of glycaemia. The hypoglycaemic activity of *A. unedo* roots, of *A. pavarii* aerial parts as well as of *A. andrachne* whole plant have been studied.

A. unedo. *A. unedo* has been used to treat several diseases including diabetes (Table 2.1). The *in vitro* and *in vivo* hypoglycaemic potential of *A. unedo* roots aqueous extract was recently studied (Mrabti et al., 2018). In particular, hypoglycaemic properties were analysed *in vitro* by using α-amylase and α-glucosidase inhibitory assays and *in vivo* by using streptozotocin (STZ)-nicotinamide induced diabetic mice. The extract inhibited both α-amylase and α-glucosidase with IC_{50} values of 730.15 and 94.81 μg/mL, respectively. Moreover, a remarkable decrease in blood glucose level in diabetic mice was found. Interestingly, the histopathologic analysis of pancreas of the diabetic mice treated with *A. unedo* revealed a restoration of pancreatic islet cell architecture.

Previously, the same extract was tested by using Oral Glucose Tolerance Test (OGTT) and Intravenous Glucose Tolerance Test (IVGTT) (Bnouham et al., 2007). In the OGTT, the extract significantly reduced the glycaemia after glucose loading; this action is not confirmed in the IVGTT. The hypoglycaemic effect is related to the modulation of peripheral glucose utilization. The same research group (Bnouham et al., 2010) tested *A. unedo* roots water extract in streptozotocin induced-diabetic rats and demonstrated a plasma glucose lowering effect comparable to the tolbutamide.

The phytochemical investigation of this extract revealed the presence of epicatechin, catechin, and gallocatechin as main constituents (Legssyer et al. 2004). These phenols are known for their anti-hyperglycaemic activity. The administration of 30 mg/kg (i.p.) of epicatechin twice daily for 4-5 days in alloxan induced diabetic albino rats normalize the

glycaemic parameter. A regeneration of the β-cell population in pancreas was evidenced by histological studies (Chakravarthy et al., 1982). Epicatechin was also able to inhibit the carbohydrates-hydrolysing enzymes α-amylase and α-glucosidase with equal and much higher potency, respectively, than acarbose.

A. pavarii. More recently, El Shibani (2017) evidenced the α-glucosidase inhibitory activity of *A. pavarii* aerial parts extract. Oral administration of *A. pavarii* methanol extract showed a potent reduction on fasting blood glucose level in STZ-induced diabetic rats. This action is probably due to the increase levels of insulin.

A. andrachne. The action against carbohydrate hydrolysing enzyme was demonstrated also by the use of the ethanol extract of the whole plant of *A. andrachne*. In fact, this extract showed an IC_{50} value of 0.44 mg/mL against α-amylase. This action was confirmed *in vivo* with an improvement in blood glucose profile (maximum rise 65%) as compared to acarbose (25%) and the control group (88%). The maximum hypoglycaemic effect was observed within the first hour after sucrose loading and appeared to be extended over at least 5 h (Hamdan et al., 2008).

2.4.4. Antibacterial and antifungal activity

Fruits, leaves, and roots of *Arbutus* species were studied for their potential antimicrobial activity.

***A. unedo*.** The essential oil of *A. unedo* fruits showed a moderate antibacterial activity against *Listeria monocitogenes* and *Enterococcus faecalis* (Kahriman et al., 2010). The most promising activity of aqueous, ethanol and methanol extracts of *A. unedo* leaves was found mainly against Gram-positive bacteria (El Ouarti et al., 2012; Orak et al., 2011), *Helicobacter pylori* and *Klebsiella pneumonia* (Ferreira et al., 2012). Salem et al. (2018) described the disk diffusion method the antimicrobial activity of ethanolic extract of fruits. They showed an intense antimicrobial activity against *Staphylococcus aureus, Bacillus subtilis* and *Pseudomona aeruginosa*, a moderate activity against *Salmonella typhimurium, Escherichia coli, Enterococcus feacium* and *Candida albicans*, and a feeble activity against *Streptococcus* strain.

Bouyahya et al. (2016) studied the antibacterial activity of the *n*-hexane extract of *A. unedo* leaves. This study demonstrated the highest antibacterial activity against *S. aureus* and *L. monocytogenes* while a moderate activity against *P. aeruginosa* and *Escherichia coli* was reported. Antifungal effects of methanol extract of *A. unedo* leaves extracts against *Aspergillus parasiticus* NRRL 2999 and NRRL 465 (Orak et al., 2011), *Candida tropicalis* (Ferreira et al., 2012), and intracellular anti-mycobacterial activity without toxic effect on macrophages (ethanol extract) (El Ouarti et al., 2012) were described.

In vitro anti-leishmanial activity (ethanol extract) (Kivçak et al., 2009), and *in vitro* activity against *Trichomonas vaginalis trophozoites* (ethyl acetate extract) (Ertabaklar et al., 2009) were also reported. Moreover, *A. unedo* leaves ethanol extract inhibited the growth of Gram-positive, Gram-negative (*E. coli, S. aureus, S. epidermidis, S.*

Typhimurium, Enterobacter cloacae and *E. faecalis*), as well as the yeast *C. albicans* (Kivçak et al., 2001a).

The aqueous extracts of *A. unedo* roots had antibacterial activity on *E. coli* comparable to that of the positive control piperacilline (Simonetti et al., 2008) whereas aqueous extract and phenolic fractions showed a moderate antibacterial activity against *E. coli* and *S. aureus* (Dib et al., 2013). The hydroalcoholic extracts of *A. unedo* fruits possessed high antifungal activity against *Aspergillus ochraceus, Aspergillus flavus* and *Cladosporium cladosporioides* (Takwa et al., 2018). A positive correlation between catechin and growth fungal was found.

A. andrachne. Abidi et al. (2016b) showed the antibacterial effects of the methanol extracts from the *A. andrachne* bark of the roots, particularly against Gram-positive bacteria. *Acne vulgaris* is one of the most common skin diseases in which the *Propionibacterium acnes* plays a role in its development when it overgrows in the pilosebaceous unit. Methanol extracts of leaves, flowers, and bark of *A. andrachne* were investigated in order to test their potential use for the treatment of acne (Amro et al., 2013). Minimum inhibitory concentration (MIC) values of 5 and 15 mg/mL for leaves and bark, respectively, were found against *P. acnes*. Flowers were not active. An interesting activity for the methanol extract of *A. andrachne* leaves and flowers against Gram-positive and Gram-negative bacteria but not against yeasts such as *Candida albicans* was reported (Ergun et al., 2014; Kivçak et al., 2001a).

A. pavarii. Habibi et al. (2015) studied the antibacterial activity of the ethanol extract of *A. pavarii* aerial parts by disc diffusion method. This extract was active against *E. coli* and *P. aeruginosa* with values of inhibition zones of 9.0 and 8.0 mm, respectively. In another work, Alsabri et al. (2013) investigated the antibacterial properties of *A. pavarii n*-hexane, chloroform, and methanol extracts, and reported that methanol extract exhibited the highest activity against *S. aureus, E. coli* and *C. albicans*. The chloroform extract was active only against *S. aureus*, while the *n*-hexane extract showed activity against *C. albicans*. Overall, these results indicated that the polarity of the solvent plays an important role in the extraction of the active ingredients and consequently in their potential antimicrobial activity.

2.4.5. Anti-proliferative activity

Only *A.unedo* and *A. andrachne* polar and apolar extracts were investigated for their potential cytotoxic activity. The chemical profile of *A. unedo* honey from different Sardinian (Italy) origins and its cytotoxic activity against human colon adenocarcinoma (HCT-116) and metastatic (LoVo) cell lines were studied (Afrin et al., 2017). IC_{50} values of 8.76 and 19.88 mg/mL after 72 h of exposure were found against HCT-116 and LoVo cells, respectively. *A. unedo* honey significantly triggered intercellular ROS accumulation in HCT-116 and LoVo cells in a concentration and time dependent manner. It is well know that remodelling of the extracellular matrix is required for cancer cell invasion. The

primary response for the degradation of extracellular matrix components is the activation of matrix metalloproteinases (MMPs).

Although MMPs are expressed in normal tissue in remodelling conditions, the abnormal expression of several MMPs has been related to pathological conditions, including cancer cell invasion and metastasis. MMP inhibitors have the capacity to suppress angiogenesis in different models (Alaseem et al., 2017).

Tavares et al. (2010) obtained hydro-alcoholic extracts from *A. unedo* fruits and leaves. These extracts were fractionated to obtain polyphenol-enriched fractions. Both crude extracts and enriched fractions were assessed to investigate the MMP-9 inhibitory activity. Fruits and leaves polyphenol-enriched fractions showed IC_{50} values similar to the ones obtained for positive controls used in this work such as blackberry and green tea. Leaves have the highest inhibitory activity with an IC_{50} value of 1.31 μg/mL.

Gallic acid derivatives are the main constituents of both fruits and leaves. Gallic acid and its analogues demonstrated a wide variety of biological activities including anticancer (Fiuza et al., 2004). Two studies (Guimarães et al., 2014; Schaffer et al., 2005) demonstrated that ethanol extracts of *A. unedo* fruits inhibited DNA synthesis and cellular proliferation in different cancer cell lines. Guimarães et al., (2014) investigated the potential anti-proliferative activity of anthocyanins-enriched extract (AE) and non-anthocyanins phenolic compounds enriched extract (PE) of *A. unedo*, *Prunus spinosa*, *R. canina*, and *Rosa micrantha*, against five human cancer cell lines namely MCF-7 (breast adenocarcinoma), HepG2 (hepatocellular carcinoma), HCT-15 (colon carcinoma), NCI-H460 (non-small cell lung cancer), and HeLa (cervical carcinoma). The most active sample was *A. unedo* PE extract against NCI-H460 and HCT-15 cell lines with GI_{50} values of 37.68 and 93.36 μg/mL, respectively. This activity could be correlated to the presence of galloyl derivatives that are exclusively found in this species in comparison to the other investigated species.

***A. andrachne*.** Abu-Dahab et al. (2007) investigated the cytotoxicity of *A. andrachne* leaves and stems against MCF-7 cell lines. Leaves and stems ethanol extracts showed a survival percentage of 103.48 and 111.05%, respectively. The anti-proliferative activity of methanol, chloroform, and *n*-hexane extracts was studied against breast adenocarcinoma (MCF7) and lung carcinoma (A549) cell lines (Alsabri et al., 2013). Both chloroform and methanol extracts significantly inhibited the proliferation of MCF7 cells with IC_{50} values of 14.96 and 29.58 μg/mL, respectively. A lower activity was found for the *n*-hexane extract with an IC_{50} value of 132.97 μg/mL. Interestingly, the chloroform extract was active against A549 cells with an IC_{50} value of 19.78 μg/mL.

2.4.6. Other biological properties

According to some authors, *A. unedo* leaves extracts could be useful for the treatment and/or prevention of cardiovascular diseases. They are able to reduce store-operated Ca^{2+}

entry induced by thrombin or to determine a selective depletion of the two Ca^{2+} stores in platelets, the dense tubular system and the acidic stores.

Aqueous, ethyl acetate, and diethyl ether extracts of strawberry tree leaves were also able to reduce both basal and thrombin-stimulated protein tyrosine phosphorylation (El Haouari et al., 2007). Some cardiovascular diseases such as hypertension are linked to an increased blood platelet activity. The aqueous extract of *A. unedo* leaves showed an inhibition of thrombin-induced platelet aggregation with an IC_{50} value of 1.8 mg/mL (Mekhifi et al., 2004). From this extract, tannins were isolated and tested. These constituents exhibited a strong antiplatelet activity. The obtained results support the use in traditional medicine of this plant in the treatment of hypertension.

The treatment of male Wistar rats with the aqueous extract of *A. unedo* roots reduced hypertension development, prevented the myocardial hypertrophy and ameliorated vascular reactivity and renal functional parameters caused by L-NG-nitroarginine methyl ester (L-NAME) (Afkir et al., 2008). An improved sensitivity of the arterial baroreceptor controlling the heart rate and acute increases of arterial pressure was also reported.

The effects on the cardiodynamics of isolated perfused rabbit hearts of *A. andrachne* methanol extract of leaves, fruits, and roots were recently studied (Abidi et al., 2016b).

Left ventricular pressure decreases by 32% with root extracts. No significant effect was observed by the tested extracts on the heart rate. The methanol extracts of leaves, fruits, and roots did not show any significant effect on the coronary flow. Moreover, the roots increased the coronary flow at a concentration of 1 and 2 mg/mL during 1 min. Electrolysis on heart tissue treated with the roots extracts decreased the MDA level from 70.51 to 48.58 nmol/g of tissue. Moreover, roots extract possessed antihypertensive effect. This last activity may be due to ability to decrease the left ventricular pressure, to do a protective role against free radical generation and the capacity to decrease the MDA level of heart tissue.

The ethanol extracts of *A. unedo* leaves collected in Greece and Montenegro were studied for their potential spasmolytic activity in rat ileum (Pavlović et al., 2011). Both extracts produced a decrease in the tone of ileal spontaneous contractions in a concentration-dependent manner. This activity, attributed to the relative high contents of phenols, tannins, arbutin, and flavonoids, probably is mediated *via* the inhibition of calcium channels.

A. unedo leaves aqueous extract possessed a vasorelaxant activity (Legssyer et al., 2004). The extract was tested on pre-contracted rat aortic rings. An endothelium-dependent relaxation of 66% was observed at a concentration of 10^{-2} mg/mL. Based on these results, the leaves were extracted successively with different solvents of increasing polarity. Methanol resulted the most active (87%). The removal of tannins from the methanol extract causes a reduction in activity (42%).

This result evidenced that the vasorelaxant activity of *A. unedo* leaves is related to polyphenols, primarily condensed tannins and catechin gallate. In a recent study, the methanol extract of the leaves of *A. pavarii* significantly decreased the level of cholesterol

in rats with hypercholesterolemia (Elmhdwi et al., 2014). This effect is due to the presence of flavonoids and phenolic compounds (Park et al., 2002). It was demonstrated that flavonoids are able to decrease low-density lypoproteins (LDL)-cholesterol and to increase high-density lypoproteins (HDL)-cholesterol. The increase of HDL concentration could protect LDL against oxidation *in vivo* (Hermansen et al., 2003).

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Chapter 3
European *Vaccinium* species

3.1. Introduction

Vaccinium L. (Ericaceae) is a genus of about 450 species of shrubs, widely found throughout the Northern hemisphere and extending south along tropical mountain ranges, especially in Malesia. The shrubs are erect or creeping, with alternate deciduous or evergreen leaves. The small flowers resemble those of the true heaths (Erica), are single, clustered, or in long spikes in the leaf axil (Kloet, 1990).

The European flora comprises *V. corymbosum*, *V. oxycoccos, V. microcarpum, V. macrocarpon, V. vitis-idaea, V. uliginosum, V. myrtillus, V. arctostaphylos*, and *V. cylindraceum.* Deciduous or evergreen dwarf shrubs, shurbs or small trees characterise the genus, and the fruits of each variety are edible.

V. corymbosum (highbush blueberry; Figure 3.1) was imported by North America, and now is cultivated in Europe for its edible fruits (Tutin et al., 1972). According to "The Plant List" *V. corymbosum* is the accepted name of the plant with three homotypic synonyms, *Cyanococcus corymbosus* (L.) Rydb., *V. corymbosum f. corymbosum, V. corymbosum var. corymbosum* (The Plant List, 2013).

Figure 3.1. *Vaccinium corymbosum* L. (adapted by Encyclopedia of Life)

V. myrtillus (bilberry) is a woody dwarf shrub, present in the forest of Northern Hemisphere. For its growth need to acid and well-drained soils and it is considered an indicator of biodiversity of forest for its abundance (Featherstone, 2002).

V. oxycoccos is an evergreen shrub. The fruit is an over-wintering berry (cranberry), globose, red (rarely white). Native, on peat bogs, usually in the wetter parts, such as north and central Europe, extending locally southwards to south-central France, north Italy and southeaster Russia (Tutin et al., 1972; Vander Kloet, 1983).

V. microcarpum is very closely related to *V. oxycoccos,* but it is sometimes considered as a subspecies or its variety (Vander Kloet, 1983). Its leaves are smaller,

often widest near the base, triangular-ovate. The fruits are lemon-shaped, ellipsoid or pyriform. *V. microcarpum* is present in north and northcentral Europe, extending southwards to northwest Ukraine; alps; carpathians (Tutin et al., 1972). *V. vitis-idaea* (lingonberry) is short evergreen shrub found in North and Central Europe (Tutin et al., 1972). Its ideal habitat is moors, heaths, coniferous wood, subalpine pastures and tundra. Fruits are more acid comparing with other berries of *Vaccinium* genus. *V. uliginosum* (bog whortleberry or bog bilberry) is a deciduous shrub and the fruits are bluish-black, with sweet taste. It is native in acid upland, heaths and bogs; rarely on calcareous soils, but it has been described as the only form of the species, which found in arctic and subarctic Europe and in all circumboreal regions. It is common in North Europe and Asia from Iceland to Japan and in the high mountains to the Sierra Nevada, Spain, northern Apennines, Albania and Bulgaria, Caucasus and Altai (Tutin et al., 1972). *V. arctostaphylos* (Caucasian whortleberry) is a deciduous shrub or small tree with the fruits purplish-black, present in Tuekey and southeaster of Bulgaria (Tutin et al., 1972).

V. corymbosum (highbush blueberry) is a deciduous shrub with blue berries grown in extreme north America, is cultivated locally in west and central Europe fot its big edible fruits (Tutin et al., 1972). The fruits are big (6.4 to 12.7 mm) if compared with other berries of *Vaccinium* species (~ 6-10 mm). The favourite soil for their grown are acid with pH compris between 4.8 and 5.2, organically rich and well drained. The roots need constant moisture, because they are shallow (Tutin et al., 1972). In this chapter, the most recent studies on chemical profile and and bioactivity of the genus, particularly of *V. corymbosum*, were reported.

3.2. Traditional uses

Several traditional uses are described for *Vaccinium* species in the world. The fruits of *V. myrtillus* are used in Europe for the treatment of stomatitis, renal stones, intestinal and liver disorders, as remedy of fevers and coughs, and for their astringent, tonic, and antiseptic properties (Kemper, 1999; Morazzoni et al., 1996). The decoction and infusion of leaves are used in southeastern Europe to treat diabetes (Frohne, 1990).

The juice of fresh fruits of *V. myrtillus* were utilised in Macedonia and Kosovo, as anti-anemic, to treat digestive and urinary tract infections and disorders, eye inflammations, hepatitis, while the infusions of leaves and fruits as lithontriptic, anti-anemic and for the respiratory inflammations (Mustafa et al., 2015). In northern Europe, *V. vitis-idaea* berries are collected for the preparation jams and sauces (Wang et al., 2005).

The fruits of *V. vitis-idaea* are effective in traditional medicine of Cree to treat frequent urination, sore eyes, abscesses, toothache, thrush and snow blind-ness (Leduc et al., 2006). In the Alaska Natives, berries are also used to treat colds, coughs and sore throats (Kari, 1985). From ancient times, stems and leaves of *V. vitis-idaea* presented anti-inflammatory properties and are noted for treat respiratory system infections in Chinese traditional medicine (Standard for the Plant Drug of Heilongjiang Province, 2001).

In Macedonia and Kosovo, leaves infusion of *V. vitis-idaea* was used for its anti-rheumatic and anti-inflammatory properties, while fruits infusion was useful to treat infections of urinary tract. Both fruits and leaves were also used as diuretic, anti-rheumatic, antipyretic, anti-diabetic, and anti-convulsant agents (Mustafa et al., 2015).

V. arctostaphylos leaves and fruits have been utilised as anti-hypertensive and anti-diabetic agents in the Iranian folk medicine (Mozaffarian, 2013).

In Quebec traditional medicine, *V. corymbosum* fruits have been mainly used to treat diabetes, but also know for the strong antioxidant and anti-inflammatory properties (Pervin et al., 2013, 2016). Highbush blueberries also have a rich source of dietary fibers, which have potential metabolic effects in the gastrointestinal tract (Branning et al., 2009). The ethnomedicinal uses of *Vaccinium* genus are listed in Table 3.1.

Table 3.1. Traditional use of *Vaccinium* genus

Species	Traditional use	Part used	Reference
V. myrtillus	Antiseptic, astringent, tonic	Fruits	Morazzoni et al., 1996
	To treat fevers and coughs,	Fruits	Kemper, 1999
	To treat intestinal and liver disorders	Fruits	Kemper, 1999
	Antidiabetic and antinflammatory	Leaves	Frohne, 1990; Morazzoni et al., 1996
	To treat eye inflammation and hepatitis	Fruits	Mustafa et al., 2015
	To treat digestive and urinary tract disorders	Fruits	Mustafa et al., 2015
	To treat respiratory inflammations and kidney stones	Leaves and fruits	Mustafa et al., 2015
	Anti-anemic	Leaves and fruits	Mustafa et al., 2015
V. vitis idaea	To treat frequent urination, sore eyes, abscesses, toothache, thrush and snow blind-ness	Fruits	Leduc et al., 2006
	To treat colds, coughs and sore throats	Fruits	Kari, 1985
	Antinflammatory	Stems and leaves	Standard for the Plant Drug of Heilongjiang Province, 2001
	To treat respiratory system infections	Stems and leaves	Standard for the Plant Drug of Heilongjiang Province, 2001
	Anti-rheumatic and antinflammatory	Leaves	Mustafa et al., 2015
	To treat infections of urinary tract and kidney stones	Fruits	Mustafa et al., 2015
	Anti-rheumatic, antipyretic, anti-diabetic, anti-convulsant, diuretic and for the wound healing	Leaves and fruits	Mustafa et al., 2015
V. arctostaphylos	Anti-hypertensive and anti-diabetic	Leaves and fruits	Mozaffarian, 2013
V. corymbosum	Antidiabetic, antioxidant and anti-inflammatory	Fruits	Pervin et al., 2013, 2016
	To treat gastrointestinal disorders	Fruits	Branning et al., 2009

3.3. Chemical constituents of fruits

Berries are an excellent source of health-promoting compounds such as anthocyanins, flavonoids, and phenolic acids (mainly hydroxycinnamic acids) (Gu et al., 2002; Howard et al., 2007; Taruscio et al., 2004; Wu et al., 2012).

Previous studies showed that phenols and iridoids are well extracted by using water or alcoholic solutions (Karikas, 1993; Taruscio et al., 2004), anthocyanins are generally

extracted by using methanol or acidified water solution (Gu et al., 2002) and apolar compounds such as satured fatty acids and carotenoids by employing acetone-petroleum ether mixture.

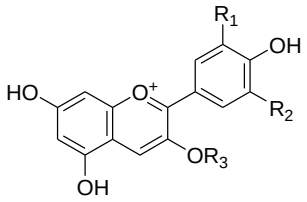
Anthocyanins. These secondary metabolites (Figure 3.2) are present in the outer layer of fruits, together with polyphenols. A small content was found also in pulp and seeds (Blumberg et al., 2013; Lee et al., 2004). The colour of berries (red, blue, purple) has been mainly attributed to glycosides of cyanidin, delphinidin and pelargonidin, respectively (Lee et al., 2005). Mono-, di-, or trisaccharide derivatives of delphinidin, cyanidin, peonidin, petunidin, and malvidin are common in *Vaccinium* berries (Beattie et al., 2005; Borges et al., 2010; Cabrita et al., 2000; Cho et al., 2004; Du et al., 2004; Gao et al., 1994; Su, 2012; Suomalainen et al., 1961; Taruscio et al., 2004; Zheng et al., 2003).

Cyanidin, delphinidin, malvinidin, petunidin, and peonidin with differents sugars (arabinoside, galactoside, and glucoside) are present in *V. corymbosum* (Beattie et al., 2005; Zheng et al., 2003). Malvidin and delphinidin derivatives represent about 75% of the total anthocyanins content of *V. corymbosum* (Scibisz et al., 2007). Cho et al. (2004) reported percentages of 27-40% for delphinidin, 22-33% for malvidin, 19-26% for petunidin, 6-14% for cyanidin, and 1-5% for peonidin in the *V. corymbosum* fruits.

The fruits of *V. oxycoccos* are one of the rare foods that comprise glycosides of the anthocyanidin family: cyanidin, peonidin, malvidin, pelargonidin, delphinidin, and petunidin (Wu et al., 2005). 3-*O*-galactosides and 3-*O*-arabinosides of cyanidin and peonidin are the most abundant anthocyanins (Beattie et al., 2005; Pappas et al., 2009).

Andersen (1985) identified cyanidin 3-*O*-glucoside and delphinidin 3-*O*-glucoside as constituents of *V. vitis-idaea*. The presence of cyanidin 3-*O*-arabinoside, peonidin 3-*O*-arabinoside, peonidin 3-*O*-glucoside, peonidin 3-*O*-galactoside, delphinidin 3-*O*-arabinoside, delphinidin 3-*O*-galactoside, petunidin 3-*O*-galactoside, petunidin 2-*O*-glucoside, malvidin 3-*O*-galactoside, malvidin 3-*O*-glucoside was also reported in the *V. vitis-idaea* fruits (Andersen, 1985; Ek et al., 2006; Hokkanen et al., 2009; Laetti et al., 2011; Madhavi et al., 1998; Pan et al., 2005).

Delphinidin-3-*O*-xyloside, delphinidin-3-*O*-glucoside, malvidin-3-*O*-galactoside, malvidin-3-*O*-glucoside, petunidin-3-*O*-galactoside, petunidin-2-*O*-glucoside, malvidin-3-*O*-xyloside and petunidin-3-*O*-xyloside were isolated from *V. arctostaphylos* (Latti et al., 2009b; Nickavar et al., 2004).



	R_1	R_2	R_3
Cyanidin 3-*O*-arabinoside	OH	H	Ara
Cyanidin 3-*O*-galactoside	OH	H	Gal
Cyanidin 3-*O*-glucoside	OH	H	Glc
Delphinidin 3-*O*-arabinoside	OH	OH	Ara
Delphinidin 3-*O*-glucoside	OH	OH	Glc
Delphinidin 3-*O*-galactoside	OH	OH	Gal
Delphinidin 3-*O*-xyloside	OH	OH	Xyl
Malvidin 3-*O*-arabinoside	OCH_3	OCH_3	Ara
Malvidin 3-*O*-galactoside	OCH_3	OCH_3	Gal
Malvidin 3-*O*-glucoside	OCH_3	OCH_3	Glc
Malvidin 3-*O*-xyloside	OCH_3	OCH_3	Xyl
Peonidin 3-*O*-arabinoside	OCH_3	H	Ara
Peonidin 3-*O*-galactoside	OCH_3	H	Gal
Peonidin 3-*O*-glucoside	OCH_3	H	Glc
Petunidin 3-*O*-arabinoside	OH	OCH_3	Ara
Petunidin 3-*O*-galactoside	OH	OCH_3	Gal
Petunidin 3-*O*-glucoside	OH	OCH_3	Glc
Petunidin 3-*O*-xyloside	OH	OCH_3	Xyl

Figure 3.2. Anthocyanins in the fruits of *Vaccinium* genus

Flavonoids. More than 50 different flavonoids (Figure 3.3), mainly flavonoid glycosides, have been isolated and identified in the *Vaccinium* genus. Glycosides are usually *O*-glycosides, with the sugar moiety bound to the hydroxyl group at C-3 or C-7 position. The most common sugar moieties include D-glucose, L-rhamnose, D-xylose, D-galactose, and L-arabinose (Su, 2012).

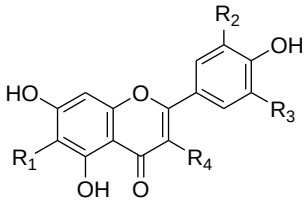
Quercetin is the most common flavonoid in *Vaccinium* berries (Su, 2012). It was found in high quantity in *V. uliginosum* and *V. myrtillus*, but the richest source is the *V. oxycoccos* with a content of 20-40 mg/100 g fresh weight (Cho et al., 2004; Pappas et al., 2009). Several glycosides of myricetin and quercetin (myricetin 3-glucoside, myricetin 3-arabinoside, myricetin 3-*O*-rhamnoside, quercetin 3-*O*-arabinoside, quercetin 3-*O*-rhamnoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, and quercetin 3-*O*-rutinoside) were identified in *V. myrtillus* (Borges et al., 2010; Cho et al., 2004; Taruscio et al., 2004; Zheng et al., 2003). Apigenin, chrysoeriol, myricetin, myricetin 3-*O*-xyloside, quercetin 3-*O*-glucuronide, quercetin 3-*O*-xyloside, isorhamnetin 3-*O*-glucoside (Hokkanen et al., 2009), and luteolin are other flavonoids described in *V. myrtillus* (Witzell et al., 2003).

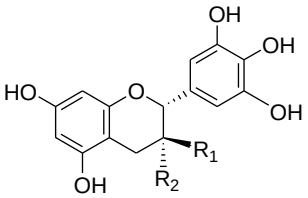
Successive studies reported also the presence of kaempferol, isorhamnetin, laricitrin, syringetin, isorhamnetin 3-*O*-galactoside, myricetin 3-*O*-glucuronide, laricitrin 3-*O*-glucoside, syringetin 3-*O*-glucoside, kaempferol 3-*O*-glucoside, myricetin 3-*O*-galactoside, and isorhamnetin 3-*O*-xyloside in *V. myrtillus* (Hokkanen et al., 2009; Laaksonen et al., 2010; Spela et al., 2011).

Flavonoids identified in *V. oxycoccos* are mainly glycosides of quercetin, myricetin, and to a lesser extent, of kaempferol (Cesoniene et al., 2011). Quercetin 3-*O*-galactoside is the predominant compound, but at least 11 other glycosides are present in lower concentrations (Pappas et al., 2009).

Epicatechin is the major constitutive unit of *V. oxycoccos*, whereas catechin and (epi)gallocatechins were present only in trace amounts (Blumberg et al., 2013; Ek et al., 2006). Kaempferol (Hokkanen et al., 2009), quercetin (Cui et al., 1992; Hokkanen et al., 2009), myricetin, myricetin 3-*O*-glucoside (Pan et al., 2005), quercetin derivatives, kaempferol 3-*O*-rhamnoside, isorhamnetin 3-*O*-galactoside (Ek et al., 2006; Lehtonen et al., 2010), isorhamnetin 3-*O*-glucoside, syringetin-3-*O*-glucoside, kaempferol 3-*O*-glucoside, and rutin (Lehtonen et al., 2010) are the principal flavonoids described in *V. vitis-idaea*.

V. uliginosum fruits are characterised by the presence of kaempferol, laricitrin, quercetin, myricetin, syringetin, quercetin 3-*O*-glucoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucuronide, isorhamnetin 3-*O*-galactoside, isorhamnetin 3-*O*-glucoside, syringetin 3-*O*-glucoside, myricetin 3-*O*-galactoside, rutin, and myricetin 3-*O*-glucuronide (Cui et al., 1992; Laaksonen et al., 2010; Latti et al., 2009a; Li et al., 2011; Yang et al., 2005). Sellappan et al. (2002) described in *V. corymbosum* the presence of catechin, myricetin, quercetin and kaempferol, but not the presence of epicatechin.





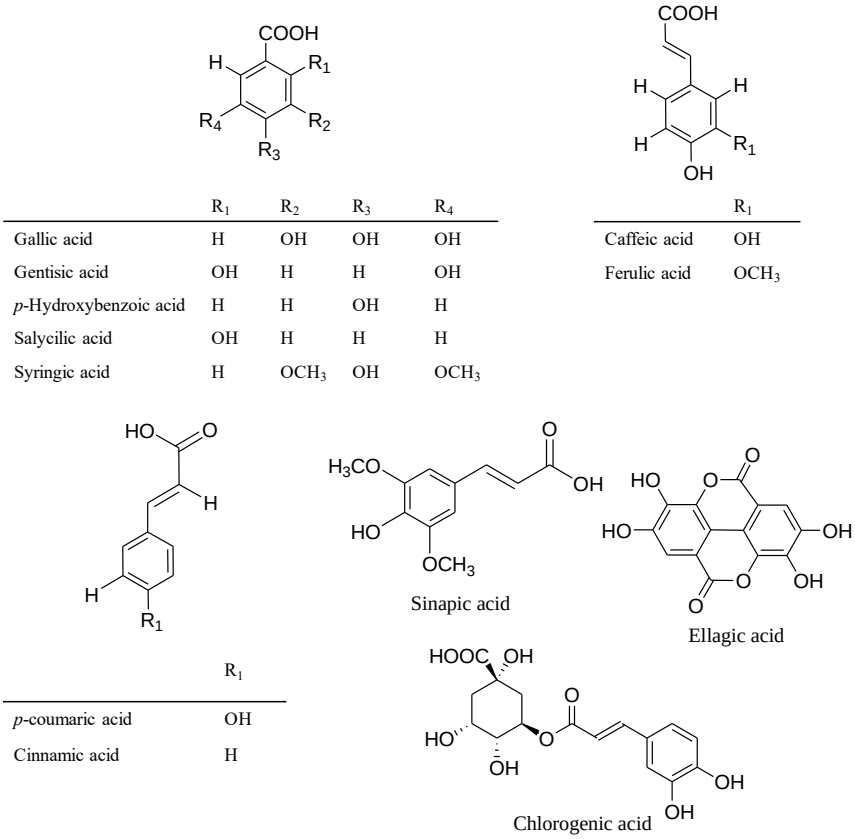
	R_1	R_2	R_3	R_4
Apigenin	H	H	H	H
Chrysoeriol	H	OCH_3	H	H
Hyperoside	H	OH	H	*O*-Gal
Isoorientin	Glc	H	OH	H
Isoquercitrin	H	OH	H	*O*-Glc
Isorhamnetin	H	OCH_3	H	OH
Isorhamnetin 3-*O*-galactoside	H	OCH_3	H	*O*-Gal
Isorhamnetin 3-*O*-glucoside	H	OCH_3	H	*O*-Glc
Isorhamnetin 3-*O*-xyloside	H	OCH_3	H	*O*-Xyl
Kaempferol	H	H	H	OH
Kaempferol 3-*O*-glucoside	H	H	H	*O*-Glc
Kaempferol 3-*O*-rhamnoside	H	H	H	*O*-Rha
Laricitrin	H	OCH_3	OH	OH
Laricitrin 3-*O*-glucoside	H	OCH_3	OH	*O*-Glc
Luteolin	H	OH	H	H
Myricetin	H	OH	OH	OH
Myricetin 3-*O*-arabinoside	H	OH	OH	*O*-Ara
Myricetin 3-*O*-galactoside	H	OH	OH	*O*-Gal
Myricetin 3-*O*-glucoside	H	OH	OH	*O*-Glc
Myricetin 3-*O*-glucuronide	H	OH	OH	*O*-GlcA
Myricetin 3-*O*-rhamnoside	H	OH	OH	*O*-Rha
Myricetin 3-O-xyloside	H	OH	OH	*O*-Xyl
Quercetin	H	OH	H	OH
Quercetin 3-*O*-arabinoside	H	OH	H	*O*-Ara
Quercetin 3-*O*-glucuronide	H	OH	H	*O*-Glc A
Quercetin 3-*O*--rhamnoside	H	OH	H	*O*-Rha
Quercetin 3-*O*-xyloside	H	OH	H	*O*-Xyl
Rutin	H	OH	H	*O*-Glc(6←1)Rha
Syringetin	H	OCH_3	OCH_3	OH
Syringetin 3-*O*-glucoside	H	OCH_3	OCH_3	*O*-Glc

	R_1	R_2	R_3
Catechin	OH	H	H
Epicatechin	H	OH	H
Epigallocatechin	OH	H	OH

Figure 3.3. The main flavonoids in the fruits of *Vaccinium* genus

Phenolic acids. Zadernowski et al. (2005) identified 17 phenolic acids (Figure 3.4) in some varieties of *V. myrtillus*. Sellappan et al. (2002) have found as phenolic acids gallic, *p*-coumaric, ferulic, ellagic and caffeic in *V. corymbosum* produced in the state of Georgia (US) as confirmed by Taruscio et al. (2004). Taruscio et al. (2004) evaluated the

phenolic acids composition of *V. corymbosum* and *V. oxycoccos*, there is a difference as composition. *V. corymbosum* was characterised of presence of chlorogenic acid as phenolic acid present in greater quantity, followed by caffeic, ferulic, *p*-coumaric and only in trace *p*-hydroxybenzoic acids. While, in the *V. oxycoccos p*-coumaric acid was the principal phenolic acid, followed by ferulic, chlorogenic, caffeic and *p*-hydroxybenzoic acids.



	R_1	R_2	R_3	R_4
Gallic acid	H	OH	OH	OH
Gentisic acid	OH	H	H	OH
p-Hydroxybenzoic acid	H	H	OH	H
Salycilic acid	OH	H	H	H
Syringic acid	H	OCH_3	OH	OCH_3

	R_1
Caffeic acid	OH
Ferulic acid	OCH_3

	R_1
p-coumaric acid	OH
Cinnamic acid	H

Figure 3.4. Principal phenolic acids in the fruits of *Vaccinium* genus.

V. oxycoccos is characterised by the presence of hydroxybenzoic acids rather than hydroxycinnamic acids (Wang et al., 2011; Zhang et al., 2004; Zuo et al., 2002). The main hydroxycinnamic acids were *p*-coumaric, sinapic, caffeic, and ferulic acids (Zuo et al., 2002). Ellagic acid and ellagitannins have not been detected in significant amounts (Blumberg et al., 2013). Thirteen phenolic acids (gallic, protocatechuic, *p*-hydroxybenzoic, *m*-hydroxybenzoic, gentisic, chlorogenic, *p*-coumaric, caffeic, ferulic, syringic, sinapic, salicylic, and trans-cinnamic acids) were identified in *V. arctostaphylos*. The dominant phenolic acids were caffeic and *p*-coumaric acids. The phenolic acid concentrations are mostly lower in *V. arctostaphylos* in comparison to the other berries of the *Vaccinium* genus (Ayaz et al., 2005).

Acids. The non-volatile acids identified and quantified in *V. arctostaphylos* and *V. myrtillus* species were malic, citric, and quinic acids, of which the latter two were found to be the major acids (Figure 3.5). It is interesting to notice that the level of malic acid in both berries increased gradually during maturation. In contrast, the level of citric and quinic acids, as well as the total acid level, decreased towards ripening in both species (Ayaz et al., 2001).

The main organic acids in *V. oxycoccos* were citric and malic acids (Huopalahti et al., 2000). In *V. corymbosum* the major acids (organic and phenolic) present are citric, malic, quinic, and chlorogenic acids. The minor acids, acetic and shikimic acid were present and their contribution to the total acid equivalents was 3.0% (Kalt et al., 1996).

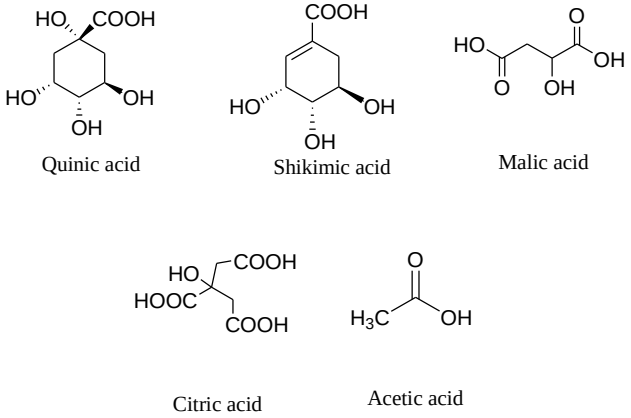


Figure 3.5. The main acids identified in the fruits of *Vaccinium* genus.

Iridoids. Some iridoids glucosides, namely monotropein, deacetylasperulosidic acid, 7,8-dihydroiridoid, scandoside, iridoid-methylester 1, iridoid-methylester 2, dihydroiridoid-methylester, *p*-coumaroyl-monotropein derivative 1, *p*-coumaroyl-monotropein derivative 2, *p*-coumaroyl-6,7-dihydromonotropein, *p*-coumaroyl-deacetylasperulosidic acid derivative 1, *p*-coumaroyl-deacetylasperulosidic acid derivative 2, *p*-coumaroyl-scandoside derivative 1, and *p*-coumaroyl-scandoside derivative 2, have identified in *V. uliginosum*, and *V. myrtillus*. (Heffels et al. (2017; Kim et al.,. 2015). The main iridoids identified in *Vaccinium* species are reported in Figure 3.6. Surprisingly, no iridoid glycosides have been identified in *V. corymbosum* (Heffels et al., 2017; Leisner et al., 2017; Ma et al., 2013).

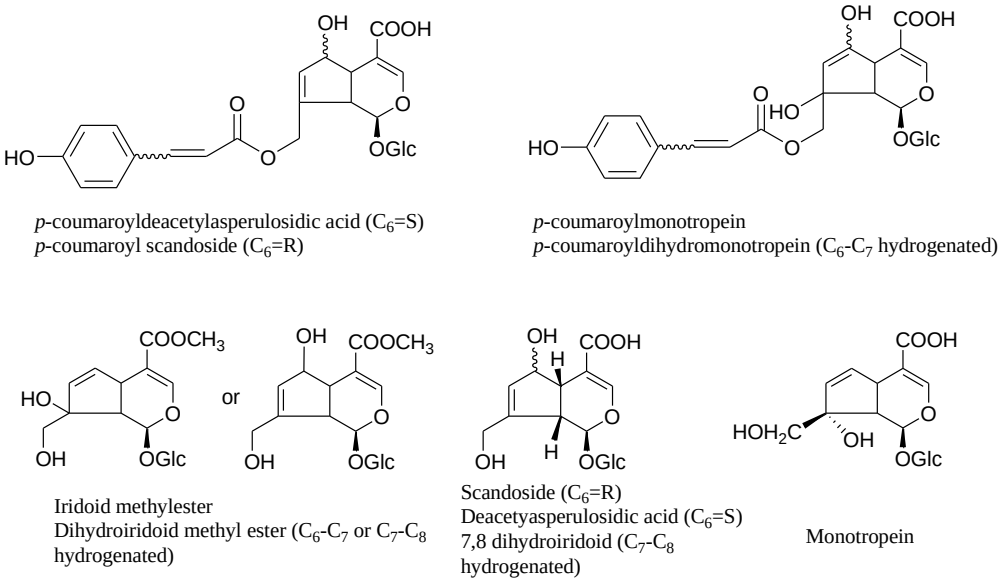


Figure 3.6. Principal iridoids identified in the fruits of *Vaccinium*.

Triterpenes and sterols. Triterpenoids were the most predominant components in cuticular wax of blueberry fruits (Chu et al., 2017).

The triterpene alcohols α-amyrin, β-amyrin, and lupeol were also detected in cuticular wax of blueberry fruits. Ursolic acid was the dominant triterpene in *V. corymbosum* (southern highbush blueberry) cultivars, whereas oleanolic acid was the most abundant in northern highbush blueberry cultivars.

For the first time, hentriacontan-10,12-dione was detected in the *V. corymbosum* (Chu et al. 2017). Ursolic acid that demonstrated to possess a strong anti-inflammatory effect (Ikeda et al., 2008) is abundant in *V. oxycoccos*, which also contains two rare derivatives of ursolic acid, *cis*-3-*O*-*p*-hydroxycinnamoyl ursolic acid and *trans*-3-*O*-*p*-hydroxycinnamoyl ursolic acid (Kondo et al., 2011) (Figure 3.7).

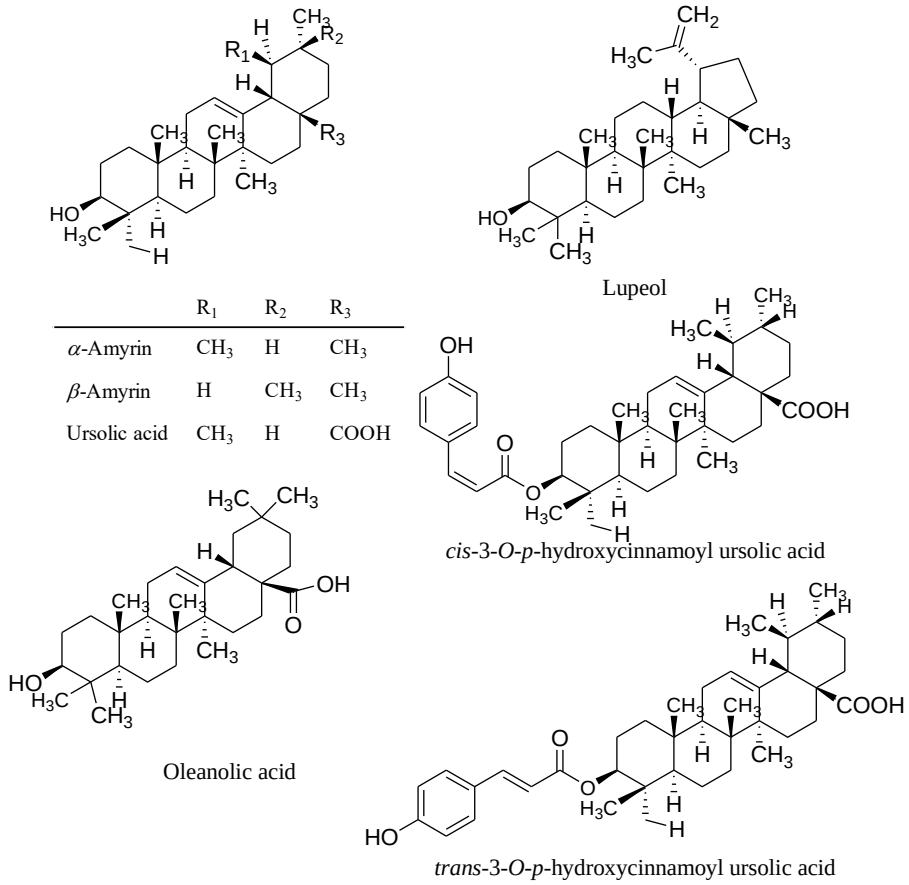


Figure 3.7. The main triterpenes and sterols identified in the fruits of *Vaccinium* species.

3.4. Chemical constituents of the leaves

The chemical constituents of leaves have been less studied than the compounds of berries. Teleszko et al. (2015) analyzed the phytochemical composition of fruits and leaves of several *Vaccinium* species; among them, *V. myrtillus* leaves present high quantity of phenolics compounds, followed by *V. oxycoccos* leaves.

Riihinen et al. (2008) showed that red leaves of *Vaccinium* genus contain anthocyanins, absent in green leaves.

Wang et al. (2015) identified in the methanolic leaves extract of *V. corymbosum* the presence of cyanidin 3-*O*-glucoside, cyanidin 3-*O*-glucuronide, and cyanidin 3-*O*-arabinoside confirming that *V. corymbosum* leaves possess a higher total anthocyanins content compared to *V. virgatum* and *V. formosum* leaves.

Both green and red leaves contain proanthocyanidins, especially procyanidin. The green leaves of *V. vitis-idaea* have similar phytochemical profile with those of *V. myrtillus* (Hokkanen et al., 2009; Ieri et al., 2013). Ieri et al. (2013) and Hokkanen et al. (2009) have quantified phenolics compounds in methanolic and hydroalcoholic leaves extracts of *V. vitis-idaea*. In general, hydroxycinnamic acids and flavonoids were the most

abundant compounds. In the methanolic extract, flavonoids content was higher than hydroxycinnamic acids, but in the hydroalcoholic extract, the opposite was observed.

The major polyphenolic group was proanthocyanidins, followed by phenolic compounds (flavonoids and phenolic acids). Phenolic compounds and proanthocyanidins, were in higher concentration than the respective fruits in the *V- myrtillus* (Teleszko et al., 2015). The collection time of *Vaccinium* leaves greatly determines their phenolic content (Ieri et al., 2013). In fact, contrary to the fruits, the flavonoids content raise during the leave development, while hydroxycinnamic acid content strongly decreases (Martz et al., 2010). Previously, Riihinen et al. (2008) have indicated that the red leaves of *V. corymbosum* have higher quantities of quercetin and kaempferol, as well as of ferulic, caffeic and *p*-coumaric, than green leaves.

Flavonoids and proanthocyanidins. Proanthocyanidins were detected in small quantities in the methanol extract of *V. vitis-idaea* leaves (Hokkanen et al., 2009). Ferlemi et al. (2015; 2016) have detected proanthocyanidin B_1/B_2 and cinchonain in the decoction extract of *V. corymbosum* leaves. The main flavonols detected in the methanol extract of *V. oxycoccos* leaves were hyperoside and quercetin-3-*O*-rhamnoside, together to quercetin-3-*O*-xyloside, quercetin-3-*O*-arabinoside and procyanidin A_2 (Neto et al., 2010). Sidorova et al. (2017) investigated about the flavonoids present in the aqueous extract of *V. myrtillus* leaves and they showed flavonoid *C*-glycosides and *O*-derivatives of apigenin and luteolin were found; the main ones are apigenin 7-*O*-glucuronide, vitexin-2-*O*-rhamnoside, and isoorientin. Flavonoids glycosides are represented mainly of quercetin derivatives, the principals are rutin and quercetin 3-*O*-glucoside-7-*O*-rhamnoside. Isorhamnetin 3-*O*-glucoside and kaempferol 3-*O*-glucuronide also were found in the extract. Additionally, free aglycones were also present (myricetin, quercetin, luteolin and kaempferol). Other identified flavonoids in the hydroalcoholic and methanol extracts of *V. myrtillus* and *V. vitis-idaea* leaves are quercetin-3-*O*-galactoside, quercetin-3-*O*-(4”- 3-hydroxy-3-methylglutaroyl)-α-rhamnoside, quercetin-3-*O*-arabinoside, quercetin-3-*O*-glucoside, quercitrin, and quercetin, as well as three kaempferol glycosides (Hokkanen et al., 2009; Ieri et al., 2013).

In addition, Hokkanen et al. (2009) detected six different isomeric flavanolignans of cinchonain (Figure 3.8) in methanol extract of *V. myrtillus* leaves, as well as other bioactive compounds such as flavan-3-ols and three proanthocyanidins.

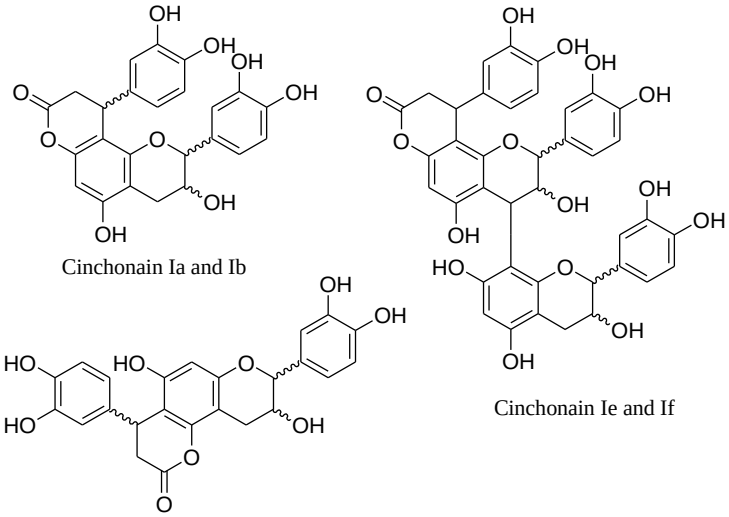


Figure 3.8. Flavanolignans in methanol extract of *V. myrtillus* leaves

Moreover, *V. vitis-idaea* leaves were characterised by coumaroyl- and caffeoyl-hexose hydroxyphenols that are not present in *Vaccinium* leaves. The most abundant flavonoid was quercetin-3-*O*-(4”-3-hydroxy-3-methylglutaroyl)-α-rhamnoside that represent 5-6% of total phenols in the hydroalcoholic extract and 32% of the methanolic extract. Rutin, hyperoside, and quercitrin were also detected in significant amounts in the methanolic extract, while traces of four quercetin glycosides and kaempferol glycosides were also found (Hokkanen et al., 2009). Quercetin 3-*O*-glucoside (Mzhavanadze, 1973), quercetin 3-*O*-rutinoside (Latti et al., 2009b), kaempferol 3-*O*-glucoside, kaempferol 3-*O*-rhamnoside (Latti et al., 2009b; Mzhavanadze, 1971) were identified in the aqueous extrct of *V. arctostaphylos* leaves. The main flavonoids detected in aqueous and methanol extracts of *V. corymbosum* leaves were hyperoside, isoquercetin and rutin. Other flavonoids founded were: myricetin (Yang et al., 2005), quercetin 3-*O*-glucoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-arabinoside (Kader et al., 1996), quercetin 3-*O*-rhamnoside (Kader et al., 1996; Scibisz et al., 2006; Spela et al., 2011), myricetin 3-*O*-glucoside, quercetin 3-*O*-rutinoside (Scibisz et al., 2006), syringetin 3-*O*-glucoside, and kaempferol 3-*O*-glucoside (Scibisz et al., 2006; Spela et al., 2011).

Phenols and phenolic acids. Qualitative and quantitative analyses lead to the identification as main bioactive compounds of hydroalcoholic and methanol extracts of *V. myrtillus* red dried leaves hydroxycinnamic acids, especially chlorogenic acid (Hokkanen et al., 2009; Ieri et al., 2013); its concentration ranges from 59 to 74% of the total hydroxycinnamic acids (Ieri et al., 2013). Sidorova et al. (2017) reported also rosmarinic acid, caffeoylquinic acid, *p*-coumaric and ferulic acid in the aqueous extract of *V. myrtillus* leaves. Hokkanen et al. (2009) analysed the methanolic extract of *V. myrtillus* leaves and identified thirty-five compounds.

Other than chlorogenic acid and its isomers, caffeoyl-shikimic acid, feroylquinic acid isomer, and traces of caffeic acid were also founded. In both hydroalcoholic and methanol extract of green leaves of *V. vitis-idaea and V. myrtillus*, the main acid was 2-*O*-caffeoylarbutin, which is not present in other *Vaccinium* leaves (Hokkanen et al., 2009; Ieri et al., 2013).

In addition, Neto et al. (2010) have performed an HPLC-MS analysis of the phenolic profile of methanol extract of *V. oxycoccos* leaves; the phenolic acids are mainly chlorogenic and neo-chlorogenic acid, as well as 3-*O*- and 5-*O*-coumaroylquinic acids. Ferlemi et al. (2015; 2016) have analysed the chemical composition of decoction extract of *V. corymbosum* red dried leaves by LC-ESI/MS and HPLC-DAD, and twenty different compounds were identified, mainly phenolic acids and flavonols. Chlorogenic acid was the main phenolic acid, present in high quantity. Same trend was observed with *V. myrtillus* leaves. LC-MS analysis showed the presence of quinic and caffeic acid in the *V. corymbosum*. Mzhavanadze et al. (2004) reported the isolation of caffeic, chlorogenic, neochlorogenic, 3- and 5-*p*-coumaroylquinic acids, and 3,5-dicaffeoylquinic acid from aqueous extract of *V. arctostaphylos* leaves together with 4-caffeoyl-quinic acid, arbutin, rosmarinic acid, caffeoylarbutin, 1-*p*-coumaroylgalactoglucose, and *p*-coumaroylarbutin (Figure 3.9).

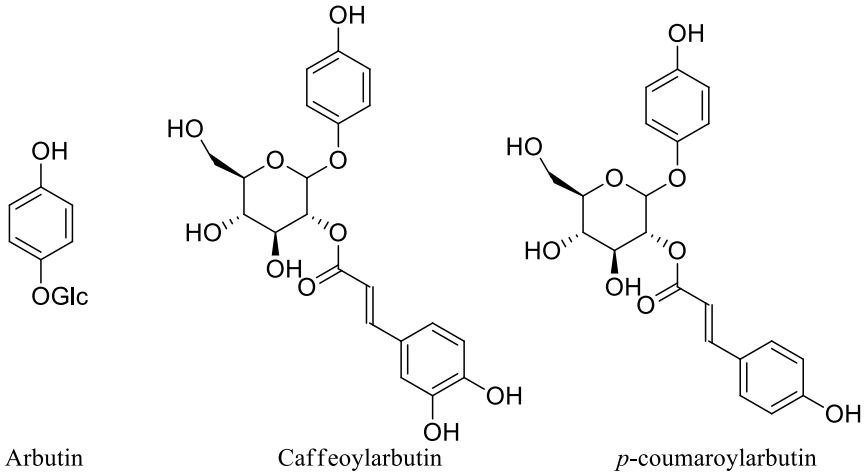


Figure 3.9. Arbutin derivatives in aqueous extract of *V. arctostaphylos* leaves

Iridoids. Hokkanen et al. (2009) have also identified two coumaroyl iridoids (*p*-coumaroyl-6,7-dihydromonotropein) in methanol extract of *V. myrtillus* leaves. While, in the methanol extract of *V. vitis idaea* the coumaroyl iridoids were quantified in small concentration (Hokkanen et al., 2009).

Triterpenes. The principal triperpene identified in the leaves of *V. myrtillus* was *β*-amyrin, together with other oleanane- and ursane-type triterpenes including oleanolic acid and ursolic acid (Szakiel et al., 2012).

3.5. Biological properties

Several studies confirmed the anti-inflammatory, antioxidant, anti-carcinogenic, and cardiovascular and neurodegenerative protective effects of *Vaccinium* species (Ramassamy, 2006; Routray et al., 2011). Among these, *V. myrtillus* and *V. corymbosum* were the most studied.

3.5.1. Antioxidant activity

Vaccinium represents an important source of ingredients with high antioxidant potential (Sellappan et al., 2002; Yi et al., 2005). Many studies focused on the antioxidant activities of blueberry leaves (Piljac et al., 2009). Ferlemi et al. (2015) have demonstrated the high antioxidant capacity of *V. corymbosum* leaves decoction and its capability to bind iron ions. Previously, Vinson et al. (2005) showed that the *V. oxycoccos* leaves extracts possessed a high level of phenolics that has been associated with significant antioxidant potential.

The antioxidant capacity is influenced by several factors, such as cultivar, genotype, growning site, cultivation techniques and condition, processing, and storage.

In contrast with some *Vaccinium* berries, the antioxidant activity of *V. corymbosum* is higher in early maturation stages and during initial pigmentation than in ripe stage. This is related to a high level of hydroxycinnamic acids and flavonols (Rodarte Castrejón et al., 2008). Del Bò et al. (2013) have studied the effect *V. corymbosum* fruits (intake of 300 g) on selected markers of oxidative stress and antioxidant protection (endogenous and oxidatively induced DNA damage) and of vascular function (changes in peripheral arterial tone and plasma nitric oxide levels).

V. corymbosum fruits considerably reduced H_2O_2-induced DNA damage. In primary cultures of rat hepatocytes, it was found that *V. myrtillus* ethanol extract protected cells against oxidative damage (Valentova et al. 2006). As described by Upton (2001), *V. myrtillus* or its anthocyanins-rich extract protects rat liver microsomes against oxidative damage and apolipoprotein B against ultraviolet (UV)-induced oxidative fragmentation. *In vivo* studies showed conflicting results. In the study of Lee et al. (2010), no significant change of urinary 8-hydroxydeoxyguanosine (8-oxodG) a biomarker of oxidative stress was reported after 14 weeks of supplementation with an anthocyanins-rich extract from mixed berries including *V. myrtillus* (Lala et al. 2006). However, a significant decrease of a biomarker of lipid peroxidation (malondialdehyde) in the brain was observed in rats OXYS (accelerated senescence rats) fed with *V. myrtillus* extract (Kolosova et al. 2006).

This strain of rats shows accelerated aging and higher oxidative stress compared to the Wistar rats and, interestingly, the Wistar rats did not show *V. myrtillus* related effects, suggesting that the antioxidant effects of *V. myrtillus* may be seen only in cases of elevated oxidative stress. In mice stressed by restraint, marked increases in liver damage and reactive oxygen species (ROS) levels associated with this stress were restored to

normal levels by administering a bilberry extract, and there was also enhanced mitochondrial complex II activity, elevated sodium/potassium ATPase activity, and elevated mitochondrial membrane potential with the bilberry treatment (Bao et al. 2010).

In contrast to the promising effects seen in animal studies, no effect on lipid peroxidation was seen in human volunteers after supplementation with mixed vegetables and fruits including bilberries (Freese et al. 2004).

3.5.2. Anti-inflammatory activity

V. corymbosum hydroalcoholic extract (300 mg/kg) showed antinflammatory activity inhibiting carrageenan and histamine oedema models. In the same study, the myeloperoxidase (MPO) activity was also evaluated. An important reduction of myeloperoxidase was observed in the rats treated with *V. corymbosum*. MPO activity is proportional with neutrophils quantities present in the inflammation site. High concentration of anthocyanins (cyanidin, delphinidin and malvidin) and flavonoids (astragalin, hyperoside, isoquercitrin and quercitrin) were founded in the *V. corymbosum* and these compounds were correlated with anti-inflammatory and antioxidant activities ascribed to these berries (Torri et al., 2007).

Another study reported the anti-inflammatory activity of an anthocyanins-rich fraction obtained from *V. corymbosum* fruits, on 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis rat model, in comparison with positive control, 5-aminosalicylic acid (5-ASA) (Pereira et al., 2017). The treatment with this fraction demonstrated increase of antioxidant defences, down-regulation of pro-inflammatory enzymes and decrease of leukocite infiltration in the inflammation site inhibiting the expression of MPO. Another sensitive marker of inflammation in the intestine is the alkaline phosphatase (ALP) that was significantly inhibited by the anthocyanins-fraction compared with positive control. The expressions of COX-2 and inducible nitric oxide synthase (*i*NOS) proteins in the colonic inflammatory status resulted increased, but the treatment with anthocyanins fraction of *V. corymbosum* strong decreased the level of COX-2, as reported also for the positive control. Surprisingly, in contrast with 5-ASA that not showed an effect against *i*NOS expression, anthocyanins fraction of *V. corymbosum* decreased drastically (95%) its levels. Considering that berries of *V. corymbosum* are edible, their consumption may be helpful for the treatment of inflammatory illnesses.

3.5.3. Antidiabetic activity

V. corymbosum berries exhibited anti-diabetic properties and protection of pancreatic *β*-cells from glucose-induced oxidative stress (Karcheva-Bahchevanska et al., 2017).

In a clinical study, 45 g of *V. corymbosum* powder as smoothie were administered to 32 volunteers (adults, obese, and insulin resistant) for breakfast and dinner, for six weeks (Stull et al., 2010). At the end of this period, participants showed improved insulin

sensitivity. Petlevski et al. (2001) tested a multi-ingredient preparation composed of *V. myrtillus* leaves and nine other plant extracts (ethanol extracts), patented as an antidiabetic remedy in Croatia founding a decrease in blood glucose and fructosamine levels in alloxan-induced non-obese diabetic mice. In studies where hydroalcoholic extract of *V. myrtillus* leaves was introduced in screening programs that aimed at identifying α-amylase inhibitors and activators of the human peroxisome proliferator-activated receptor gamma (PPARγ), *V. myrtillus* leaves extract showed activity in the last model (Rau et al., 2006). The hydroalcoholic leaves extract activated PPARγ, a receptor involved in the insulin resistant because it is capable to decrease the fatty levels increasing their uptake with improved sensitivity to insulin in the liver and muscle tissue.

In addition, it modulates of adipokine production, enhancing insulin sensitivity and inhibits the synthesis of cytokines their associated. Finally, the flavanolignans cinchonains might play significant role in the blood glucose lowering effect as they have been found to induce insulin secretion in both *in vitro* and *in vivo* experiments in rats (Cignarella et al., 1996).

V. myrtillus leaves possessed excellent antidiabetic properties, as demonstrated by Bljajić et al. (2017). The aqueous extract was able to restore glutathione concentration in HepG2 cells subjected to glucose-induced oxidative stress. The hydro-ethanolic extract strongly inhibited α-glucosidase. Both solvents produced extracts with excellent antioxidant properties. The enzymes involved in glucose metabolism and type 2 diabetes were also inhibited by the juices; this is the first time that *V. myrtillus* and *V. oxycoccos* juices are reported to inhibit α-glucosidase and dipeptidyl peptidase-4 (DPP4) in a dose-dependent manner. The anthocyanins content of fruits has been related to α-glucosidase inhibition also in previous studies (McDougall et al., 2005) and other authors have shown that polyphenols can enhance the insulin response and attenuate secretion of glucose-dependent insulinotropic polypeptide and glucagon-like peptide 1 (GLP-1). The DPP-4 enzyme also regulates glycaemia and its inhibitors such as sitagliptin represent some of the new treatments for type 2 diabetes (Cásedas et al., 2017).

3.5.4. Anticancer activity

Fruits and leaves ethanol extracts of *Vaccinium* species (*V. myrtillus* and *V. oxycoccos*) were found to induce apoptosis in cancer cells and to inhibit human leukemia (Katsube et al., 2003; Neto et al., 2005). Various studies confirmed the apoptotic activity of *Vaccinium* extracts *in vitro* about breast (Faria et al., 2010), colon (Katsube et al., 2003; Neto et al., 2005; Yi et al., 2005; Zhao et al., 2004), lung (Neto et al., 2005), and prostate cancer (MacLean et al., 2007). In *in vitro* and *in vivo* studies, berry anthocyanins demonstrated antioxidant, anti-proliferative, apoptotic, anti-angiogenic, and anti-inflammatory effects (Benzie et al., 2010; Lala et al., 2006; Matsunaga et al., 2010; Seeram, 2009). *V. corymbosum* water and ethanol extracts showed anti-proliferative and

anti-metastatic potential against breast and colon cancer cells (Adams et al., 2010; Samad et al., 2014).

The effects of *V. uliginosum* anthocyanins extract on cell growth, membrane permeability, and cell cycle of Caco-2 and Hep-G2 cancer cell lines were investigated (Liu et al., 2010). Cyanidin 3-*O*-glucoside was the most abundant anthocyanin, followed by malvidin 3-*O*-glucoside and malvidin 3-*O*-galactoside. Hep-G2 LC_{50} was calculated to be 0.563 mg/mL, Caco-2 LC_{50} was 0.390 mg/mL.

LDH release, a marker of membrane permeability, was significantly increased in Hep-G2 cells and Caco-2 cells after 48 and 72 h compared to 24 h. The increase was 21% at 48 h and 57% at 72 h in Caco-2 cells and 66 and 139% in Hep-G2 cells compared to 24 h. Treatment increased sub-G1 in both cell lines without influencing cells in the G2/M phase. Previously, cyanidin 3-*O*-glucoside, delphinidin, and peonidin 3-*O*-glucoside demonstrated to suppress cell growth and apoptosis of colon and breast cell lines (Chen et al., 2005; Yun et al., 2009).

3.5.5. Antimicrobial activity

Antimicrobial effects of herbs and natural products can be highlight *via* inhibition of bacterial binding (adhesion) to cell walls, direct antimicrobial killing, or by effects that potentiate antibiotics, as demostrated by lowered minimum inhibitory concentration (MIC) of antibiotics in the presence of an herb compared to that of the antibiotic alone. Several natural products have been found to have antimicrobial effects (Lee et al., 2006).

Ripe *V. myrtillus* and other berry fruits, as well as purified berry phenolics have been reported to show direct antimicrobial effects against human pathogens, including *Salmonella* and *Staphylococcus aureus* (Puupponen-Pimiä et al., 2005a; 2005b). Berry extracts were found to inhibit the growth not only of *Helicobacter pylori* but also of gram-positive organisms such as *Bacillus, Clostridium,* and *Staphylococcus* bacteria. *V. myrtillus* leaves, extracted with acetone:water solution (7:3), demonstrated a significant effectiveness against *S. aureus* enhancing, at the same time, when used in association the bactericidal potential of vancomycin and linezolid (Sadowska et al., 2014). A high phenols content with a predominance of hydroxycinnanimic acids was reported.

V. oxycoccos has powerful anti-adhesion properties and it is widely used to prevent urinary tract infections. The anti-adhesion effects of *V. oxycoccos* berries on *H. pylori*, and the increased antimicrobial effects of clarithromycin against *H. pylori* were also reported (Chatterjee et al., 2004). Hydroalcoholic extract of *V. corymbosum* leaves acts as an antimicrobial agent, especially against *Salmonella typhymurium* and *Enterococcus faecalis* (Pervin et al., 2013).

3.5.6. Neuroprotective activity

V. corymbosum fruits have been showed to be more active neuroprotective agents compared with other berries (Joseph et al., 1999). Epidemiological and *in vitro* studies suggested that *Vaccinium* berries helps maintaining the health and acts as a barrier to the

effects of aging, particularly with respect to neurodegeneration and cognitive defects. Many beneficial effects seem to be related to the antioxidant properties of phenols present in the fruit (Kalt et al., 1997). Malin et al. (2011) demonstrated that a *V. corymbosum*-enriched diet could reverse age-related memory loss in rats.

Ripe *V. myrtillus* berries are used in diabetes-related research to try to prevent and repair some of the brain damage caused by diabetes, particularly tissue loss and reduction in cognitive function and memory (Matysek et al., 2017). It was found that *V. myrtillus* berries were able to modulate neurodegeneration in diabetic rats by increasing neurotransmitter release and reducing the quantity of ROS in the brain (Matysek et al., 2017). The researchers also note that the berries were able to promote healthy morphological modulations in αCaMKII in hippocampal neurons. Casadesus et al. (2004) analyzed the effects of polyphenols on hippocampal plasticity and cognitive behavior, specifically age-related CNS deterioration associated with loss of learning and memory. *V. corymbosum* fruits diet administered to aging rats improved hippocampal neuroplasticity and had a positive effect on spatial memory by promoting the activation of insulin-like growth factor 1 (IGF1) and IGF-1 receptor levels, which mediate the rate of neurogenesis (Casadesus et al., 2004).

In a study conducted by Joseph et al. (1999), in which rats were fed a diet containing *V. corymbosum* berries, spinach or strawberry extract for eight weeks, it was demonstrated that *V. corymbosum* fruits supplementation was able to significantly reverse some neurodegenerative effects related to aging. Successively, a clinical study investigated the effects of daily wild *V. corymbosum* juice consumption for 12 weeks in adults experiencing early memory decline (Krikorian et al., 2010). Regarding list recall and paired associate learning the group administered the berry juice performed better than the placebo group. Although these results are encouraging, the study represents a starting point for further studies since the sample size was limited.

A recent study showed that a diet rich in *V. corymbosum* berries could improve cognitive abilities in older adults (Miller et al., 2018). In this study, 24 women and 13 men were recruited for 3 month to investigate the effects of *V. corymbosum* on cognition capacity and long and short term-memory. The addition of blueberry to the diet of healthy older adults improved some aspects of executive function, and these improvements were most evident in the most challenging cognitive tests, as long term. However, further trials and clinical experiments need to be conducted to evaluate the effects a longer intervention among healthy older adults.

V. corymbosum and *V. oxycoccos* juices revealed inhibitory activities of two main groups of enzymes; one group in relation with neurotransmitter metabolism (monoamine oxidase A (MAO-A), tyrosinase (TYR) and acetylcholinesterase AChE), the other group in relation to diabetes (α-glutamic acid (α-GLU) and DPP-4).

Both juices were able to inhibit TYR and MAO-A. TYR is the enzyme that convertes tyrosine in levodopa, a precursor of dopamine. These lattest have an important role in the neurological disorders, as Parkinson's disease. MAO-A is involved in deamination of

catecholamines and serotonin and certain polyphenols as anthocyanins have been described to be involved in this inhibition, that may result in an anxiolytic and antidepressant effects (Nabavi et al., 2015). Juices showed also inhibition of enzymes linked to diabetes, α-GLU and DPP-4.

Data obtained by Cásedas et al. (2017) revealed that berry juices may have potential as neuroprotective agents *via* the inhibition of MAO-A and/or TYR (Les et al., 2015). Indeed, a study by American researchers showed that consumption of *V. corymbosum* juice riched in anthocyanins for 12 weeks improves cognition and memory in older adults with no accentuate dementia (Krikorian et al., 2010). Hong et al. (2018) have found that aqueous extracts (BE) and vinegar (BV) of *V. corymbosum* significantly inhibited malondialdehyde levels and rescued the activities of ROS and catalase in the cortex, suggesting that the neuroprotective effects of BE and BV might be able to directly remove free radicals or inhibit oxidative stress in the brain. Analysis phytochemical reported the presence of chlorogenic acid, cyanidin chloride and epicatechin. These bioactive compounds could be contributed to *V. corymbosum* activity, regulating oxidative stress as antioxidant and reduce lipid peroxidation in brain. The acetone extract of *V. vitis idaea* leaves exhibited neuroprotective effects, as demonstrated by Vyas et al. (2013). In this study, brain-derived cell cultures from rats cells, after treatment with glutamate, were treated with *V. vitis idaea* leaves extract. A high protection against glutamate toxicity was demonstrated.

3.5.7. Other activities

V. oxycoccos berries are known to be effective against urinary tract infections (Blumberg et al., 2013). A randomized experimental trial with 12 participants showed that also the consumption of *V. oxycoccos* leaves beverage can be useful to improve urinary tract health inhibiting the adhesion of bacteria presents in urine (Mathison et al., 2014). The consumption of a mixture of anthocyanins isolated from *V. oxycoccos* fruits (320 mg/day) showed lowered LDL and increased HDL on 150 hypercholesterolemic participiants. Good results were observed also with cranberry juice in obese men, hypertriglyceridemic or diabetes patients. Positive effects were not obtained in patients with cardiovascular disorders (Blumberg et al., 2013).

The consumption of cranberry anthocyanins improved lipid profiles, increasing HDL and decreasing LDL in rats, hamsters fed a high-fat diet and hypercholesterolemic swine. The same trend was observed in clinical studies. *V. corymbosum* berries could also be used for lower blood pressure, decreasing of blood cholesterol and, therefore, reducing of cardiovascular risk and atherosclerosis prevention (Basu et al., 2010; Prior et al., 2009; Wu et al., 2010). *V. corymbosum* berries (fresh or jams or juice) protected hepatocytes from oxidative stress and could modulate the function of T cells (Madrigal-Santillán et al., 2014). Prevention of cataract was showed for *V. corymbosum* leaves (Wang et al., 2015). Responsible for *V. corymbosum* biological properties were diverse phenolic-type

phytochemicals as proanthocyanidins, phenolic acids and flavonoids (flavonols, flavanols and anthocyanins)

Hydroalcoholic extract of *V. vitis-idaea* leaves were mainly used as diuretics as well as for their antiseptic activity in urinary tract, probably due to the high content of tannins, especially arbutin and its derivatives (Ieri et al., 2013). The proanthiocyanidins of *V. vitis-idaea* berries exhibit significantly inhibition of *in vitro* adhesion of *Escherichia coli* to uroepithelial cells (Howell et al., 2005).

The ethanolic extract of *V. vitis-idaea* leaves has shown significant antitussive and anti-catarrhal properties in rats (Wang et al., 2005).

Ravan et al. (2017) demonstrated that methanol extract of *V. arctostaphylos* prevents acute CCl_4-induced hepatotoxicity in rats in a dose-dependent manner. The hepatoprotective effect of this extract may be attributed to the antioxidant capacity of extract, which is due to phenolic and flavonoid contents of compound. Moreover, *V. myrtillus* berries have been reported to have a pharmacological impact against ophthalmologic disorders. They improve blood and oxygen delivery to the eye and scavenge free radicals, which contribute to cataract and macular degeneration (Calò et al., 2014).

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Chapter 4
The genus *Cornus* L.: chemistry, traditional uses and biological properties

4.1. Introduction

The genus *Cornus* L. (Cornaceae), which consists of about 65 species, is widely diffused in central and southern Europe, southwest Asia, east Africa, and America (Dinda et al., 2016). *Cornus mas*, *C. officinalis*, *C. controversa*, and *C. kousa* have edible fruits consumed in different parts of Europe and Asia (Seeram et al., 2002). *C. mas* (cornelian cherry) (Figure 4.1) is native to southern Europe and southwest Asia (Rop et al., 2010). This plant comes from the foothills of the Caucasus and from there it spreads over Romania, Bulgaria, Italy, and Turkey, and to the inland European continent (Asadov et al., 1990). It is a small tree or medium to large deciduous shrub growing to 5-12 m tall with opposite leaves, dark brown branches, greenish twigs, tetramerous hermaphrodite yellow flowers on short stalks.

The flowers bloom in early spring before the leaves emerge in dense and olive-shaped single seeded fruits of 10-23 mm long. Ripe (cherry red or dark) fruits are used for fresh consumption or for the production of marmalade, jam, yoghurt, compote, liquor, soup with rice, syrup, juice, and wine (Brindza et al., 2007). According to "The Plant List" *C. mas* f. *conica* Jovan., *C. mas* var. *oblongifolia* Jovan., *C. mas* f. *oxycarpa* Jovan., *C. mas* var. *nana* Dippel, *C. mas* f. *macrocarpa* Dippel, *C. mas* f. *pyriformis* Sanadze and *C. mas* f. *microcarpa* Sanadze are synonims of *C. mas* (The Plant List., 2013)

Figure 4.1. *Cornus mas* (left) and *Cornus sanguinea* (right). (adapted by Encyclopedia of Life)

C. sanguinea (commonly known as European dogwood, blood twig dogwood, or common dogwood) is a species, widely distributed in the temperate regions of Europe (Krüsi et al., 1988). It is a small tree (1-5 m tall), with leaves that have a characteristic dark red color in senescence (Kollmann et al., 2001). It can be found in most vegetation types, but usually in different mixed temperate broad-leaved forests dominated by oak,

lime, maple, ash, elm and hornbeam. Usually, this species is cultivated for ornamental purposes for its decorative flowers and colourful leaves. Dogwood berries have a tart flavour that makes them unpleasant for raw consumption. They are used in jams and juices. According to "The Plant List" *C. sanguinea* L. is the only accepted name of the plant with two homotypic synonym *C. sanguinea subsp. czerniaewii* Grosset, *C. sanguinea subsp. sanguinea*, and five heterotypic synonym *C. citrifolia* Wahlenb., *C. latifolia* Bray, *C. sylvestris* Bubani, *Swida sanguinea* (L.) Opiz, *Thelycrania sanguinea* (L.) Fourr (The Plant List, 2013).

4.2. Traditional medicine uses

Fruits, leaves, and flowers of *C. mas* have been traditionally used for more than 1000 years for the treatment of measles, digestion problems, rickets, anaemia, hepatitis A, and pyelonephritis diseases (Table 4.1) (Asadov et al., 1990; Damirov et al., 1983; Lewis et al., 1977; Tzitzin et al., 1963).

Table 4.1. Ethno-medicinal uses of *Cornus* species.

	Traditional use	Country	Part used	Ref.
C. sanguinea				
	Diarrhea	Turkey	Fruits	Bulut, 2011
	Externally sore eyes, astringent	Serbia	Bark	Popović et al., 2014
C. mas				
	Colds and flu, urinary inflammation	Turkey	Fruits and leaves	Polat et al., 2013
	Urinary inflammation	Iran	Fruits and leaves	Zargari, 1996
	Asthmatic problems	Albania	Fruits	Pieroni et al., 2014
	Fever	Albania	Fruits	Pieroni et al., 2014
	Fever	Iran	Fruits	Zargari, 1996
	Fever	Slovakia	Fruits	Bertova, 1984
	Gastrointestinal disorders	Turkey	Fruits	Guler et al., 2015
	Diarrhea	Iran	Fruits	Zargari, 1996
	Diarrhea	Serbia	Fruits	Jaric et al., 2007; Savikin et al., 2013
	Diarrhea	Romania	Fruits	Tita et al., 2009
	Diarrhea	Azerbaijan	Fruits	Miraldi et al., 2001
	Cholera	Armenia	Fruits	Chevallier, 1996
	Wound healing, stomach ulcers and colitis	Iran, Azerbaijan, Armenia, Georgia and Turkey	Fruits	Damirov et al., 1983; Lewis et al., 1977; Tzitzin et al., 1963
	Rheumatism, anemia, blood circulation	Kasova	Fruits	Mustafa et al., 2012
	Gout, anemia, skin diseases,	Greece	Fruits	Reich, 1996
	Diabetes	Ukraine, Russia	Fruits	Sokolov et al., 1985
	Diabetes	Kasova	Fruits	Mustafa et al., 2012
	Diabetes	China	Fruits	Jia et al., 2003
	Bowel disease, malaria, kidney stone, cancer, sunstroke, kidney infections	Iran	Fruits	Zargari, 1996
	Digestive disorders and inflammation	Slovakia	Fruits	Bertova, 1984

Digestive disorders and inflammation	Albania	Fruits	Pieroni et al., 2014
Obesity	Croatia	Fruits	Pieroni et al., 2003
Bruises and Headache	Croatia	Fruits	Pieroni et al., 2003
Laxative	Serbia	Fruits	Jaric et al., 2007
Cough	Turkey	Fruits	Genc et al., 2006
Excessive urination, sweating and menstrual bleeding	USA	Fruits	McGuffin et al., 1997
Cosmetic to exert favourable human complexion	Italy	Fruits	Polinicencu et al., 1980
Dyspepsia and colitis	Italy	Fruits	Egea et al., 2015
Appetizer	Italy	Fruits	Di Novella et al., 2013
Bronchitis	Turkey	Fruits	Altundag et al., 2011
Constipation	Turkey	Fruits	Baytop, 1963
Kidney function	China	Fruits	Hsu et al., 2014
Removal of kidney stones and rheumatic disorders	Albania	Fruits	Rexhepi et al., 2013
Vermifuge, febrifuge, dysentery	Romania	Fruits, leaves and bark	Tita et al., 2009
Gastrointestinal disorders, tuberculosis, menstrual problems	Greece	Fruits, leaves and bark	Reich, 1996
Diarrhea, intense menstrual bleeding, skin ailments	Bosnia and Herzegovina	Fruits and bark	Saric- Kundalic et al., 2011
Intestinal diseases, anemia, immune system strengthening	Serbia	Fruits and flowers	Zlatkovic et al., 2014
Sore throat, digestion problems, measles, chicken pox, anemia, rickets	Azerbaijan, Russia	Fruits, leaves and flowers	Damirov et al., 1983; Tzitzin et al., 1963
Diarrhea, diabetes	Turkey	Leaves	Yesilada et al., 1999
Cardiac problems	Romania	Leaves	Dragan et al., 2014
Diabetes	Turkey	Seed	Genc et al., 2006
Antipyretic	Azerbaijan	Bark	Miraldi et al., 2001

C. mas is used in Turkey and Azerbaijan to treat diarrhoea, haemorrhoids, and gastrointestinal disorders, in Slovakia to treat fever, digestive disorders, and inflammation, and in Iran for the treatment of malaria, diarrhoea, inflammatory bowel disease, fever, kidney stones, urinary tract infections, and cancer (Asadov et al., 1990; Bertova, 1984; Celik et al., 2006; Damirov et al., 1983; Zargari, 1996).

C. sanguinea is used for the treatment of externally sore eyes and gastrointestinal disorders (Bulut, 2011; Popović et al., 2014).

4.3. Chemical constituents

Phytochemical investigations of *Cornus* species revealed the presence of polyphenols (Forman et al., 2015b), anthocyanins (Vareed et al., 2006), flavonoids (Pawlowska et al., 2010), iridoids (Deng et al., 2013), and carboxylic acids as the most abundant compounds (Krivoruchko, 2014). In literature, there are several works in which the chemical composition and the biological activity of *Cornus* species are previous described, with the exception of *C. controversa*.

Herein we decided to report data only on *C. mas* and *C. sanguinea* because of their distribution in Europe.

Few studies are present in literature on *C. sanguinea*. Therefore, its chemical and biological evaluation attract scientific interest. Flavonoids, iridoids, monoterpenes, triterpenes, and fatty acids are the main classes of phytochemicals identified in both *C. mas* fruits and leaves (Table 4.2).

Essential oil. Only the essential oil of *C. mas* has been analysed. Monoterpenoids are the most abundant constituents of the essential oil of *C. mas* flowers. Generally, the major compound is camphor (9.1-16.2%), followed by verbenone (5.7-14.8%). Salycilic acid in found in the essential oil of *C. mas* flowers (Krivoruchko et al., 2011). In addition, also hydrocarbons (undecane, pentadecane, and dodecane) are described in the essential oil.

Carbohydrates. Glucose, fructose and sucrose are present in high concentration in the hydroalcoholic extract of *C. mas* fruits. Major carbohydrate is glucose, followed by fructose and sucrose (Bijelic et al., 2011; Perova et al., 2014).

Minerals. *C. mas* fruits are rich in potassium, calcium, magnesium and sodium content, other minerals as phosphorus, iron, zinc, copper and manganese in smaller quantities. A variability was found in dependence of the site of collection (Bijelic et al., 2011; Dokoupil et al., 2012; Sotiropoulos et al., 2011; Juranovic-Cindric et al., 2012). For example, fruits from Czech Republic showed a variability in sodium (82-58 mg/kg), potassium (3798-3411 mg/kg), calcium (656-301 mg/kg), magnesium (290-241 mg/kg) depending of type of cultivars (Dokoupil et al., 2012). Fruits from the cultivars of Serbia had 315-40 mg/kg of sodium, 5609-1845 mg/kg of potassium, 466-27 mg/kg of calcium, 161-10 mg/kg of magnesium (Bijelic et al., 2011).

A variability in mineral content was found also in the samples collected in Croatia and Greece. In the *C. mas* fruits collected in Croatia, main mineral is potassium (4019 mg/kg), followed by calcium (2074 mg/kg), magnesium (288 mg/kg) and sodium (22.9 mg/kg; Juranovic-Cindric et al., 2012). A different composition is reported for fruits from Greece, potassium (1320-880 mg/kg), phosphorus (90-80 mg/kg), magnesium (50-40 mg/kg), iron (45-19 mg/kg) and calcium (30-20 mg/kg) as main minerals identified (Sotiropoulos et al., 2011). Compared with other fruits juices (pear, plum and apple juices), the *C. mas* fruits juice resulted more rich in various minerals as potassium, calcium, sodium, iron, zinc, copper and manganese (Krosniak et al., 2010).

Fatty acids. Seven fatty acids are identified in both fruits and leaves of *C. mas*. Linoleic acid and oleic acid are the most abundant in the fruits. The main fatty acid in the leaves is 2,4-heptadienoic acid, followed by palmitic acid (Krivoruchko, 2014).

Table 4.2. The main chemical constituents of *C. mas and C. sanguinea*.

	Class	Chemical constituent	Part of plant	Reference
C. mas				
	Anthocyanins	Cyanidin 3-*O*-galactoside, cyanidin 3-*O*-glucoside cyanidin 3-*O*-robinobioside, cyanidin 3-*O*-rutinoside, delphinidin-3-*O*-galactoside, pelargonidin 3-*O*-galactoside, pelargonidin 3-*O*-glucoside, pelargonidin 3-*O*-robinobioside, pelargonidin 3-*O*-rutinoside, peonidin 3-*O*-glucoside	Fruits	Jayaprakasam et al., 2006; Pawlowska et al., 2010; Sozanski et al., 2014; Tural et al., 2008
	Acids	Quinic acid, shikimic acid	Fruits	Drkenda et al., 2014
	Carbohydrates	Glucose, fructose, sucrose	Fruits	Bijelic et al., 2011; Perova et al., 2014
	Carotenoids	*β*-Carotene, *β*-Carotene-5,6-monoxide, *β*-Cryptoxanthin, lutein, lutein-5,6-epoxide, (9Z, 9'Z)-lutein, (13Z, 13'Z)-lutein, (all-E)-neoxanthin, (9'Z)-neoxanthin, luteoxanthin	Fruits	Horvath et al., 2007
	Fatty acids	Lauric acid, myristic acid, pentadecenoic acid, vaccenic acid	Fruits	Brindza et al., 2007
		α-Linoleic acid, oleic acid, α-Linolenic acid, palmitoleic acid, palmitic acid, stearic acid, 2,4-heptadienoic acid	Fruits and leaves	Krivoruchko, 2014
	Hydrocarbons	Undecane, pentadecane, dodecane	Flowers	Krivoruchko et al., 2011
	Iridoids	Loganin, loganic acid, cornuside, sweroside	Fruits	Deng et al., 2013; Szumny et al., 2015
		Secologanin	Leaves	Jensen et al., 1973
	Phenolic acid	Gallic acid, ellagic acid	Fruits, leaves and flowers	Deng et al., 2013; Milenkovic-Andjelkovic et al., 2015; Savikin et al., 2009
		Neochlorogenic acid	Fruits	Szummy et al., 2015
		Chlorogenic acid	Fruits and leaves	Deng et al., 2013; Milenkovic-Andjelkovic et al., 2015; Szummy et al., 2015
		Ferulic acid, vanillic acid	Fruits	Krivoruchko et al., 2014
		p-Coumaric acid	Fruits	Behrangi et al., 2015

		Salicylic acid	Flowers	Krivoruchko et al., 2011
	Flavonoids	Quercetin 3-*O*-xyloside, quercetin 3-*O*-rhamnoside, quercetin 3-*O*-galactoside, kaempferol 3-*O*-galactoside, aromadendrin 7-*O*-glucoside	Fruits	Pawlowska et al., 2010
		Quercetin 3-*O*-glucoside, quercetin 3-*O*-glucuronide, kaempferol 3-*O*-glucoside, Catechin	Fruits and leaves	Badalica- Petrescu et al., 2014; Milenkovic-Andjelkovic et al., 2015; Pawlowska et al., 2010
		Quercetin 3-O-robinobioside	Fruits	Drkenda et al., 2014
		Quercetin 3-*O*-rutinoside (=rutin)	Fruits, leaves and flowers	Milenkovic-Andjelkovic et al., 2015; Pawlowska et al., 2010; Savikin et al., 2009
		Kaempferol 3-*O*-glucuronide, isorhamnetin 7-*O*-rhamnoside, quercetin 3-*O*-galactosyl 7-*O*-rhamnoside	Leaves	Badalica-Petrescu et al., 2014
		Quercetin	Fruits	Sochor et al., 2014
		Aromadendrin, myricetin, naringenin 3-*O*-methyl ester, 7,3'-dihydroxy-5,4'-dimethoxyflavanone, 4-acetoxy-5,2',4',6'-β-pentahydroxy-3- methoxychalcone	Fruits	Rudrapaul et al., 2015
	Minerals	Potassium, calcium, magnesium and sodium, phosphorus, iron, zinc, copper, manganese	Fruits	Bijelic et al., 2011
	Monoterpenoids	Borneol, camphor, carvacrol, carvone, limonene, verbenone, *β*-thujone, α-terpeneol, 1,8-cineol	Flowers	Krivoruchko et al., 2011
	Proanthocyanidins	Epicatechin, epicatechin- 4,8-epicatechin, epicatechin-4,6-catechin	Fruits and leaves	Badalica-Petrescu et al., 2014; Milenkovic-Andjelkovic et al., 2015
	Triterpenoid	Ursolic acid	Fruits and flowers	Jayaprakasam et al., 2006; Savikin et al., 2009
	Vitamins	Ascorbic acid, *a*-tocopherol, biotin, riboflavin	Fruits	Zargari (1997)
C. sanguinea				
	Flavonoids	Quercetin 3-*O*-rhamnoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-galactoside,	Fruits	Popović et al., 2017

	quercetin 3-*O*-glucuronide, quercetin 3-*O*-rutinoside

Vitamins. Vitamins identified in the aqueous extracts of *C. mas* fruits are ascorbic acid, α-tocopherol, biotin (vitamin B_8), and riboflavin (vitamin B_2) (Figure 4.2). Their concentration varies in function of cultivar and climatic conditions (Zargari, 1997).

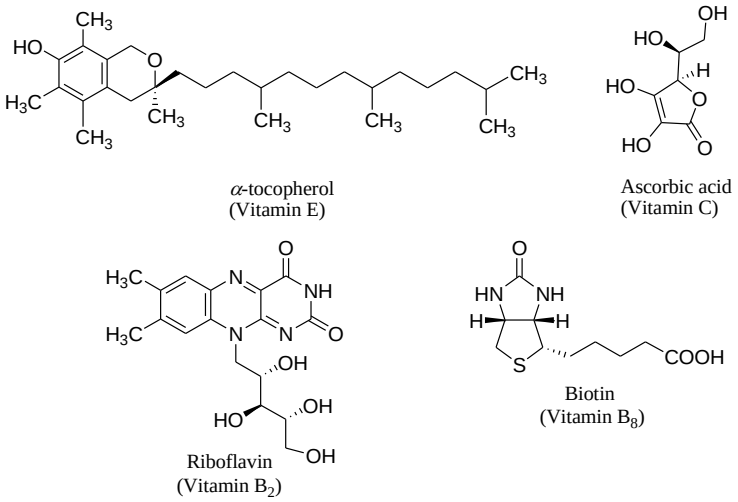


Figure 4.2. Vitamins found in aqueous extract of *C. mas*

Carotenoids. Ten carotenoids (Figures 4.3 and 4.4), namely β-carotene, β-carotene-5,6-monoxide, β-cryptoxanthin, lutein, lutein-5,6-epoxide, (9*Z*, 9'*Z*)-lutein, (13*Z*, 13'*Z*)-lutein, (all-*E*)-neoxanthin, (9'*Z*)-neoxanthin, luteoxanthin are reported in the hydroalcoholic extract of *C. mas* fruits treated with hexane (Horvath et al., 2007).

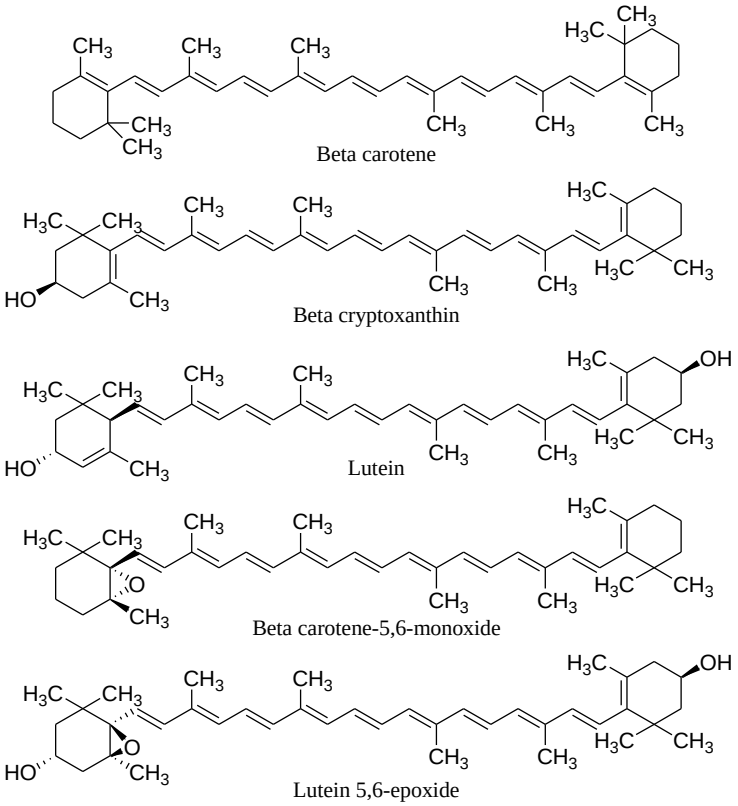


Figure 4.3. Carotenoids identified in *C. mas*

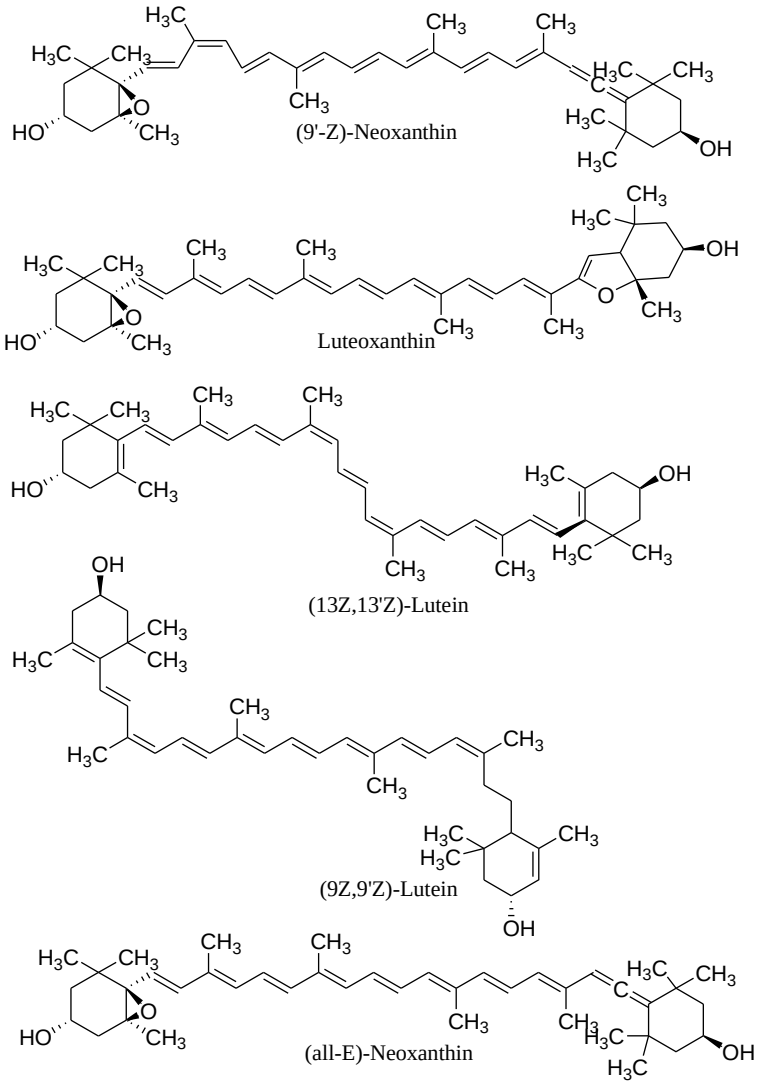


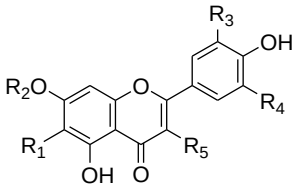
Figure 4.4. Carotenoids identified in *C. mas* (*continued*)

Flavonoids and proanthocyanidins. Among flavonoids, flavonol glycosides are the major constituents (Figures 4.5 and 4.6). Most of these compounds contain kaempferol or quercetin as aglycone. Quercetin 3-*O*-glucuronide is the major constituent of methanol extract of *C. mas* fruits and aqueous extract of leaves (Badalica-Petrescu et al., 2014; Pawlowska et al., 2010). Some of those flavonoids are specifically found in fruits as aromadendrin, myricetin, naringenin 3-*O*-methyl ester, 7,3'-dihydroxy-5,4'-dimethoxyflavanone, 4-acetoxy-5,2',4',6'-β-pentahydroxy-3-methoxychalcone.

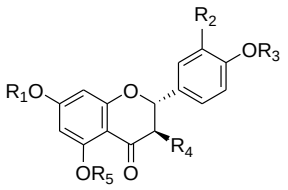
Among proanthocyanidins and flavonoids, catechin is predominant in hydroalcoholic fruits extract, followed by epicatechin and procyanidin B2, while, epicatechin is the most abundant compound in aqueous and hydroalcoholic leaves extracts (Badalica-Petrescu et al., 2014; Milenkovic-Andjelkovic et al., 2015).

The only study that investigated the chemical profile of *C. sanguinea* reported the presence of quercetin 3-*O*-glucuronide, followed by quercetin 3-*O*-glucoside, and

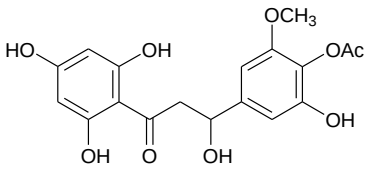
quercetin 3-*O*-galactoside, quercetin 3-*O*-rutinoside, and quercetin 3-*O*-rhamnoside in methanol extract of *C. sanguinea* fruits (Popović et al., 2017).



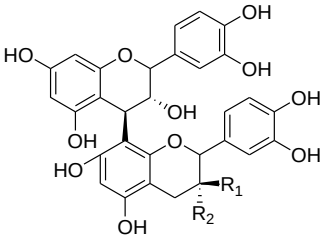
	R_1	R_2	R_3	R_4	R_5
Hyperoside	H	H	OH	H	*O*-Gal
Isoquercitrin	H	H	OH	H	*O*-Glc
Isorhamnetin 7-*O*-rhamnoside	Glc	Rha	OCH_3	H	H
Kaempferol 3-*O*-galactoside	H	H	H	H	*O*-Gal
Kaempferol 3-*O*-glucoside	H	H	H	H	*O*-Glc
Myricetin	H	H	OH	OH	OH
Quercetin	H	H	OH	H	OH
Quercetin 3-*O*-galactosyl 7-*O*-rhamnopyranoside	H	Rha	OH	H	*O*-Gal
Quercetin 3-O-glucuronide	H	H	OH	H	*O*-Glc A
Quercetin 3-*O*--rhamnopyranoside	H	H	OH	H	*O*-Rha
Quercetin 3-O-robinobioside	H	H	OH	H	*O*-Gal(6←1)Rha
Quercetin 3-*O*-xyloside	H	H	OH	H	*O*-Xyl
Rutin	H	H	OH	H	*O*-Glc(6←1)Rha



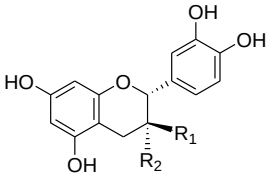
	R_1	R_2	R_3	R_4	R_5
Aromadendrin	H	H	H	OH	H
Aromadendrin 7-O-glucoside	Glc	H	H	OH	H
Naringenin 7-*O*-methyl ether	CH_3	H	H	H	H
7,3'-Dihydroxy-5,4'-dimethoxyflavanone	H	OH	CH_3	H	CH_3



4-Acetoxy-5,2',4',6'-β-pentahydroxy-3-methoxychalcone



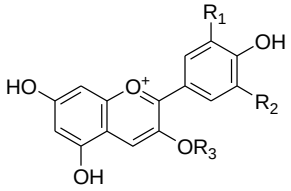
	R_1	R_2
Epicatechin-4,8-epicatechin	H	OH
Epicatechin-4,6-catechin	OH	H



	R1	R2
Catechin	OH	H
Epicatechin	H	OH

Figure 4.5. The main flavonoids and proanthocyanidins identified in *C. mas*

Anthocyanins. Among anthocyanins, cyanidin 3-*O*-galactoside and pelargonidin 3-*O*-galactoside are the most abundant in the acidified hydroalcoholic extract of *C. mas* fruits (Pawlowska et al., 2010; Sozanski et al., 2014; Jayaprakasam et al., 2006). Peonidin 3-*O*-glucoside and cyanidin 3-*O*-galactoside are the major components of acidified aqueous extract of *C. mas* fruits collected in Bosnia and Herzegovina (Drkenda et al., 2014). Other anthocyanins found in the *C. mas* fruits are cyanidin 3-*O*-robinobioside, cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, pelargonidin 3-*O*-robinobioside, pelargonidin 3-*O*-glucoside, pelargonidin 3-*O*-rutinoside, delphinidin 3-*O*-galactoside (Sozanski et al., 2014; Tural et al., 2008). Anthocyanins isolated in *C. mas* were reported in Figure 4.6.



	R_1	R_2	R_3
Cyanidin 3-*O*-galactoside	OH	H	Gal
Cyanidin 3-*O*-glucoside	OH	H	Glc
Cyanidin 3-*O*-robinobioside	OH	H	Gal(6←1)Rha
Cyanidin 3-*O*-rutinoside	OH	H	Glc(6←1)Rha
Delphinidin 3-*O*-galactoside	OH	OH	Gal
Peonidin 3-*O*-glucoside	OCH_3	H	Glc
Pelargonidin 3-*O*-galactoside	H	H	Gal
Pelargonidin 3-*O*-glucoside	H	H	Glc
Pelargonidin 3-*O*-robinobioside	H	H	Gal(6←1)Rha
Pelargonidin 3-*O*-rutinoside	H	H	Glc(6←1)Rha

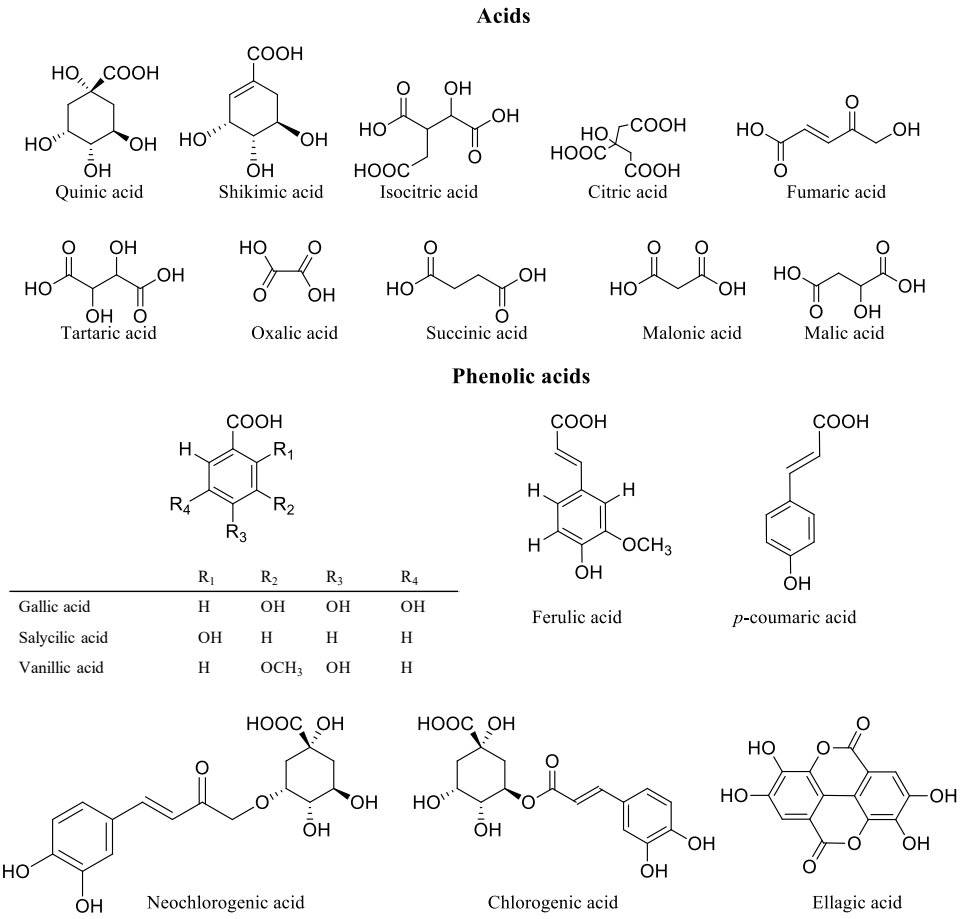
Figure 4.6. The main anthocyanins of *C. mas*

Acids and phenolic acids. Ten organic acids, namely maleic acid, oxalic acid, succinic acid, citric acid, tartaric acid, fumaric acid, malonic acid, isocitric acid, quinic acid, and shikimic acid, are identified in fruits and leaves extracts (Drkenda et al., 2014; Ognjanov et al., 2009; Hassanpour et al., 2012).

Malic acid, followed by tartaric acid, and citric acid are the principal acids reported in the acidified aqueous extract of *C. mas* fruits collected in Bosnia and Herzegovina (Drkenda et al., 2014). The fruits of *C. mas* collected in Czech Republic possessed citric acid a the major organic acid (Sochor et al., 2014). Ellagic acid is main phenolic acid found in hydroalcoholic extracts of *C. mas* fruits and leaves collected in Serbia, together with gallic acid (Leskovac et al., 2007; Milenkovic-Andjelkovic et al., 2015) while fruits from Czech Republic reported chlorogenic acid as principal phenolic acid.

Other phenolic acids, neochlorogenic acid, *p*-coumaric acid, vanillic acid, and ferulic acid are reported in the methanol extract of *C. mas* fruits (Behrangi et al., 2015;

Krivoruchko et al., 2014; Szummy et al., 2015). Structure of acids and phenolic acids found in the *C. mas* extracts were summarised in Figure 4.7.

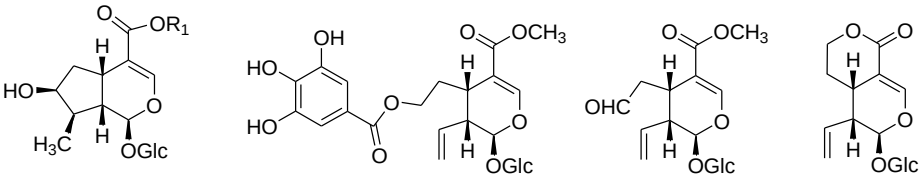


	R_1	R_2	R_3	R_4
Gallic acid	H	OH	OH	OH
Salycilic acid	OH	H	H	H
Vanillic acid	H	OCH_3	OH	H

Figure 4.7. The main acids and phenolic acids of *C. mas*

Iridoids. Five iridoids (Figure 4.8) which three of them are secoiridoids are identified in *C. mas*. Loganic acid, cornuside, loganin, and sweroside were detected in hydroalcoholic extract of fruits and in the juice (Deng et al., 2013; Szumny et al., 2015).

Secologanin was found in the leaves (Jensen et al., 1973).



	R_1
Loganin	CH_3
Loganic acid	H

Figure 4.8. The main iridoids of *C. mas*

4.4. Biological activities

C. mas possess several biological activities such as antioxidant, antibacterial, antidiabetic, hypolipidemic, anti-inflammatory, anticancer, and anticoagulant properties (Dinda et al., 2016). Conversely, few studies assessed the biological properties of *C. sanguinea* (Hozoori et al., 2012; Stanković et al., 2012).

4.4.1. Antioxidant activity

C. mas fruits are a good source of antioxidants (Behrangi et al., 2015; Demir et al., 2003; Pantelidis et al., 2007; Popović et al., 2012; Rop et al., 2010; Serteser et al., 2009; Tural et al., 2008). In the study of Serteser et al. (2009), hydroalcoholic extracts of fruits and leaves from *C. mas* plants collected in Turkey were analysed. Both fruits and leaves reported an interesting inhibition against DPPH radical and H_2O_2 activity. Furthermore, these extracts presented high Fe^{2+} chelating activity with percentages of 54.24 and 65.42%, respectively. Hydroalcoholic extract obtained by *C. mas* fruits from Northern Greece showed high protection (98.6%) in the deoxyribose protein assay. Similar value to that of blackberry cultivar "Hull thornless" (red-black, *Rubus fruticosus*) (98.9%) (Pantelidis et al., 2007).

The antioxidant activity is influenced by the phytochemicals content that in turn is influenced by different factors including genotype, site of collection, maturity stage, climatic conditions, and extraction procedures. Methanolic extracts of *C. mas* fruits air dried at 55 °C showed low FRAP value (83.9 μM ascorbic acid equivalent (AAE)/g, dry weight (DW)), compared with lyophilized fruits (FRAP value of 190 μM AAE/g, DW) (Pantelidis et al., 2007; Behrangi et al., 2015).

In drying process (60 °C), there is a decomposition of bioactive compounds as anthocyanins and ascorbic acid with reducing of antioxidant activity. Tural et al. (2008) investigated various *C. mas* genotypes of Turkey and reported different values (16.21-94.43 μM AAE/g, FW) in FRAP assay, in addition the presence of pelargonidin 3-*O*-glucoside was the main pigment found in this fruits. Stanković et al., (2014) used solvents of different polarity, such as methanol, water, ethyl acetate, petroleum ether, and acetone to obtain different *C. mas* extracts. The highest antioxidant activity was reported for the

methanol extract with IC_{50} values of 39.40 and 27.58 μg/mL, respectively, for leaves and flowers extracts. The activity reported for flowers could be associated to high phenolic content (187.94 mg gallic acid equivalent/g). In the CCl_4-treated rats, the methanol extract of the leaves of *C. mas* increased the antioxidant enzymes activities and decrease the level of lipids peroxidation. This trend is confirmed in the normal healthy rats, reporting the increase of antioxidant capacities The antioxidant activity of methanol extract of the leaves of *C. mas* was resulted of gallic acid presence, isolated in this extract as bioactive compound (Celep et al., 2013).

Serteser et al. (2009) compared the antioxidant activity of hydroalcoholic leaves and fruits extracts of *C. sanguinea* with other plants collected in Turkey, demonstrating that *C. sanguinea* extracts have high DPPH radical scavenging activity, with an IC_{50} values of 1.48 mg/g and 1.2 mg/g for leaves and fruits, respectively. Similar trend was observed with Fe^{2+} chelating and H_2O_2 inhibition activities. The hydroalcoholic extracts of fruits and leaves of *C. sanguinea* exhibited percentages of 51.2 and 44.6%, respectively, of Fe^{2+} chelating activity. Moreover, these extracts resulted more active in the H_2O_2 inhibition test than extracts of other plants. The methanol extract of *C. sanguinea* fruits collected in Iran showed an interesting antioxidant activity in DPPH test with an IC_{50} value of 94.83 μg/mL (Hozoori et al., 2012). *C. sanguinea* leaves were more active in DPPH test in comparison to the fruits (Stanković et al., 2012). In particular, the methanol and water extracts showed IC_{50} values of 19.84 and 22.37 μg/mL, respectively.

4.4.2. Antidiabetic and anti-obesity activities

The hydroalcoholic extract of *C. mas* fruits (100 mg/kg, i.p.) showed antidiabetic activity in alloxan-induced diabetic rats by reduction of serum glucose, low density lypoproteins (LDL), triglycerides (TG), and very low density lipoprotein (VLDL) levels, and by increasing high density lypoproteins (HDL) level in comparison to the control group (Mirbadalzadeh et al., 2012).

The oral administration of *C. mas* fruits (5, 10 and 15 g/meal) as diet supplement for 20 days in hamsters decreased the body weight and increased the insulin levels (Rasoulian et al., 2012). In another work, the oral administration in alloxan-induced diabetic rats of *C. mas* fruits (2 g/kg/d) for 4 weeks exhibited antidiabetic effects comparable to that of the positive control glibenclamide (0.6 mg/kg/d) (Asgary et al., 2014). In diabetic rats, the administration of *C. mas* fruits powder (2 g/kg/d) for 4 weeks showed an improvement of pancreatic damage (Shamsi et al., 2011).

In a clinical study, 60 patients having type-2 diabetes were randomly assigned to two groups that receive *C. mas* anthocyanins-rich extract or placebo capsules (2 capsules twice daily after main food) orally for 6 weeks along with their usual diet and physical activity. Each capsule contained 150 mg of anthocyanins isolated from the aqueous ethanol extract of *C. mas* fresh fruits. After 6 weeks, significant increase in insulin level and decrease in HgbAIC, and TG levels were observed in treated group in comparison to placebo. These results suggested that the consumption of *C. mas* fruits might improve the

glucose intolerance in type-2 diabetic patients by increasing the insulin levels and by reducing the HgbAIC and TG levels (Soltani et al., 2015). Anthocyanins and ursolic acid were responsible to antidiabetic and anti-obesity activities reported by *C. mas* fruits. Anthocyanins were able to reduce significantly the development of obesity through suppressed of fat accumulation increased the enzyme activity responsible to lipolysis of triglicerides (Tsuda et al., 2003, 2005). In another study (Jayaprakasam et al., 2005) cyanidin and delphinidin glucosides stimulated *in vitro* the insulin production. Through phosphorilation of receptor and stimulation of glucose intake by tissues, ursolic acid decrease the blood glucose (Zhang et al., 2006).

4.4.3. Hypolipidemic and anti-atherosclerotic properties

The effects of *C. mas* fruits administration on hypercholesterolemic rabbits were evaluated by Asgary et al. (2010). This study has demonstrated that a somministration of *C. mas* fruits (1 g/kg b.w./d for 60 days) decrease of serum fibrinogen level. This effect was better than the effect of anti-fibrinogenic drug, lovastatin (10 mg/kg b.w./d for 60 day). Therefore, the consumption of *C. mas* fruits as diet supplement might be beneficial to reduce their risk of cardiovascular diseases in atherosclerotic patients. Also, the same study was do with rabbits with same dose of fruits was do for evaluated atherogenic index parameter and it was observed a significant decrease of LDL, MDA, TG, TC, fibrinogen and atherogenic index parameter and also un increase of antioxidant activity in this group compared with the group of hypo-chlolesterolemic of rabbits. Probably, this effect is related to the quantity of phenolic compounds and vitamins (ascorbic acid and tocopherol) (Rafieian-Kopaei et al., 2011).

In another work (Sozanski et al., 2014), the protective effect of *C. mas* fruits against atherosclerosis and hypertriglyceridemia was investigated in a rabbit model by oral administration of fruits (100 mg/kg body weight (b.w.)) or simvastatin (5 mg/kg b.w.) for 60 days. A significant increase of liver peroxisome proliferator-activated receptor α (PPARα) protein expression and a significant decrease of serum triglycerides level (44%), pro-inflammatory cytokines, IL-6, and TNF-α was demonstrated. Based on these results, *C. mas* fruits showed protective effects against diet-induced hypertriglyceridemia and atherosclerosis by across increased PPARα protein expression and by regulating oxidative stress and inflammation (Sozanski et al., 2014). In fact, it was demonstrated that the activation of PPARα leads to increased tissue-specific expression of crucial genes that are involved in fatty acids uptake and β-oxidation (Fruchart, 2009). PPARα decreases triglycerides by increasing free fatty acid β-oxidation, hepatic lipoprotein lipase expression, and expression of apolipoprotein V, and by decreasing expression of apolipoprotein CIII. Furthermore, PPARα activation regulates HDL metabolism by stimulating hepatic expression of apoA-I and -II and thus raising HDL production in the liver, promoting HDL-mediated cholesterol efflux from macrophages, and inhibiting cellular cholesteryl ester formation activity, as well as contributing to enhanced efflux of

free cholesterol to extracellular receptors. PPARα activation may influence the development of atherosclerosis indirectly through effects on glucose and lipid homeostasis in adipose tissue, liver and skeletal muscle. In addition, PPARα activation may have a direct effect on inflammation by modification of NF-κB and activator protein-1 (AP-1).

Through the regulation of oxidative stress and inflammation, loganin and cornuside could be responsible to the hypolipidemic activity of extract. Loganin reported anti-inflammatory capacities in various disease models including acute pancreatitis and Parkinson's disease (Kim et al., 2015; Xu et al., 2017). The anti-inflammatory effects of loganin was ascribed to the inhibition of inflammatory cytokines and deactivation of the NF-kB signaling pathway. Moreover, it was demonstrated that the administration of loganin can improve memory enhancing long term memory in hippocampal tissues (Hwang et al., 2017).

Cornuside demonstrated to dilate vascular smooth muscle through endothelium dependent nitric oxide signaling (Kang et al., 2007a) and showed anti-inflammatory activity in LPS-induced inflammation model *via* the inhibition of NF-κB activity (Choi et al., 2011). Previously, Kang et al. (2007b) observed that the addition of cornuside to human umbilical vein endothelial cell cultures reduced the mRNA expression of VCAM-1 and ICAM-1, and diminished the TNFα-induced NF-κB activation. In the carrageenan-induced mouse paw edema model, loganic acid exhibited a strong anti-inflammatory activity (Recio et al., 1994). Loganic acid and cornuside showed increase of PPAR-α levels with reducing atherosclerotic plaque formation in cardiovascular diseases (Gervois et al., 2012).

The hypocortisol and hypolipidemic effects of *C. mas* fruits were studied in hamsters model (Lotfi et al., 2014). Different supplementations (5-15 g/daily) of *C. mas* fruits were evaluated. The highest dose allows decreasing the levels of cholesterol (108.3 mg/mL) and LDL (21 mg/mL), and allows increasing HDL (54 mg/mL) and triglycerides (191.7 mg/mL) levels, compared with untreated hamsters. The hamsters treated with *C. mas* fruits showed also a decrease of cortisol levels (28.8 ng/mL) compared with control (45.8 ng/mL).

4.4.4. Antimicrobial activity

The hexane extract of *C. mas* fruits showed significant antibacterial activity against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* at the tested dose of 10 μL/disc (Mamedov et al., 2004). Methanol and ethanol extracts of *C. mas* fruits, leaves, seeds, and bark revealed antibacterial activity against *E. coli*, *S. aureus*, and *Pseudomonas aeruginosa* and antifungal activity against *Candida albicans* and *Aspergillus fumigates* (Krzysciak et al., 2011). In another study, the hydroalcoholic extracts from the fruits and leaves of *C. mas* exhibited antimicrobial activity against 13 species of bacteria and yeast. Gram-positive strains *Listeria monocytogenes*, *Clostridium*

perfringens, *Staphylococcus aureus*, and *Sarcina lutea*, and Gram-negative strains *Proteus vulgaris*, *Shigella sonnei*, and *Salmonella enteritidis* are the most sensitive (Milenkovic-Andjelkovic et al., 2015). The antimicrobial activity of these extracts could be related with their high total phenols content. The antibacterial activity justified the use in traditional medicine of *C. mas* fruits in the treatment of gastrointestinal disorders and diarrhea, skin diseases, and urinary infections (Balcht et al., 1994; Hansson et al., 1995; Kyriakopoulos et al., 2015; Tong et al., 2015).

4.4.5. Other activities

The hydroalcoholic extract of *C. mas* fruits (5 μg/mL) exhibited cytotoxic activity against breast adenocarcinoma (MCF-7), prostate adenocarcinoma (PC-3), ovarian cancer (SKOV-3), and non-small cell lung cancer (A549) cells (Yousefi et al., 2015).

The mean growth inhibition was 81.8, 81.9, 81.6, and 79.3% in SKOV3, MCF-7, PC-3, and A549 cell lines, respectively. Previously, the methanol extract of leaves and flowers of *C. mas* and the isolated compounds gallic acid, ellagic acid, and ursolic acid showed a moderate cytotoxic activity against human colon carcinoma (LS174) and human cervix adenocarcinoma (HeLa) cell lines (Savikin et al., 2009). The most active constituents were ursolic acid and gallic acid with IC_{50} values of 0.7 and 2.6 μg/mL against HeLa and LS174 cells, respectively.

The aqueous extract of *C. mas* leaves showed cytotoxicity against MCF-7 cells at 750 μg/mL. (Forman et al., 2015b). The administration of freeze-dried *C. mas* fruits protected the brain tissue by reducing the quantity of free radicals generated in the brain tissue. These effects were demonstrated in three types of diet-control, fructose and high fat-diets in rats with an increased activity of catalase and paraoxonase (PON) in brain tissue and decreased levels of protein carbonyl group and thiol groups in brain tissue as well as in plasma (Francik et al., 2014). PON is responsible for anti-atherogenic of HDL in blood and anti-inflammatory properties and prevented the oxidation of lipoproteins (Unsal et al., 2013). Probably, phenolic compounds and ascorbic acid in *C. mas* fruits play a significant role of increase the PON-1 levels (Jarvik et al., 2002).

The hydroalcoholic extract of *C. mas* fruits (200 and 500 mg/kg b.w.) displayed significant hepatoprotective effects in CCl_4-induced hepatotoxicity in rats by ameliorating the levels of AST (aspartate transaminase), ALT (alanine transaminase) and ALP (alkaline phosphatase) and by reducing the oxidative stress and elevated hepatic MDA (malondialdehyde) content (Alavian et al., 2014). Another research group demonstrated that the hydroalcoholic extract of *C. mas* fruits showed renal-protective effects in CCl4-induced nephrotoxicity in rats by improving renal lesions, antioxidant enzymes, lipid peroxidation-induced MDA levels, and other biochemical parameters including creatinine, uric acid and serum urea levels (Haghi et al., 2014).

Oral administration of hydroalcoholic extract of *C. mas* fruits (50, 200 and 400 mg/kg b.w./d) for 3 weeks in male rats exhibited a significant reduce in the platelet distribution width (PDW) (Abdollahi et al., 2014), a specific marker of platelet activation (Vagdatli

et al., 2010). Because of blood coagulation, there is an increase of PDW that decrease with the inactivation of platelets. Pre- and post- treatments with the hydroalcoholic extract of *C. mas* fruits at dose of 300 and 700 mg/kg b.w./d, for 16 day, in CCl4-induced toxicated rats showed cardioprotective effects by attenuating myocardial lipid peroxidation level and recovering enzymatic defence system by increasing the levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and modulating the bioenergetics state of cardiac tissue (Haghi et al., 2012).

The phenols-rich leaves extract of *C. mas* (0.01 mg/mL) demonstrated cardioprotective property on neonatal rat cardiomyocytes in both normoxic and hypoxic conditions by ameliorating cell viability of 19 and 11%, respectively (Dragan et al., 2014). The administration of loganic acid, isolated from *C. mas* fruits, at dose of 0.7% aqueous solution containing 0.15% sodium hyaluronate significantly reduced (25%) the conjunctivitis-induced intraocular pressure (IOP) of eye in rabbit model (Szumny et al., 2015). This effect, with increase of blood flow in the eye, could be beneficial in the treatment of diabetic retinopathic and venous thrombotic patients. Probably, the decrease of IOP is ascribed to reduction of nitric oxide (Chiou et al., 2011; Lv et al., 2012). The same effects were observed in glaucoma treated with loganic acid. Glaucoma is characterised by high levels of nitric oxide that decrease after loganic acid treatment (Chiou et al., 2011; Lv et al., 2012).

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Chapter 5

Oxidative stress, inflammation, and diabetes: beneficial effects of flavonoids and iridoids

5.1. Introduction

Increasing evidences have suggested that the cellular damage arising from reactive oxygen species (ROS) is one of the essential mechanisms in the pathogenesis of a number of human diseases with high social impact including diabetes, atherosclerosis, cancer, obesity, dementia, and metabolic syndrome (Krishnaiah et al., 2011).

The term oxidative stress refers to excessive production of ROS (Ito et al., 2019a). ROS are radical derivatives such as superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen, produced in the normal cellular metabolism that have a crucial role in activation of signaling pathways in plant and animal cells, which alter the intra- and extracellular metabolism. In the mithocondrial respiratory chain, the majority of ROS are produced (Poyton et al., 2009; Reuter et al., 2010). In the aerobic cells, ROS are produced by reduction of molecular oxygen during endogenous metabolic reactions (Figure 5.1).

In the state of oxygen deficiency, the mitochondrial respiratory chain also produced nitric oxide, that it is the responsible for the production of reactive nitrogen species (RNS) (Poyton et al., 2009). The formation of ROS is amplified when the body is under a stress situation, but it is induced, also, by exogenous factors such as UV radiations (Black, 1987), air polluants (Boffetta et al., 2003), tobacco (Youn et al., 1992), and industrialized lifestyle (Grattagliano et al., 2008).

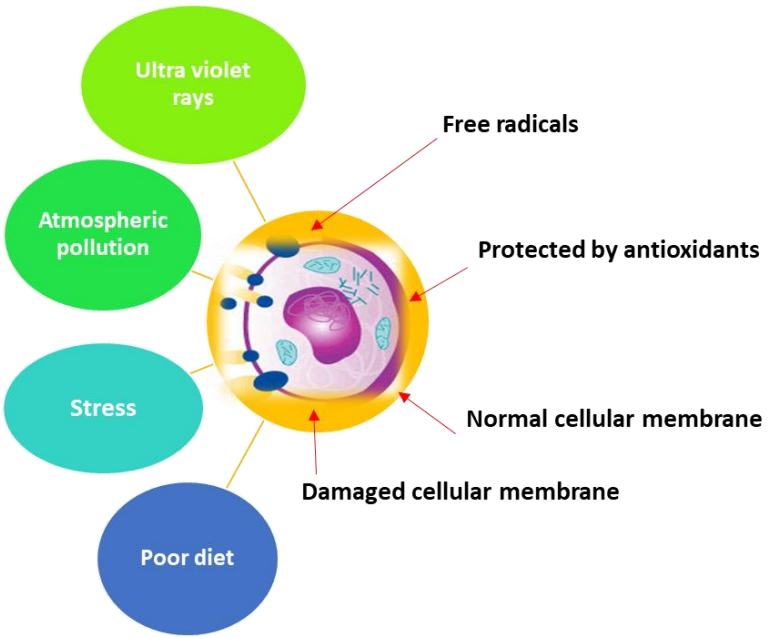

Figure 5.1. ROS production and their effects

The control of ROS levels is given by their production and elimination. The imbalance among the elimination and production in favour of the last has been called oxidative stress (Lushchak, 2014). Lipids and proteins are target of ROS and RNS and their alteration can improve the mutagenesis procedure (Coussens et al., 2002; Hussain et al., 2003).

When in excess, ROS interfere with the metabolism, because they can to deactivate important cellular molecules which are important for the homeostasis and its regulation. In inflammatory process, the production and liberation of ROS increase the presence of leukocytes and mast cells (Coussens et al., 2002; Hussain et al., 2003). Inflammatory cells produce inflammatory mediators, such as arachidonic acid, cytokines, and chemokines that promote signal transduction cascades and modifications of transcription factors, such as signal transducer and activator of transcription 3, NF-E2 related factor-2, hypoxia-inducible factor-1α (HIF1-α), nuclear factor of activated T cells, nuclear factor kappa B (NF-κB), that mediate vital cellular stress reactions.

Activation of cytokines (tumor necrosis factor-α (TNF-α), IL-1β, IL-6), chemokines (CXC chemokine receptor 4), some enzymes and compounds (COX-2, *i*NOS) are known to have an important role in the inflamattion, in fact were implicated in the trasformation in the espression of specific microRNAs (Hussain et al., 2007; Federico et al., 2007).

Under oxidative stress and inflammation conditions, healthy cells of organism can damaged with consequent development of different disorders (Federico et al., 2007; Reuter et al., 2010).

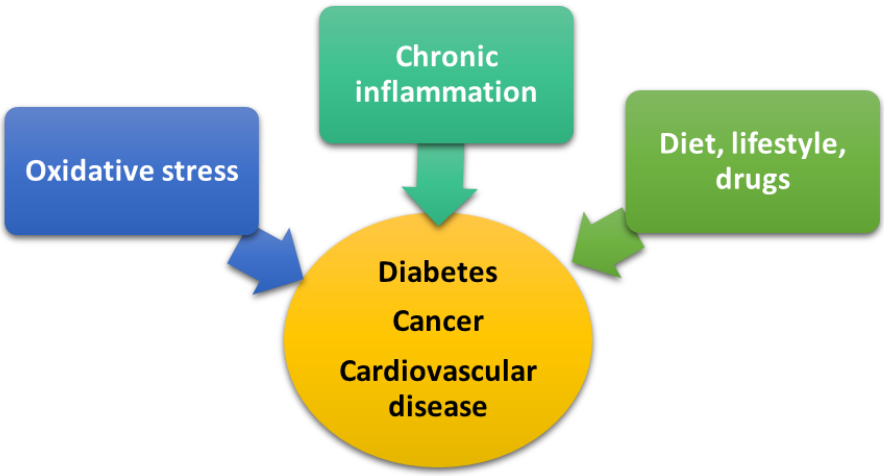

Figure 5.2. Consequences of inflammation and oxidative stress

Usually, there is an equilibrium between antioxidant defence mechanism and formation of ROS. This balance can be alter leading to development of oxidative stress that can compromise the structure of every vital cellular compounds and may carry to cell death (Tandon et al., 2005). This condition underpin to different illnesses such as cardiovascular and degenerative disorders, diabetes, inflammation and cancer (Ravipati

et al., 2012). The use of natural antioxidants may be a resolution to the human health disorders, representing a preventive medicine and alternative to synthetic antioxidants. Synthetic antioxidants as BHA and BHT are widely used in food industry but present adverse effects (Krishnaiah et al., 2011), thus the search of natural antioxidants is increasing. Moreover, it was demonstrated that there is an inverse correlation between the incidence of human disorders and intake of antioxidants rich foods (Sies, 1993).

5.1.1 Defence against oxidative stress

To counteract the action of free radicals, the body has a defence system much effective. This system varies depending for localization (intra or extracellular), tissues and cell types (Bonnefont-Rousselot et al., 2003). The term antioxidant is used to describe any substance present at low concentration that significantly retards or inhibits oxidation of the substrate (Halliwell et al., 1990). The antioxidants can be classified in endogenous and exogenous (Figure 5.3); the latter can be obtained from diet or *via* the assumption of food supplements (Rahman, 2007).

Antioxidants are molecules with enzymatic and not enzymatic activities. The enzymatic antioxidants contain a limited number of proteins such as catalase, glutathione peroxidase, and superoxide dismutase (Uttara et al., 2009). Catalase is principally present in peroxisomes and in erythrocytes (Alfonso-Prieto et al., 2009); the catalysed reaction is the decomposition of hydrogen peroxide to water and molecular oxygen. When hydrogen peroxide levels are too low to activate catalases, the decomposition of these reactive chemical species occurs by activation of glutathione peroxidase, an enzyme present in two different forms, a selenium-dependent (GPx), the other independent selenium (glutathione-*S*-transferase, GST).

In the human organism, there are four different types of GPx, which have the function of reducing peroxides. GPx acts in association with glutathione, a molecule present at high concentrations in cells that represents one of the most important endogenous mechanisms of defence against free radicals. Superoxide dismutase is an enzyme belonging to the metalloprotein family whose function is to convert two superoxide anion molecules into hydrogen peroxide and molecular oxygen (Rahman, 2007). In the mammals are described three isoforms for the superoxide dismutase (SO): the manganese superoxide dismutase in the mitochondria, copper or zinc in the mitochondria and in the cytoplasm, and both copper/zinc extracellular superoxide dismutase in vessels (Fukai et al., 2011). Under ideal conditions, superoxide dismutase, catalase and glutathione peroxidase act in an orderly and sequential manner, enhancing their important role as antioxidants.

Non-enzymatic antioxidants can be distinguishing with direct or indirect action. The first ones can be synthetized by the cells. The second ones include chelating agents that reduce metals and prevent the formation of radicals (Uttara et al., 2009).

Some of the most active antioxidants are vitamin C, E, A, carotenoids, and polyphenols. Polyphenols are the most abundant antioxidants in our diet and are present

in fruits, vegetables, cereals, olives, dried vegetables, chocolate and beverages, such as tea, coffee and wine. Different hydroxyl groups on aromatic rings characterise the classical polyphenolic structure, but also molecules with a single phenolic ring, such as acids and phenolic alcohols, can be considered polyphenols (D'archivio et al., 2007).

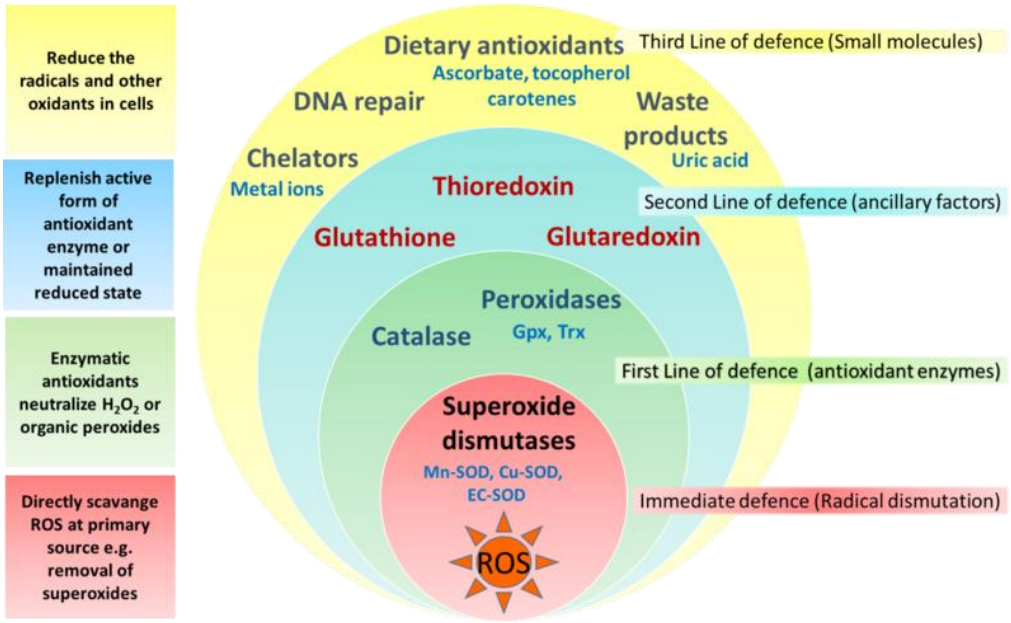

Figure 5.3. Defence against reactive oxygen species (ROS) (adapted by Maurya et al., 2017).

Natural polyphenols, depending on the number of phenolic rings they contain and the structural elements that bind each ring with another, are divided into hydroxybenzoic acids, hydroxycinnamic acids, stilbenes, lignans, and flavonoids (Laguerre et al., 2007).

The antioxidant action of the polyphenols is of the chain-breaking type: they react with the radicals yielding them a hydrogen radical (Laguerre et al., 2007).

Flavonoids are the most abundant polyphenols in plants. Structurally they are characterised by two benzene rings linked by a linear chain of three carbon atoms, which can form a ring with 6 atoms with a hydroxyl group of one of the benzene rings.

More than 4000 individual flavonoids have been identified, divided into subclasses depending on the substitution scheme and the oxidation state of the central ring (D'archivio et al., 2007). Depending on the type of heterocycle, its substituents and the substituents of the benzene rings, flavonoids are subdivided into anthocyanidins, flavonols, flavanes, flavanones, flavones, isoflavones, tannins (Harborne, 1989).

5.2. Inflammation

Oxidative stress and inflammation are closely related pathophysiological events that are linked with one another. Inflammation is a reaction of the body to the attack of pathogens and it is linked to many pathogenic illnesses as chronic and autoimmune

diseases, obesity, viral and microbial infections, exhibition to allergens, toxic and radiation chemicals, tobacco use, high calorie diet. Majority of chronic illnesses is associated with high production of ROS result in oxidative stress and protein oxidations (Berlett et al., 1997). The protein oxidations can release inflammatory signals molecules as peroxiredoxin 2 (PRDX2), recognized as an inflammatory signal. PRDX2 act as a redox dependent inflammatory mediator and activates macrophages to produce and release TNF-α (Salzanoa et al., 2014). The inflammation has been classified in two different processes: acute and chronic (Figure 5.4). The classification change based on duration and different immune factors involved in the processes.

The acute inflammation is a short process, can last from minutes to a days and it is characterised for loss of fluid or plasma proteins and movement of leukocytes under an extravascular zone (Markiewski et al., 2007). These reactions are mediated by chemical factors that are liable of the classic symptoms of inflammation as leakage of function, pain, swelling, warmth, reddening. Principally, is involved the vascularized connective tissue (Markiewski et al., 2007). The inflammatory process includes three phase: rise blood flow to inflame zone, subsequently vasodilatation and increase vascular permeability with loss of plasma from the microcirculation, and phagocytic leukocyte movement to the near tissue (Kobayashi et al., 2014; Markiewski et al., 2007; Schmid-Schönbein, 2006).

During inflammation, there are modifications in the vascular flow and in the diameter of small blood vessels. The capillaries and arterioles increase blood flow in inflamed area, moreover gradual alterations in the endothelium boost the vascular permeability of the microvasculature bringing a leakage of the fluid into an extravascular zone (Markiewski et al., 2007). The fluid reduces quantity in the lumen of blood vessels, enhance the viscosity of the blood, and reduce the flow rate. Successively, the margination and the adhesion of leukocytes to the endothelium start. Finally, leukocytes begin a process of transmigration through vascular wall direction the interstitial tissue. The final step of acute inflammation is the presence of leukocytes and plasma mediators to the inflamed zone (Kobayashi et al., 2014; Markiewski et al., 2007). The mediators, target tissues and sensors are different; depend on the genre of infection (parasitic, virulent, bacterial, etc.) (Medzhitov, 2010).

For example, bacterial pathogens are identified through receptors of the innate immune system like Toll-like receptors (TLRs) that are found in tissue-resident macrophages and stimulate the formation of inflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6), chemokines (e.g., chemokine C-C motif ligand 2 and chemokine CXC motif ligand 8), and prostaglandin E2 (PGE2) (Pecchi et al., 2009).

These inflammatory products moved on target tissues (blood vessels) to promote vasodilation, extravasation of neutrophils, and loss of plasma into the infected area (acute inflammation). Therefore, neutrophils tissue-resident macrophages and mast cells discover the pathogens to remove them. Components of plasma and antibodies are responsible of this process.

An effective acute inflammatory response carries to the removal of infectious factors followed by a repair and resolution phase that is mediated through tissue-resident macrophages (Serhan et al., 2005), because the mediators do not discriminate between host targets and microbial; therefore, damage to host tissues is unavoidable (Medzhitov, 2008; Nathan, 2002). For the resolution phase it is necessary the movement of lipid markers from proinflammatory prostaglandins to lipoxins, that present anti-inflammatory capacity. Lipoxins reduce the activity of neutrophils and at the same time active the activity of monocytes that remove dead cells and actuate tissue reshaping.

An important role in the resolution of inflammation is ascribed to the protectins and resolvins that are part of class of lipid marker together to transforming growth factor beta **(**TGF-β) and other growth factors produced from macrophages, because they are responsible to the tissue repair (Serhan et al., 2005, Serhan, 2007).

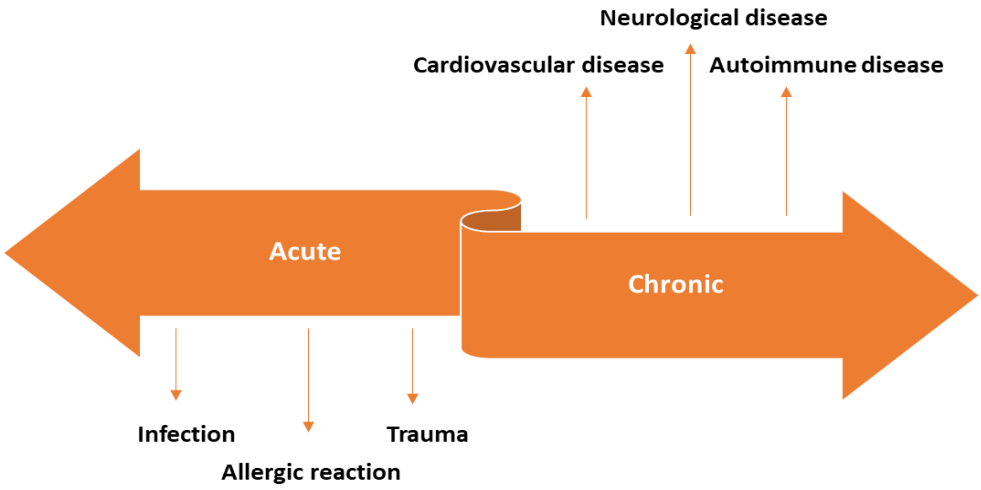

Figure 5.4. Acute and chronic inflammation

Chronic inflammation is also referred to as slow, long-term inflammation lasting for prolonged periods of several months to years. Generally, the duration and effects of chronic inflammation vary with the cause of the injury and the ability of the body to repair and overcome the damage (Arulselvan et al., 2016). Normally, chronic inflammation in tissue take place when do not presence real stimulus, but because the previous infection was not cured with any way (Eaves-Pyles et al., 2008).

Furthermore, the genetic predisposition, continuous exposition to physical and chemical agents, recurring acute inflammation and specific pathogens can contribute to onset of chronic inflammation (Ferguson, 2010). However, the state of chronic inflammation can have different collateral effects in biological response linked with increased danger of chronic disorders and diseases.

The chronic inflammation process depends rely on the type of organ and inflamed cells involved (Weber et al., 2011). Different diseases are characterised by inflammatory

response as arterial hypertension, cancer (Suematsu et al., 2002), myocardial ischemia (Anselmi et al., 2004; Entman et al., 1991), venous and chronic arterial illnesses (Engler et al., 1983; Schmid-Schönbein et al., 2001; Schmid-Schönbein et al., 2005), Alzheimer's disease, acute cerebral stroke (Schmid-Schönbein et al., 2005), osteoarthritis (Benito et al., 2005; Stürmer et al., 2004). Diseases correlated with inflammation are numerous and increasing. The inflammation is related also to insufficient physical activity (Colbert et al., 2004) and to obesity (Calabro et al., 2005; Nicklas et al., 2004). Previously, inflammation had been associated to immune system and infection. Recently, evidence suggest that inflammatory markers are present in many illnesses, thus it is simple deduce that anti-inflammatory compounds used for one specific illnesses can be valid in other disorders (Schmid-Schönbein, 2006).

5.3. Diabetes

Diabetes mellitus (DM) is a metabolic disorder characterised by high levels of blood sugar (hyperglycaemia) due to defects in the secretion or action of insulin or both. The chronicity of hyperglycaemia is correlated with dysfunction, damage, and insufficiency of target systems such as central nervous and cardiovascular systems (Uazman et al., 2014). The defects of synthesis and secretion of insulin is due to alteration of β-pancreatic cells, this condition along with the resistance of insulin in peripheral tissue characterise DM. Insulin secretion depends to different factors, such as hormones, nutrients and neural factors (Carvalho et al., 2018).

Type 1 diabetes is the form autoimmune where there is beta cell destruction and absolute insulin deficiency, as final result. Type 1 diabetes is typical of young age, but occasionally appears in non-obese adults and is characterised by a severe or total reduction in insulin production due to the immune-mediated destruction of pancreatic beta cells. Type 1 diabetes is a severe form and is associated with ketosis; in fact, since the body does not get enough energy from glucose in the tissues, it begins to use the reserve fat. This causes an increase in the blood of ketone bodies, compounds that make the blood acidic and interfere with breathing. If left untreated, this type of diabetes is lethal. Coma and death are the inevitable conclusions of the disease, if insulin therapy is not followed.

Type 2 diabetes, which is the principal form (90% of cases) of the illness, is one of the 4 major categories of diabetes (American diabetes association, 2019). The increased insulin resistance and a progressive decrease of insulin secretion characterise type 2 diabetes. In the world, the incidence of type 2 diabetes is correlated with increase of obesity. Type 2 diabetes is the most common form of diabetes and develop predominantly in adults although it may occasionally begin in childhood. In this type of diabetes the pancreas produces a considerable amount of insulin which is, however, insufficient for the body's needs, especially since the tissues are often resistant to the effects of the hormone. A high level of blood sugar, for example, makes insulin target receptors in the body's cells inactive. Patients are not dependent on exogenous insulin therapy for their

survival. The causes triggering the onset of the disease are to be found in hereditary and environmental factors.

Over the years the existence of a hereditary transmission factor, not yet very clear, has been discovered that exposes to these pathologies some populations more than the others. In these cases, there is the predisposition to have diabetes linked to characteristic aspects as obesity. The sedentary life, stress and diseases that lead the body to important responses fall, instead, into the list of environmental triggers. Age also plays an important role: the aging of the organism, in fact, reflects on the functionality of all the organs and, above all, on the pancreas, which is no longer able to respond promptly to requests received. Therefore, people with type 2 diabetes are generally people of the second or third age, with a weight greater than the ideal one, often with hereditary to diabetes. The symptoms are not as obvious as in type 1 diabetes and are ignored.

The discovery of diabetes usually occurs during a check-up. For this reason, the diagnosis of this form of diabetes is rather late and it is easy to find the presence of complications in an advanced state of development.

Gestational diabetes mellitus is diabetes diagnosed during the pregnancy. Gestational diabetes is an alteration of carbohydrate metabolism that occurs during pregnancy, stimulated by hormonal and metabolic changes of this period. There are serious risks associated with gravidic diabetes for both the mother and the child. In particular, in the mother diabetes can be associated with infections, diabetic ketoacidosis and hypertension. In the child, in addition to a higher risk of death at the time of delivery or post-partum, congenital malformations, macrosomia, respiratory and metabolic problems may occur.

Ultimately, there is the diabetes due to other causes, such as, cystic fibrosis, after use of glucocorticoid or organ transplantation, neonatal diabetes and chemically induced diabetes (American diabetes association, 2019).

In both forms of diabetes (type I and II), the continuation for many years of a condition in which blood sugar levels remain moderately high may eventually cause kidney disease, impaired vision and lens opacification. The reduction of blood flow to the limbs, with disorders such as their numbness and loss of function, in severe cases may require amputation. Other diseases associated with diabetes are the increased risk of heart attack and stroke.

5.4. Linkage between oxidative stress, inflammation and diabetes mellitus

Inflammation and oxidative stress are strictly correlated pathophysiological processes (Figure 5.5). One can be readily induced by the other, and both are described in several pathological conditions (Biswas, 2016). Different studies reported oxidative stress as main cause of chronic inflammatory illnesses.

Glutathione (GSH) is an intracellular thiol antioxidant that takes part in the redox regulation of immunity (Ghezzi, 2013). When the its levels are low, the production of ROS increase making more susceptibility to infection because there are disequilibrium between immune response and inflammation (Ghezzi, 2011).

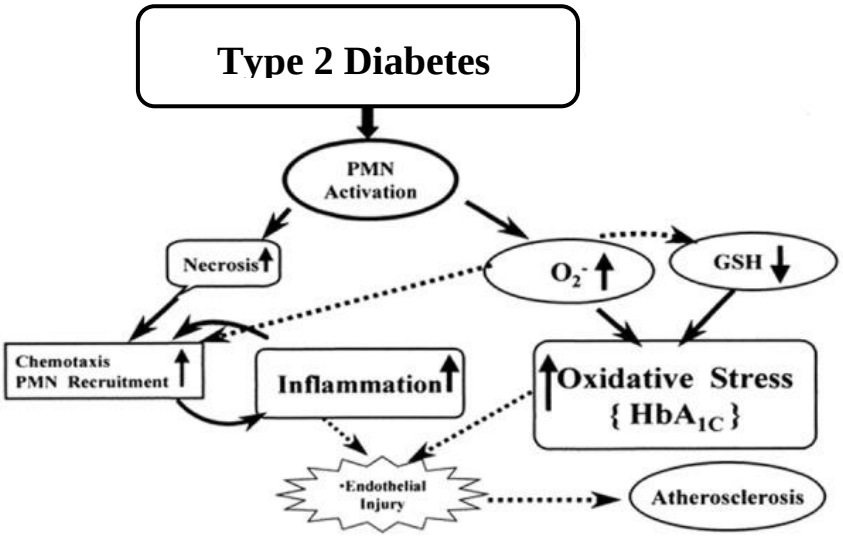

Figure 5.5. Relationship between type 2 diabetes, oxidative stress and inflammation (adapted by Oguntibeju, 2019).

Moreover, oxidative stress can induced grave cellular harm of the brain in diabetes (Reagan et al., 1999). Diabetes induced oxidative stress upregulates inflammatory molecules like vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and nuclear factor-kappa B (NF-κB), and also increases the level of proinflammatory cytokines such as TNF-α and IL-6 (Prabhakar et al., 2013), which evolves to degeneration of neurons and then in diabetic encephalopathy.

C-reactive protein (CRP) is a biomarker used for determination of inflammation state, it is an immunomodulatory protein produced by the liver. Acute inflammation induces IL-6-dependent transcription of CRP (Pradhan et al., 2001).

Cottone et al. (2006) demonstrated the correlation between CRP and oxidative stress. In the hypertension, oxidative stress is associated with increase to levels of inflammatory molecules, as CRP. Different studies have showed the presence of high levels of CRP and IL-6 in the patients with diabetes of type 2 and insulin resistance syndrome (Festa et al., 2000; Ford, 1999; Ito et al., 2019b). Thus, inflammation cannot be describe as a disease but should be rather evaluated as a biological process.

Insulin plays a crucial role in the inflammatory reactions by it acting on oxidative stress and on the production of cytokines (Qiu et al., 2018).

The nuclear factor-κB (NF-kB) family is comprised of DNA-binding protein factors required for the transcription of most pro-inflammatory molecules (Lin et al., 2018).

Several studies performed in *in vitro* and *in vivo* systems suggest that the activation of NF-κB is a key event early in the pathobiology of diabetes.

Normally, insulin stimulates insulin receptor signalling pathway, including the phosphorylation of insulin receptor substrate-1 (IRS-1), phosphoinositide 3-kinases

(PI3K), and phosphatidylinositol 4,5-bisphosphate (PIP2) and the activation of protein kinase B (Akt), eventually leading to the translocation of glucose transporter 4 (GLUT4) to the plasma membrane. Obesity-associated inflammation of the adipose tissue and liver induces macrophage infiltration and increase in pro-inflammatory cytokines and ROS generation. Increased ROS inhibits the insulin receptor-signalling pathway, leading to insulin resistance and hyperinsulinemia (Ito et al., 2019a).

The "mechanism" used by oxidative stress for the development of diabetes complications is complex. The insulin resistance is due by different mechanisms linked to inflammation (Ye, 2013). One of the risk factors for the development of insulin resistance is oxidative stress; the increase of oxidative stress stimulates the insulin receptor and then increases the absorption of glucose from the blood *via* glucose transporter 4 (GLUT4). Moreover, there is activation of the c-Jun *N*-terminal kinase (JNK) pathway that increases phosphorylation of serine residue of IRS-1, which no longer functions PI3K protein (Hurrle et al., 2017). Therefore, GLUT4 not functioning and the levels of glucose in the blood increase. Moreover, between oxidative stress and diabetes there is a relation cause-effect, in fact the oxidative stress is involved in complications linked of diabetes.

Renal damage is due to production of hydrogen peroxide by mesangial cells and lipid peroxidation, but also to activation of protein kinase C (PKC), mitogen-activated protein (MAP) kinases, and cytokine (Anjaneyulu et al., 2004). Cataract was characterised by presence of NF-κB and collection of advanced-glycation end-products (AGEs) in sub-retinal membranes (Yamagishi et al., 2012).

More note is the haemoglobin A1c (HbA1c), product from glycation of haemoglobin and it is a biomarker typical between oxidative stress and diabetes. The increase of HbA1c can evolve in microvascular complications in diabetes of type 1 (Kilpatrick et al., 2008; Marcovecchio et al., 2011; Waden et al., 2009) and in nephropathy and cardiovascular illnesses in diabete of type 2 (Penno et al., 2013; Sugawara et al., 2012).

In addition, inflammation plays a role in diabetic complications (Ishibashi et al., 2013; Touyz, 2005; Wellen et al., 2005). Hyperglycaemia carries to a systemic and local inflammation because the degree of pro-inflammatory proteins is high (Wellen et al., 2005) and the macrophages discharge inflammatory cytokines. More specifically, the release of cytokines (monocyte chemoattractant protein and interleukins) is linked with a higher body mass index (Derosa et al., 2013). The obesity-related insulin resistance and abnormal vascular reactivity is correlated by the high production of TNF and to diabetic complications (Moriwaki et al., 2007; Westermann et al., 2007).

5.5. Potential multitargeting agents to treat oxidative stress-related diseases

In the last years, the interest on natural antioxidants is growing. Some toxicological studies have shown unwanted or adverse effects of synthetic antioxidants. These works have urged the researchers to focus their attention on exploring the natural sources with antioxidant potential. Natural antioxidants are distributed in all parts (leaves, fruits,

flowers, seeds, stems, bark, wood, and roots) of plant-foods and medicinal plants (Chanwitheesuk et al., 2005). Among these antioxidant compounds, especially flavonoids and iridoids exhibited a wide range of biological effects.

5.5.1. Flavonoids

Flavonoids are a large group of polyphenolic compounds omnipresent in plants. Flavonoids are synthesized by plants in response to microbial infection, but have several important functions as protection activity against harmful UV radiation or plant pigmentation (Dixon et al., 1983). Available reports showed different of pharmacological activities of these secondary metabolites (Mahomoodally et al., 2005; Pandey, 2007).

Chemically, flavonoids have a 15 carbon skeleton that consists of a heterocyclic ring and two phenyl rings (aglycone). As products of secondary metabolism, flavonoids are identified in plants as glycoside and methylated derivatives (Kumar et al., 2013).

Their antioxidant activity can be explicated through their ability to scavenge ROS, to suppress ROS formation and to up-regulate or to protect antioxidant defences (Halliwell et al., 1998; Mishra et al., 2013). Moreover, flavonoids block ROS generation through the inhibition of enzymes such as glutathione *S*-transferase, nicotinamide adenine dinucleotide phosphate (NADP) oxidase and others (Brown et al., 1998).

The activities reported are structure dependent, in fact a carbonyl group on C-4, the unsaturation of the C ring, the position and number of hydroxyl groups, and glycosylation condition are responsible for the antioxidant and anti-inflammatory activities of flavonoids (Lago et al., 2014; Theoharides et al., 2001).

Glycosylation of flavonoids has been associated to a reduction of the inhibitory effects on inflammation because glycoside derivatives are more easily absorbed than aglycones. Furthermore, the presence of a catechol group in the B ring of the quercetin impart powerful anti-inflammatory properties, whereas the insertion of one hydroxyl group on position 20 of the B ring of morin eliminate any anti-inflammatory activities (Lago et al., 2014). Moreover, the hydroxylation of the B ring inhibits the liberation of cytokines by macrophages and mast cells (Kim et al., 2004).

The hydroxyl groups of flavonoids complexing with oxidant species can stabilise and remove free radicals, decrease oxidative damage, which is the basis of several chronic illnesses (Kumar et al., 2013; Yao et al., 2004).

Flavonoids have various properties, but one of more important is their high radical scavenging activity, that was linked to different disorders as inflammation and diabetes.

Flavonoids have the ability to sequester free radicals, are natural antioxidants derived from plants and are commonly found in foods and beverages.

The main structural features of flavonoids required for antioxidant activity can be determined by three fundamental factors (Figure 5.6):

1) a 3′,4′-dihydroxy (catechol) structure in the B ring favors the electron delocalization (A);

2) an unsaturated 2-3 bond in conjugation with a 4-keto group provides electron delocalization from the B ring (B);
3) hydroxyl groups at positions 3 and 5 form intramolecular hydrogen bonding to the keto group (C).

These effects lead to the increases of the radical scavenging by delocalization of electrons or by donation of hydrogen (Stefek, 2011).

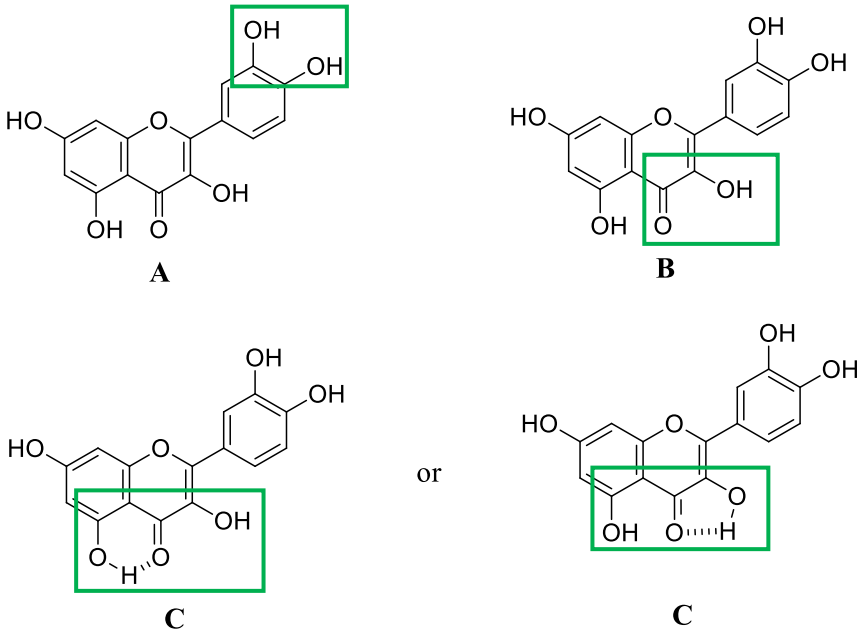

Figure 5.6. Structural groups important for radicals scavenging activity.

In vivo other mechanisms of action are described such as the regulation nuclear transcription factors and fat metabolism, and the modulation of the synthesis of inflammatory mediators including cytokines TNF-α, IL-1β, and IL-6.

Flavonoids can control transcription of much proinflammatory proteins and enzymes, but also influence pathways of inflammasome activation and autophagy and promote the resolution phase for prevent the chronic inflammation (Lim et al., 2019).

Flavonoids are able to stimulate the immune system, to inhibit the prostaglandins synthesis and to promote the protein kinase cascades with consequently cytokine production (Havsteen, 2002). Flavonoids, such as hesperidin, quercetin, luteolin, and apigenin, demonstrated analgesic and anti-inflammatory activities.

Flavonoids could be influence the enzyme systems activities involved in the inflammation processes, in fact flavonoids inhibit the nitric oxide synthase, lipooxygenase and cyclooxygenase, that are responsible for the production of a high amount of leukotrienes, nitric oxide, prostanoids, and other inflammatory mediators as chemokines, cytokines, or adhesion molecules (Tunon et al., 2009). One of the most studied flavonoids is quercetin. Quercetin and its glycosides were showed be potent anti-

inflammatory agents on sarcoidosis patients and *in vivo* models of allergic airway inflammation and arthritis (Boots et al., 2008; Oliveita et al., 2018). Inflammation is correlated with the insurgence of diabetes (Pollack et al., 2016). Metabolic dysregulation correlated with diabetes induce a proinflammatory response in macrophages (Garcia et al., 2010) with release of proinflammatory cytokines (Vinayagam et al., 2015). Various studies have reported the beneficial properties of flavonoids in the treatment for diabetes mellitus (Testa et al., 2016). Flavonoids can modulate carbohydrates and lipids metabolism, reduce hyperglycaemia and insulin resistance, and minimize oxidative stress and inflammation (Choi et al., 2009).

Flavonoids can stimulate insulin effect influencing the protein phosphokinases with consequently GLUT-4 translocation (Havsteen, 2002). However, diabetes has complications that attack different organs as nerves, heart, kidneys and eyes (Jadhav et al., 2012). The role of flavonoids is to not only help to reset glucose homeostasis decreasing the diabetic state, but also normalize the damages to the different peripheral organs (Ginwala et al., 2019). Cataract is a complication of diabetes mellitus type II, characterised by progressive loss of transparency of the crystalline lens, which leads to a decrease in sight. Aldose reductase is involved in this process because reduce glucose and galactose in hexitols. These latter exert a high osmotic activity that at eyes levels creating visual disturbance. Flavonoids inhibiting aldose reductase are able to reduce the risk to develop cataract (Havsteen 2002).

5.5.2. Iridoids

Iridoids constitute a large group of cyclopenta[*c*]pyran monoterpenoids; they are present in plants and animals; in plants they are frequently tie to glucose, as iridoid glycosides (Villaseñor et al., 2007). A six-member ring containing an oxygen connected to a cyclopentane ring characterises the chemical structure of iridoids. Iridoids have been extensively investigated for their potential anti-inflammatory activity.

Recio et al. (1994) studied twelve iridoid glycosides for their anti-inflammatory properties by using the TPA-induced mouse ear oedema and the carrageenan-induced mouse paw oedema models. Obtained results allow establishing relationships between the chemical structure and anti-inflammatory activity based on the different patterns of substitution. In particular, introducing a hydroxyl function in the iridoid structure, the topical activity decreases, while the conversion of a –COOH moiety to its –COOMe analogue intensify topical activity. The topical properties are increased also by hydroxyl substitution at C-5, unsaturation at C7-C8, and methyl substitution of carboxyl C-11 (Recio et al., 1994).

A double bond between C-7 and C-8 with additional hydroxyl substituents in skeleton of iridoids (such as aucubin) is positive to increase the biological activity.

The introduction of a hydroxyl on C-8 decreases the topical activity as confirmed with harpagide and harpagoside (Ahmed, 2003). In the structure of iridoid glucosides, the presence of a hydroxyl group on C-8 showed potent anti-inflammatory activity as

demonstrated with ixoroside and shanzhiside. On the contrary, the presence of -OH in position C-7 in the aglycon skeleton decreases the immune stimulating property, as reported for loganin (Mathad 1998). None effects were observed in relation to the presence of a keto group on the cyclopentane ring.

The integrity of the cyclopentane ring plays an important role on anti-inflammatory activity. In fact, iridoids with a cyclopentane open showed a decrease in topical anti-inflammatory activity (Recio et al., 1994).

Zhang et al. (2011) showed that the activity of iridoid glycosides increase after hydrolysis, for example harpagide and harpagoside after hydrolysis demonstrated inhibit COX-2 because their structure is similar to prostaglandin. The same trend was observed with other iridoid glycosides, such as loganin and catalpol (Park et al., 2010).

Geniposide modulated the activity of cytochrome P-450 and GSH in the rat liver. Genipin (the aglycone of geniposide) exhibited a potent topical anti-inflammatory activity (Koo et al., 2004, 2006; Zhang et al., 2011). For delaying the process of cell injury inducted by oxidative stress, geniposide increase expression of heme oxygenase (HO-1) upregulate the expression of some antioxidative proteins and some antioxidant enzymes including glutathione peroxidase (GPx), hepatic lipid peroxidation (LPO), glutathione-*S*-transferase (GST) and glutathione (GSH) (Wang et al., 2015).

Another iridoid potential candidate as anti-inflammatory agent is cornuside that showed inhibition of several pro-inflammatory cytokines and increase of leukocyte-endothelium adhesion *via* increase of endothelial cell adhesion molecules (ICAM-1; VCAM-1) (Kang et al., 2007). In addition, cornuside demonstrated a good inhibition of the production of IL-6, NO, IL-1β and TNF-α, with percentages of 75.7, 67.6, 55.4, and 50.8%, respectively (Choi et al., 2011).

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Experimental part

Chapter 6
Materials and methods

6.1. Chemicals and reagents

Solvents of analytical grade were purchased from VWR International s.r.l. (Milan, Italy). Solvents used for liquid chromatography-electrospray ionization-quadrupole-time of flight-mass spectrometry (LC-ESI-QTOF-MS) were purchased from Carlo Erba s.r.l. (Milan, Italy). Tween 20, ascorbic acid, Folin-Ciocalteu reagent, sodium carbonate, butylated hydroxytoluene (BHT), propyl gallate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic)acid diammonium salt (ABTS) solution, 3(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT), Griess reagent, interleukin-2 (IL-2), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), *β*-carotene, doxorubicin, linoleic acid, dimethyl sulfoxide (DMSO), *o*-dianisidine color reagent (DIAN), peroxidase-glucose oxidase (PGO), Tris Buffer Saline (TBS), bovine serum albumin, chlorogenic acid, aucubin, quercetin, acetic acid, Dulbecco's modified Eagle's medium, fetal bovine serum, lipopolysaccharides (LPS), glucose, penicillin-streptomycin, potato starch, sodium acetate, sodium phosphate, sodium potassium tartrate, sodium chloride, *α*-amylase from porcine pancreas (EC 3.2.1.1), *α*-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), IgG-TRITC, maltose, 3,5-dinitrosalicylic acid, gallic acid, protocatechuic acid, catechin, *p*-coumaric acid, quinic acid, caffeic acid, syringic acid, gentisic acid, ellagic acid, salicylic acid, cornuside, isoquercitrin, rutin, quercetin, and kaempferol were purchased from Sigma-Aldrich S.p.a. (Milan, Italy).

Geniposide, ferulic acid, chlorogenic acid, loganin, myricetin 3-*O*-glucoside, and hyperoside were purchased from Extrasynthese (Lyon, France). XAD-16 and XAD-4 were purchased from Alfa-aesar, Thermo Fisher Scientific (Kandel, Germany). Chromabond HLB was purchased by Macherey-Nagel (Hoerdt, France).

Anti-NF-κB p65 monoclonal antibody were purchased from Santa Cruz Biotechnology, (CA, USA). Acarbose from *Actinoplanes* sp. was purchased from Serva (Heidelberg, Germany). RAW 264.7 cells were obtained from American type culture collection (VA, USA).

6.2. Plant materials

Fruits and leaves of *A. unedo* were collected in the Botanic Garden of the University of Calabria, Rende (Cosenza, Italy) (WGS84: 39°21′36″N, 16°13′50″E) (plant n. 103) in November 2016.

Fruits and leaves of *V. corymbosum* were collected in Balzata San Giorgio, Rogliano (Cosenza, Southern Italy) (WGS84: 39°17′22″N, 16°38′17″E) (Voucher number CLU26258) in June 2017.

Fruits and leaves of *C. sanguinea* were collected in the Botanic Garden of the University of Calabria, Rende (Cosenza, Italy) (WGS84: 39°24′33″N, 16°13′43″E) (plant n. 165) in September 2017.

In the same period, fruits and leaves of *C. mas* were collected in the "Parco della Cessuta", Cerchiara di Calabria (Cosenza, Italy) (WGS84: 39°51′41″N, 16°21′47″E) (Voucher number CLU26257).

Fruits and leaves were randomly selected and examined for integrity and absence of dust and insects contamination. Fruits were harvested at maturity stage, defined by visual colour and size measurement.

All samples were identified by Dr. N.G. Passalacqua, Natural History Museum of Calabria and Botanic Garden (CLU), University of Calabria (Italy).

6.3. Extraction procedures

Once harvested, *A. unedo*, *V. corymbosum*, *C. sanguinea*, and *C. mas* fruits and leaves were separated in two parts to prepare fresh and dried materials in order to investigate not only the impact of different solvents and extraction procedures, but also the impact of the use of fresh or dried materials in the phytochemicals content of selected species and consequently in their bioactivity. In fact, drying, frequently applied to inactivate oxidizing and hydrolysing enzymes, is one of the most important factors that could affect plant benefits (Lim and Murtijaya, 2007). Indeed, it was been reported that phytochemical compounds may be affected chemically or physically during drying and in this case, the assessment of the bioactivity of the extracts could be erroneous. Herein, fruits of selected plant species were dried in a gravity convection oven (Thermo Scientific Heraeus, Germany) at 50 °C for 7 days. During the drying process, the temperature was stable, and its distribution is based on warm air moving upwards. The benefit of this technology is very low air turbulences for gentle drying and heating. Daily determination of weight was measured with weight electronic scale until the weight was stable. Leaves were dried at room temperature for 7 days in the dark.

Fresh and dried fruits and leaves of *A. unedo*, *V. corymbosum*, *C. mas*, and *C. sanguinea* were then subjected to four procedures such as maceration, decoction, Soxhlet apparatus, and ultrasound for the extraction of bioactive components. Previously, leaves and fruits dried were finely grind to increase the surface of contact, while fresh materials were extracted with no preparation to prevent enzymes liberation with consequent modification of chemical composition.

Ethanol and a hydroalcoholic solution ($EtOH/H_2O$ 6:4 *v/v*) were used as solvents. The choice of the solvent is a prominent factor influencing the success of a particular extraction procedure. According to the principle "like dissolves like", the solvent with the polarity value near to the polarity of target compound is likely to be dissolved better.

Flavonoids and iridoids extraction is commonly performed by using polar solvents such as methanol and ethanol. In this study, ethanol was chosen as solvent because it is a

GRAS (Generally Recognized As Safe) solvent widely used for the extraction of polar compounds.

Maceration is a very simple and widely used method for the extraction of phytochemicals at room temperature. The use of room temperature is a great advantage for the extraction of thermolabile compounds. The disadvantage is the long extraction time. The extract from decoction presents a large content of water-soluble impurities. This method is adapted for compounds water-soluble, heat-stable constituents extraction.

Compared with maceration, decoction could increases the dissolution of bioactive compounds (Zhang et al., 2013). In addition, use of high temperature deactivate *β*-glucuronidase, enzyme that catalyses the hydrolysis of the glucuronic acid group from glycosides to transform into aglycones. Another method that use high temperature is Soxhlet apparatus, an automatic continuous extraction method with use a lower quantity of solvent than maceration (Chin et al., 2013). Reduced extraction times and high extraction efficiency were also described. However, the use of Soxhlet apparatus increases the possibility of thermal degradation of thermolabile compounds.

Ultrasound-assisted extraction uses ultrasonic wave energy. Ultrasound (with levels greater than 20 kHz) is used to disrupt plant cell walls, which helps improve the ability of solvent to penetrate the cells and obtain a higher extraction yield. Therefore, ultrasound can use a low operating temperature through processing, maintaining a high extract quality. Extraction of phytochemicals by ultrasound has grown during recent years due to its role in reducing the amount of solvent and energy used (Khan et al., 2010).

Herein both fresh and dried plant materials were exhaustively extracted by the following procedures:

a) maceration (3 × 72 h) using ethanol as solvent (1:1 *w/v* and 1:3 *w/v* for fresh and dried fruits of *A. unedo*, 1:2 *w/v* and 1:5 *w/v* for fresh and dried fruits of *V. corymbosum*, 1:2 *w/v* for fresh and dried fruits of *C. sanguinea*, 1:3 *w/v* and 1:10 *w/v* for fresh and dried fruits of *C. mas*, 1:1 *w/v* and 1:3 *w/v* for fresh and dried leaves of *A. unedo*, 1:1.5 *w/v* and 1:4 *w/v* for fresh and dried leaves of *V. corymbosum*, 1:1.15 *w/v* and 1:3 *w/v* for fresh and dried leaves of *C. sanguinea*, 1:5 *w/v* for fresh and dried leaves of *C. mas*);
b) maceration (3 × 72 h) using a hydroalcoholic solution (EtOH/H_2O, 6:4 *v/v*) (1:1 *w/v* and 1:3 *w/v* for fresh and dried fruits of *A. unedo*, 1:2 *w/v* and 1:5 *w/v* for fresh and dried fruits of *V. corymbosum*, 1:2 *w/v* for fresh and dried fruits of *C. sanguinea*, 1:3 *w/v* and 1:10 *w/v* for fresh and dried fruits of *C. mas*, 1:1 *w/v* and 1:4 *w/v* for fresh and dried leaves of *A. unedo*, 1:1.5 *w/v* and 1:5 *w/v* for fresh and dried leaves of *V. corymbosum*, 1:1.15 *w/v* and 1:4 *w/v* for fresh and dried leaves of *C. sanguinea*, 1:5 *w/v* for fresh and dried leaves of *C. mas*);
c) Soxhlet apparatus (conventional glass with an extraction chamber with a diameter of 8 cm and a height of 30 cm, accompanied by a flask of capacity of 1 L; 7 cycles) using ethanol as solvent (1:4 *w/v* and 1:10 *w/v* for fresh and dried fruits respectively

of *A. unedo*, *V. corymbosum*, *C. sanguinea*, 1:6 *w/v* and 1:12 *w/v* for fresh and dried fruits of *C. mas*, 1:15 *w/v* for all fresh leaves, 1:20 *w/v* for all dried leaves);

d) ultrasound-assisted extraction (3 × 1 h) using ethanol as solvent (1:4 *w/v* and 1:1 *w/v* for fresh and dried fruits of *A. unedo*, 1:2 *w/v* and 1:1.5 *w/v* for fresh and dried fruits of *V. corymbosum*, 1:0.5 *w/v* for fresh and dried fruits of *C. sanguinea*, 1:0.75 *w/v* and 1:2.5 *w/v* for fresh and dried fruits of *C. mas*, 1:0.75 *w/v* and 1:3 *w/v* for fresh and dried leaves of *A. unedo*, *V. corymbosum*, *C. sanguinea*, 1:5 *w/v* for fresh and dried leaves of *C. mas*) using a Branson 3800 ultrasonic system, series CPXH (130 W, 40 kHz frequency) (Milan, Italy);
e) decoction (1:1 *w/v*, 30 min for all fruits, 1:10 *w/v*, 10 min for all fresh leaves, 1:20 *w/v*, 10 min for all dried leaves).

Both extraction time and solvent to feed ratio were defined by previous experiments.

Extractive solutions, after being filtered (using Whatman N. 1 filter paper) and combined, were evaporated under reduced pressure in order to obtain dry crude extracts. Extraction yields (%) are reported in Tables 7.1, 8.1, 9.1, and 9.19.

6.4. Total phytochemicals content

6.4.1. Determination of total phenols content

The total phenols content (TPC) was determined by using the Folin-Ciocalteu method (Gao et al., 2000). Briefly, 100 μL of extract (1.5 mg/mL) was mixed with 0.2 mL of Folin-Ciocalteu reagent, 2 mL of water, and 1 mL of 15% aqueous solution of Na_2CO_3. After 2 h of incubation at 25 °C, the absorbance was measured at 765 nm using a UV-Vis Jenway 6003 spectrophotometer (Milan, Italy). The results were expressed as milligrams of chlorogenic acid equivalents (CA)/g of dry extract.

6.4.2. Determination of total flavonoids content

The total flavonoids content (TFC) was determined as previously described method (Yoo et al., 2008). In this assay, 1 mL of extract (1.5 mg/mL for extracts) was added to 4 mL of distilled water and 0.3 mL of 5% (*w/v*) sodium nitrite. After 5 min, 0.6 mL of 10% (*w/v*) $AlCl_3$ were added, and 6 min later, 2 mL of 1 M NaOH and 2.1 mL of distilled water. Absorbance was read at 510 nm. The total flavonoids content was determined in triplicate and expressed as milligrams of quercetin equivalents (QE)/g of dry extract.

6.4.3. Determination of total iridoids content

The total iridoids content (TIC) was determined according to a colorimetric method based on the Trim and Hill reaction. In this assay, 400 μL of extract at the concentration of 1.5 mg/mL was mixed with 4.0 mL of Trim-Hill reagent (acetic acid/0.2% $CuSO_4$/HCl aq, 10:1:0.5; *v/v/v*). Then, the sample had been heated at 100 °C for 5 min, the absorption was read at 609 nm, a blue colour indicating the presence of iridoids. The total iridoids

content was determined in triplicate and expressed as milligrams of aucubin equivalents (AU)/g of dry extract.

6.5. Liquid Chromatography/Mass Spectrometry (LC-MS) analyses

A liquid chromatography (LC) method with mass spectrometry (MS) and diode array detectors (DAD) has been developed to obtain the profile of unknown samples and sample fractions. The HPLC system Thermo Scientific Dionex UltiMate 3000 Series with DAD and MS simple quadrupole (MSQ plus-Surveyor) detections (Courtaboeuf, France) included a quaternary pump, a vacuum degasser, an automatic sampler and a PDA spectrophotometer detector, which detects at 210, 240, 270 and 340 nm and between 200 and 600 nm. The selected analytical column was a C18 (Acclaim Polar Advantage II (Thermo Scientific, Courtaboeuf, France), 100 × 2.1 mm, 3 μm) thermostated at 35 °C. The flow rate was set at 0.5 mL/min, the sample injection volume was 10 μL. The solvents used were: solvent A: 0.1% formic acid in ultrapure water containing 10% LC-MS grade methanol (*v/v*), solvent B: 0.1% formic acid in acetonitrile LC-MS grade (*v/v*). A gradient in the mobile phase was applied as follow: at the time of injection and for 2 min, the volume *ratio* A:B was set at 100:0, increases to 95:5 between 2 and 5 min analysis time, then to 80:20 between 5 and 20 min, then to 60:40 between 20 and 25 min, and finally to 20:80 between 25 and 30 min; for 3 min this *ratio* is maintained and returns to initial conditions between 33 and 36 min.

The gradient ensured absence of impurity coelution and took into account late eluting impurities. All samples and solvents were filtered through 0.2 and 0.45 μm respectively, nylon filter disk prior injection. The chromatographic effluent stream is directed into the mass spectrometer (MS) interface, which consisted of an electrospray ionization (ESI) source, in positive and/or negative ionization mode. The conditions are as follows: capillary voltage was set at 3 kV, the flows of nebulizing and drying gas (nitrogen) were respectively set at 9.0 L/min and 4.0 bar and drying gas was heated at 500 °C, generated by a nitrogen generator (F DGSi Alliance Innovation Gas System Company). Mass spectra were acquired in the range 100-1300 thomson where the cone energy is 50 V in scanning mode. The ions produced are formed by collision-induced dissociation (CID). The software Chromeleon 6.8, provided by Thermo Scientific (91940 Les Ulis, France) was used for data treatment.

6.6. Liquid Chromatography-Electrospray Ionization-Quadrupole-Time Of Flight-Mass Spectrometry (LC-ESI-QTOF-MS) analyses

Standards (1mg/mL) and extracts (20 mg/mL) of each plant were solubilised in methanol, filtered, and analysed using an HPLC (U-3000, Thermo) coupled to an ESI-QTOF mass spectrometer (Maxis II, Bruker). The chromatographic separation is performed on a C18 column (Acclaim RSLC polar advantage II, 100 × 2.1 mm, 2.2 µm) maintained at a temperature of 35 °C, with a flow of 0.3 mL/min. The mobile phase

consists of a mixture of 0.1% formic acid, 10% methanol and water (phase A), and 0.1% formic acid and acetonitrile (phase B). The elution gradient is as follows: 0 to 2 min 95% A; 2 to 7 min, 95 to 85% A; 7 to 15 min, from 85 to 50% A; 15 to 18 min, 50 to 20% A; 18 to 19 min, 20% and 19 to 21 min, 20 to 95% A. The injection volume was 2 μL and the flow rate was 0.3 mL/min. Chromatograms were acquired at four different wavelengths: 240, 270, 340 and 510 nm. Mass spectra were acquired in positive mode using the following parameters: ESI 3500 V, *m*/*z* 50-1200, MS 2 Hz.

6.7. Separation techniques

Three different systems (HLB SPE, XAD-4 and XAD-16) were used to obtain the best separation of flavonoids and iridoids from the total matrix of the most active extracts. Extracts more active were obtained by *Cornus* genus, in particular hydroalcoholic maceration of dried leaves and dried fruits of *C. mas*, hydroalcoholic maceration of dried fruits of *C. sanguinea* and ethanol maceration of *C. sanguinea* fresh leaves. Chromabond HLB extraction products contain a resin made from a co-polymer of divylbenzene and vinyl pyrrolidinone. The pyrrolidinone functionality acts as an integrated hydrophilic group that provided better retention for some polar analytes. Thus, HLB phase has a hydrophilic-lipophilic balance allowing a high retention of neutral acidic basic compounds and a high stability in organic solvents. Amberlite XAD-4 and XAD-16 are polystyrene resins. Amberlite XAD-4 is a neutral resin consisting of an aromatic polymer cross-linked macroreticular, indicated for the adsorption of substances organic with a relatively low molecular weight. Amberlite XAD-16 is a neutral resin consisting of an aliphatic polymer cross-linked macroreticular, recommended for the adsorption of phenols from aqueous solvents; its porosity allows adsorbing organic molecules with a molecular weight from relatively low to medium.

Amberlite XAD-16 is the most suitable for the recovery and fractionation of flavonoids from plant extracts and foods rich in sugars and other polar compounds such as honey and fruit jams. The structural differences of separation methods are reported in Table 6.1.

HLB column (150 mg), XAD-4 (40 mL) and XAD-16 (160 mL) resins were activated using methanol, and then water to remove traces of solvent. Resins were left in methanol for 24 h before analysis. The sample was dissolved to distilled water. For HLB column it was deposit 5 ml of sample (40 mg), it was started elution with mQ-water (9 mL) for remove unattached compounds to resin, after elution with 70% ethanol and 100% ethanol for collected all compounds that present affinities with the column.

Table 6.1. Differences between extraction techniques

Separation method	Particles size (mm)	Surface area (m^2/g)	Porosity (mL/g)
HLB	0.03-0.06	750	0.65
XAD-4	0.490- 0.690	$\geq$ 750	$\geq$ 0.50
XAD-16	0.560-0.710	$\geq$ 800	$\geq$ 0.55

The samples with XAD-4 (200 mg) and XAD-16 (4g) have been placed on resins, after their activation, into glass bottle and shaken for 30 min, for allow sample to attacked to resin, by mechanic agitation and no magnetic agitation for prevent break of resins.

After each elution (15 min), resins were filtered to remove the solvent, their avoiding deactivation (dry resin). After deposit, sequential elution done with water (30 mL XAD-4 and 840 mL XAD-16); 20 mL of 70% ethanol for XAD-4 and 420 mL of 80% ethanol for XAD-16 and 100% ethanol (30 mL XAD-4 and 700 mL XAD-16). Obtained fractions were evaporated under reduced pressure and analysed by LC-MS.

6.8. Antioxidant activity

An antioxidant is defined as a molecule capable of slowing or preventing the oxidation of other molecules, whereas a biological antioxidant has been defined as "any substance that when present at low concentrations compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substrate". Recently, polyphenols/flavonoids found in plants have begun to receive much attention among researchers as a new natural antioxidant. Several methods were developed for measuring the total antioxidant capacity of a matrix; these assays differ in their chemistry (generation of different radicals and/or target molecules) and in the way end points are measured.

Because different antioxidant compounds may act *in vivo* through different mechanisms, no single method can fully evaluate the antioxidant activity of a matrix. For this reason, in this project the antioxidant properties of plant extracts were investigated using different methods. To this aim, three methods, i.e., ABTS, DPPH, FRAP, and β-carotene bleaching tests, were selected.

6.8.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) test

DPPH radical scavenging assay measured the ability of antioxidants directly react with DPPH radicals, monitoring the decrease of absorbance. DPPH is a stable free radical, but when find an antioxidant, there is a transfer of proton from antioxidant to radical and consequently decrease of absorbance. This assay is simple, rapid and allow to evaluated pure compounds, extracts, and both hydrophilic and lipophilic antioxidant compounds, because it is compatible with polar and no polar organic solvents (Charles, 2013).

The DPPH radicals scavenging activity was determined according to the method previously described (Loizzo et al., 2010). The mixture of DPPH methanol solution (800 μL at concentration of 1.0×10^{-4} M), and methanolic solutions of samples (extracts and fractions) (200 μL, at concentration in the range 1-1000 μg/mL) were prepared. The pure compounds were analysed at concentrations in the range 0.4-100 μg/mL.

The absorbance was read at 517 nm. The positive control was ascorbic acid. The scavenging activity (%) was calculated as follows:

DPPH radicals scavenging activity (%)= $[(A_0 - A_1)/A_0] \times 100$,

where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the samples/fractions/standards.

6.8.2. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) test

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay measured the ability of antioxidant to react with ABTS cation radicals formated by a chemical method. Radical formation was due to potassium persulfate or manganese dioxide used as radical initiator. $ABTS^{\bullet+}$ is a cation radical, characterized by a blue-green colour that becomes colourless when reduced to its non-radical form (ABTS) by antioxidants. Scavenging mechanism occur *via* electron donation or hydrogen atom transfer. As DPPH assay, ABTS test is simple, rapid, and versatile with hydrophilic and lipophilic compounds (Charles, 2013). In brief, the ABTS solution (2 mM) was mixed with potassium persulfate (500 μL) and left in the dark for 12 h before use (Loizzo et al., 2010) and an absorbance of 0.70 at 734 nm was measured. Samples (10 μL) (1-400 μg/mL) and pure compounds (0.15-10 μg/mL) were added to the ABTS solution, and the absorbance was measured after 6 min. Ascorbic acid was the positive control. The scavenging ability (%) was calculated with this equation:

ABTS radicals scavenging activity (%) = $[(A_0-A_1)/A_0] \times 100$,

where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the samples/fractions/standards.

6.8.3. β-Carotene bleaching test

This method is based of discoloration of a β-carotene solution due to the breaking of π-conjugation by addition reaction of lipid or lipid peroxyl radical, or to a C=C double bond of β-carotene. Use of antioxidant compound prevent the discoloration, because it's competing with the radical species to reaction with β-carotene.

This assay was done following the procedure previously described (Amin et al., 2004). Concisely, the β-carotene solution (1 mL) was mixed with 20 μL of linoleic acid and 200 μL of 100% Tween 20. After evaporation of chloroform and dilution with water (100 mL), the emulsion (5 mL) was mixed with 200 μL of extracts (at concentrations in the range 1-100 μg/mL) or with 200 μL of pure compounds (at concentrations in the range 1-40 μg/mL). Tubes were placed in a water bath at 45 °C. The absorbance was read at 470 nm against a blank at t= 0 and after 30 and 60 min of incubation.

Propyl gallate was used as positive control. The antioxidant activity (AA) was calculated as follow:

$$AA = \left(\frac{A_0 - A_t}{A_0^* - A_t^*} \times 100 \right) \pm S.D.$$

where A_0 and A_0^* are the absorbance values obtained at the time 0 for samples and control, respectively, while A_t and A_t^* are the absorbance values obtained after 30 and 60 min of incubation for samples and control, respectively.

6.8.4. Ferric Reducing Activity Power (FRAP) assay

The FRAP method measures the change in absorbance that occurs when the TPTZ (2,4,6-tripyridyl-*s*-triazine)–Fe^{3+} complex is reduced to the TPTZ-Fe^{2+} form in the presence of an antioxidant (Benzie and Strains, 1996). Concisely, the FRAP reagent was freshly prepared with 2.5 mL of 10 mM TPTZ solution, 40 mM HCl, 2.5 mL of 20 mM $FeCl_3$, and 25 mL of 0.3 M acetate buffer (pH 3.6) (Loizzo et al., 2010). Extracts were dissolved in methanol at a concentration of 2.5 mg/mL.

Concentrations of 1.0 mg/mL were prepared for fractions and pure compounds. An aliquot of 0.2 mL of sample solution was mixed with 1.8 mL of FRAP reagent and the absorption was measured at 595 nm. Methanolic solutions of known Fe(II) concentration were used for obtaining the calibration curve. The FRAP value represents the *ratio* between the slope of the linear plot for reducing Fe^{3+}–TPTZ reagent by extract compared to the slope of the plot for $FeSO_4$. The positive control was butylated hydroxytoluene (BHT).

6.8.5. Relative Antioxidant Capacity Index (RACI)

Relative Antioxidant Capacity Index (RACI) is used as an integrated approach to evaluate and compare the antioxidant capacity of different samples (Sun and Tanumihardjo, 2007). Herein, data obtained from ABTS, DPPH, FRAP, and *β*-carotene bleaching tests were used to calculate RACI value for *A. unedo*, *C. mas*, *C. sanguinea*, and *V. corymbosum* samples. Standard scores were derived from data from different chemical methods without unrestricted units and no variance between the methods. The standard score is calculated using the following equation: $RACI = (x - \mu)/\sigma$
where x is the raw data, μ is the mean, and σ is the standard deviation.

6.8.6. Global Antioxidant Score (GAS) calculation

Global Antioxidant Score (GAS) is a correlation index of results obtained from different *in vitro* assays that allows evaluating the total antioxidant activity of analysed samples. The T-score is calculated from the equation:

$$T - score = (X - min)/(max - min),$$

where min and max represent the smallest and largest values, respectively, of the variable X between the studied extracts (Leeuw et al., 2014).

6.9. *In vitro* inhibitory activity of carbohydrates-hydrolysing enzymes

Modulation of hyperglycaemia is an important tool in the management of the diabetic patient. *α*-Amylase is an endoglucanase, which hydrolyses the internal *α*-1,4 glucosidic

linkages in starch while α-glucosidase is one of the glucosidases located in the brush border surface membrane of intestinal cells. Both enzymes are involved in the carbohydrates digestion and absorption. For the above reason, both enzymes have been recognized as therapeutic targets for modulation of post-prandial hyperglycaemia in patients with type 2 diabetes mellitus (Kim et al., 2014).

6.9.1. α-Amylase inhibitory activity assay

The α-amylase inhibition assay was performed as previously described (Tundis et al., 2016). Briefly, the α-amylase (EC 3.2.1.1) solution was prepared by mixing 25.3 mg of enzyme in 100 mL of cold distilled water. The starch solution (0.5% *w/v*) was obtained by stirring 0.125 g of potato starch in 25 ml of 20 mM sodium phosphate buffer (PBS) with 6.7 mM sodium chloride, pH 6.9 at 65 °C for 15 min.

The colorimetric reagent was prepared mixing a sodium potassium tartrate solution (12.0 g of sodium potassium tartrate, tetrahydrate in 8.0 mL of 2 M NaOH) and 96 mM 3,5-dinitrosalicylic acid solution. Extracts (at concentrations in the range 1-1000 μg/mL), pure compounds (at concentrations in the range 1-50 μg/mL), and the positive control (acarbose) were added to the starch solution and left to react with α-amylase solution at 25 °C for 5 min. The generation of maltose was quantified by the reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid, the product being detectable at 540 nm. The percentage of enzyme inhibition (% I) was calculated by using the following equation:

$$\% \text{ Inhibition} = 100 - \left(\frac{[\text{Maltose}]\ \text{test}}{[\text{Maltose}]\ \text{control}} \times 100 \right) \pm \text{S.D.}$$

6.9.2. α-Glucosidase inhibitory activity assay

In the α-glucosidase inhibition assay a maltose solution was prepared mixing 12 g of maltose in 300 mL of 50 mM sodium acetate buffer (Tundis et al., 2016). The enzyme solution was prepared by 1 mg of α-glucosidase (10 units/mg) in 10 mL of ice cold distilled water. The *o*-dianisidine (DIAN) solution was prepared by dissolving one tablet in 25 mL of distilled water. Peroxidase-glucose oxidase (PGO) system-colour reagent solution was obtained by dissolving one capsule in 100 mL of ice cold distilled water.

Extracts, fractions (at concentrations in the range 1-1000 μg/mL), pure compounds (at concentrations in the range 1-50 μg/mL) and control were stirred to maltose solution and left to equilibrate for 5 min at 37 °C. The reaction was started with addition of α-glucosidase solution. The reaction was stopped by adding a solution of perchloric acid, after 30 min of incubation at 37 °C. The supernatant of tube of step one was mixed with

DIAN and PGO and was left to incubate at 37 °C for 30 min. Acarbose was used as positive control.

The percentage of enzyme inhibition (% I) was calculated by using spectrophotometric data at 500 nm and by the equation:

$$\% \text{ Inhibition} = 100 - \left(\frac{[\text{Glucose}]\ \text{test}}{[\text{Glucose}]\ \text{control}} \times 100 \right) \pm \text{S.D.}$$

6.10. Cell viability assay

6.10.1. Cell culture

Human Foreskin Fibroblast (HFF1), obtained from the American Type Culture Collection (Rockville, MD, USA), were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Life Technologies) supplemented with 15 % fetal bovine serum, 4.5 g/L glucose, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were plated at a constant density to obtain identical experimental conditions in the different tests, and to achieve a high accuracy of the measurements.

6.10.2. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

MTT assay is a colorimetric assay for assessing cell metabolic activity. This assay measured the conversion of tetrazolium salt (MTT) to yield coloured (purple colour) formazan in the presence of metabolic activity (Malfa et al., 2014). MTT assay was performed to assess cell viability on a 96 multiwell plate (8×10^3 cells/well).

After 24 h of incubation in humidified atmosphere of 5% CO_2 at 37 °C to allow cell attachment, the cells were treated with different concentrations of extracts (12.5-250 μg/mL) for 24 h. This assay measures the conversion of tetrazolium salt to yield coloured formazan in the presence of metabolic activity. The amount of formazan is proportional to the number of living cells. The optical density was measured with a microplate spectrophotometer reader (Titertek Multiskan, Flow Laboratories, Helsinki, Finland) at λ= 570 nm. Results are expressed as percentage cell viability respect to control (untreated cells).

6.11. Inhibitory effects on nitric oxide (NO) production

Nitric oxide (NO) is as a potent mediator in several cellular processes such as regulation of neurotransmission, vascular tone, host defence mechanisms, and inflammation (Sharma et al., 2007). The use of NO inhibitors represents an important therapeutic approach in the management of inflammatory diseases.

Herein, the inhibitory effects of extracts on NO production were investigated by using the assay based on the reaction of diazocopulation of nitrite with the Griess reagent

(Saijo et al., 2010). HFF1 cells, pre-treated with 12.5 μg/mL of sample for 90 min, were stimulated with interleukin-2β (IL-2β) (10 μg/mL) for 30 min. The method is based on the reaction of diazocopulation of nitrite with the Griess reagent. The total nitrite concentration in the cells was measured by adding 250 μL of Griess reagent to 250 μL of medium. The optical density of each well was measured with a microplate spectrophotometer reader (Titertek Multiskan, Flow Laboratories, Helsinki, Finland) at λ= 546 nm. Results were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in H_2O and expressed as percentage of nitrite production respect to untreated and interleukin stimulated cells.

6.12. NF-κB immunolocalization

RAW 264.7 cells were seeded on coverslip in 6-well plates at a density of 1×10^5 cells/well, and cultured overnight in complete medium. Then, they were treated for 1 h with LPS (1 μg/mL) and selected fractions from *C. mas* namely TDB (II) and MDB (II), and from *C. sanguinea* namely PF1 (II), and SD2 (II) at their respective IC_{50} value. Ice cold methanol was used to fix RAW 264.7 cells for 20 min at – 20 °C. Cells were washed with Tris buffer saline (TBS) and incubated for blocking with 5% bovine serum albumin in TBS for 40 min at 37 °C. Next, cells were incubated in anti-NF-κB p65 monoclonal antibody diluted 1:200, for 40 min at 37 °C. Then, they were washed with TBS and incubated in anti-mouse IgG-TRITC, diluted 1:300, for 40 min at 37 °C in TBS. Finally, cells were washed with TBS before microscope visualization.

Images at 20× magnification were taken on Olympus BX41 microscope with CSV1.14 software, using a CAMXC-30 for image acquisition.

6.13. Haemolysis assay in human blood

Fresh human blood from healthy volunteers was collected in sodium citrate tubes and centrifuged at 2000 rpm for 10 min to isolate red blood cells (RBCs) as a pellet. RBCs were washed three times with cold PBS pH 7.4, and resuspended in the same buffer (10% *v/v*). Further, 10 μg/mL of the different fractions, namely TDB (II), MDB (II), PF1 (II), and SD2 (II), were added to the erythrocyte suspension and incubated for up to 24 h at 37 °C. The release of haemoglobin was determined after centrifugation (2000 rpm for 10 min) by photometric analysis of the supernatant at 540 nm at two different endpoints (1 and 24 h), using a microplate reader (Synergy H1 microplate reader, BioTek).

Complete haemolysis was achieved by using 0.1% (*v/v*) Triton X-100 which yielded the 100% positive control value. DMSO 0.5%, used as solvent for the fractions, gave the negative control value.

6.14. Statistical analysis

Data are expressed as mean ± standard deviation (SD) of the mean and subjected to statistical analysis using Graphpad Prism 4 statistical software package (Graphpad, San

Diego, CA, USA). Differences within and between groups were evaluated by one-way analysis of variance test (ANOVA) followed by multicomparison Dunnett's test that was used to compare each groups with the positive control. A p value < 0.05 was considered statistically significant. Obtained data were processed using statistical procedures to highlight any significant relationship between the chemical composition of the extracts and/or fractions and bioactivity. *Pearson's* correlation coefficient (r) and linear regression, assessment of repeatability, calculation of average, and relative standard deviation was performed using Microsoft Excel 2010 software.

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Chapter 7

Arbutus unedo: Results and discussion

7.1. Effect of extraction procedures on chemicals content

A. unedo fruits (5.9 kg) and leaves (4.1 kg) were separated in two parts for the preparation of fresh and dried materials. Fruits (2.4 kg) were dried at 50 °C for 7 days. Leaves (1.8 kg) were dried at room temperature for 7 days in the dark. At the end of these procedures, 3.5 kg of fresh fruits, 950 g of dried fruits, 2.3 kg of fresh leaves, and 674 g of dried leaves were subjected to extraction. The best extraction yield was obtained using hydroalcoholic maceration of dried plant materials with values of 42.32 and 39.21% for fruits (DF2) and leaves (DL2), respectively (Table 7.1 and Figures 7.1 and 7.2).

Interestingly, the use of ethanol or hydroalcoholic solution at room temperature gave yields between 11.44 and 14.72% for fresh materials (both fruits and leaves) without significant differences between the two solvents/solution. On the other hand, remarkable differences can be observed by using dried material of both fruits and leaves.

Table 7.1. Extractive yield (**%**) of *A. unedo* extracts.

	Yield (%)[a]			
Extraction procedure	Fruits		Leaves	
	Fresh	Dried	Fresh	Dried
Maceration (EtOH)	12.53 ± 1.31	19.80 ± 2.03	10.74 ± 1.11	21.51 ± 2.50
Maceration (Hydroalcoholic 60%)	11.44 ± 1.20	42.32 ± 4.25	14.72 ± 1.54	39.21 ± 4.21
Decoction (H_2O)	9.60 ± 0.92	5.70 ± 0.51	6.60 ± 0.70	10.84 ± 1.82
Soxhlet apparatus (EtOH)	15.92 ± 1.64	25.44 ± 2.50	4.70 ± 0.51	21.43 ± 2.10
Ultrasound-assisted extraction (EtOH)	10.02 ± 1.01	9.50 ± 0.94	8.10 ± 0.82	7.30 ± 0.72

Data are reported as mean ± standard deviation (n= 3). [a] Expressed as (g dried extract/ g plant materials) × 100

Extraction yields in the range 9.60-15.92% were obtained with fresh fruits. The direct extraction of fresh fruits in ethanol Soxhlet apparatus employing ethanol gave the highest yield (15.9%). In a previous work, Isbilir et al. (2012) confirmed that ethanol (70.3%) has greater efficacy as a solvent than water (50.3%) for the extraction in fresh fruits.

Except for decoction and ultrasound-assisted extraction (EtOH), the extraction of the fruits after drying allows to obtain greater yields.

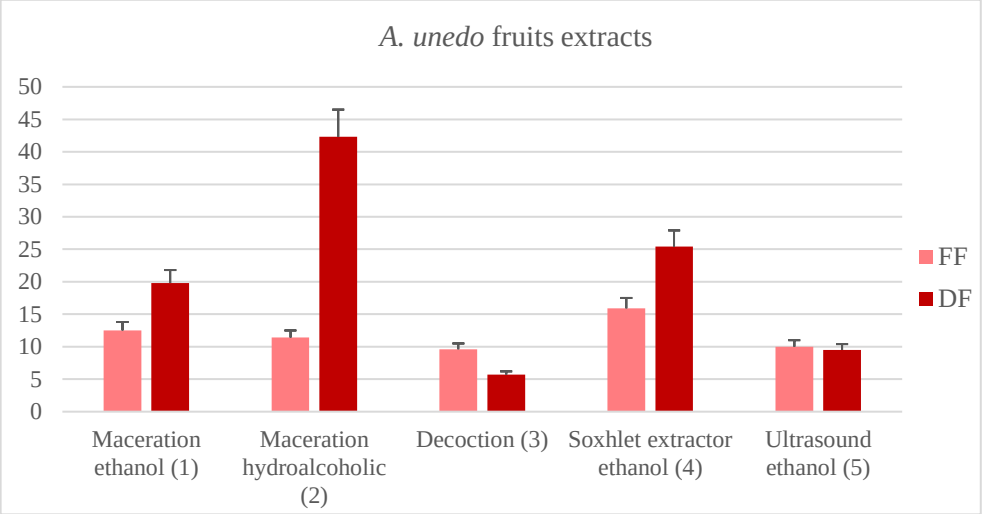


Figure 7.1. Extraction yield (%) of *A. unedo* fruits extracts. FF: fresh fruits; DF: dried fruits.

Hydroalcoholic maceration is confirmed as the technique that allows to obtain higher yields (14.72%) compared to the other extraction procedures used for fresh leaves (4.70-10.74%). However, the best extractive results in terms of yield were obtained with dried leaves. In fact, except only for the ethanol ultrasound-assisted extraction, values ranged from 10.84 to 39.21%.

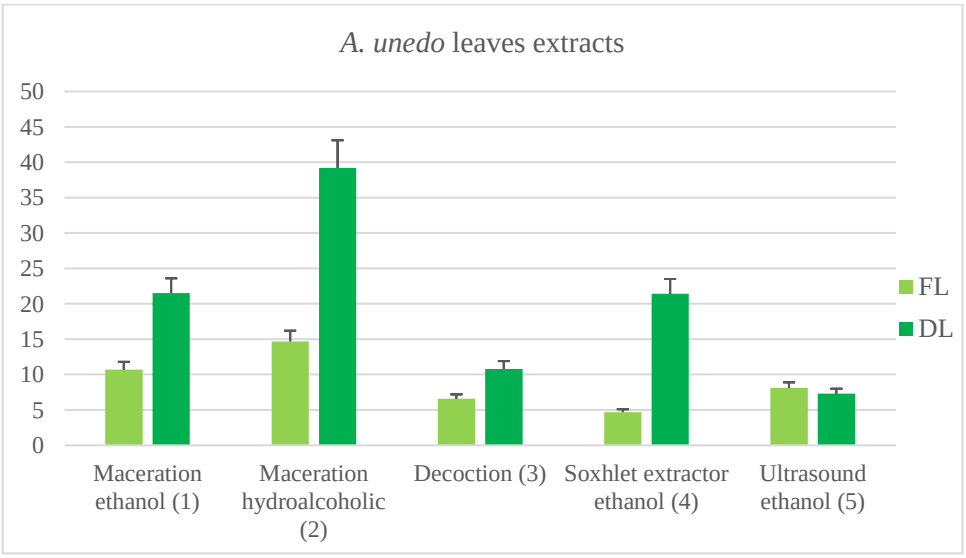


Figure 7.2. Extraction yield (%) of *A. unedo* leaves extracts. FF: fresh leaves; DF: dried leaves.

In a previous work, Oliveira et al. (2009) reported water as more efficient in the extraction of *A. unedo* dried leaves (32.1%) compared with ethanol (15%). On the other hand, Orak et al. (2011a) reported similar yields by using ethanol and water for the extraction of dried leaves.

A. unedo extracts were preliminarily investigated in order to evaluate the total content of the most important classes of constituents such as phenols, flavonoids, and iridoids.

Data are shown in Table 7.2 and 7.3. Results show that extracts prepared starting from fresh fruits by using hydroalcoholic maceration (FF2) showed the highest content of both total phenols and flavonoids with values of 40.06 mg chlorogenic acid (CA) equivalents/g extract and 29.13 mg quercetin (QE) equivalents/g extract, respectively. An interesting total iridoids content (104.11-158.67 mg aucubin (AU) equivalents/g dry extract) was found for all extract. The highest TIC was obtained using Soxhlet apparatus (FF4) (158.67 mg AU equivalents/g extract).

Table 7.2. Total phytochemicals content of *A. unedo* fruits and leaves extracts

A.unedo	Total Phenols Content (TPC)[a]	Total Flavonoids Content (TFC)[b]	Total Iridoids Content (TIC)[c]
Fresh fruits			
FF1	39.93 ± 0.41	26.07 ± 0.13	119.33 ± 1.30
FF2	40.06 ± 0.35	29.13 ± 0.20	104.67 ± 1.02
FF3	34.53 ± 0.44	25.13 ± 0.34	104.11 ± 1.23
FF4	35.87 ± 0.53	25.22 ± 0.23	158.67 ± 1.60
FF5	35.02 ± 0.55	26.61 ± 0.25	108.21 ± 1.24
Dried fruits			
DF1	82.20 ± 1.03	27.73 ± 0.22	147.33 ± 1.21
DF2	42.27 ± 0.21	26.02 ± 0.31	176.66 ± 1.94
DF3	81.73 ± 1.15	26.86 ± 0.20	116.61 ± 1.20
DF4	39.27 ± 0.64	27.27 ± 0.22	166.22 ± 1.01
DF5	36.13 ± 0.73	26.13 ± 0.13	195.30 ± 1.92

FF: fresh fruits, DF: dried fruits; 1. Ethanolic maceration; 2. Hydroalcoholic (60%) maceration; 3. Decoction; 4. Ethanol Soxhlet extraction; 5. Ethanol ultrasound-assisted extraction. Data are reported to mean ± Standard Deviation (SD) (n = 3). [a]mg chlorogenic acid (CA) equivalents/g extract; QE: [b]mg quercetin (QE) equivalents/g extract; [c]mg aucubin (AU) equivalents/g extract.

Among dried fruit samples, ethanol maceration and decoction allow to obtain the highest total phenols content with values of 82.20 and 81.73 mg CA equivalents/g extract for DF1 and DF3, respectively. This trend was not found for TFC. In fact, all extracts evidenced a similar flavonoids content (26.02-27.73 mg QE equivalents/g extract).

Fortalezas et al. (2010) have reported for the hydroalcoholic (50% ethanol) dried fruits extract a value of TPC of 16.46 mg gallic acid (GAE) equivalents/g. Results obtained by Bouzid et al. (2014) indicated lower values of TPC and TFC in aqueous extracts of dried fruits from Turkey compared with our results.

Orak et al. (2011b) reported for the fresh fruits TPC value of 14.29 mg GAE/g that decrease with different drying treatment (ethyl oleate and water blanching application). Compared with our results, low TPC and TFC values were found by Turker et al. (2012) that investigated fresh fruits from Turkey. In a recent study, Salem et al. (2018) have analysed and compared the efficiency of methanol and ethanol for the extraction of *A. unedo* dried fruits founding higher phenols and flavonoids contents in ethanol extract compared with methanol extract.

If we consider the total iridoids content, the highest value was obtained by ethanol ultrasound-assisted extraction (195.30 mg AU equivalents/g extract), followed by DF2 and DF4 samples (176.66 and 166.22 mg AU equivalents/g extract).

To the best of our knowledge, this is the first report that reports the total iridoids content of *A. unedo* extracts.

Table 7.3. Total phytochemicals content of *A. unedo* leaves extracts

A.unedo	Total Phenols Content (TPC)[a]	Total Flavonoids Content (TFC)[b]	Total Iridoids Content (TIC)[c]
Fresh leaves			
FL1	305.87 ± 1.74	178.67 ± 1.50	105.01 ± 0.75
FL2	173.33 ± 1.20	87.07 ± 1.02	102.70 ± 0.72
FL3	376.01 ± 1.93	153.62 ± 1.80	115.33 ± 0.63
FL4	298.67 ± 2.50	99.87 ± 0.62	211.31 ± 1.31
FL5	320.21 ± 3.24	137.33 ± 1.41	220.14 ± 1.02
Dried leaves			
DL1	272.67 ± 2.20	152.02 ± 1.52	118.15 ± 1.26
DL2	329.33 ± 2.32	98.01 ± 1.20	170.67 ± 2.05
DL3	290.66 ± 1.90	83.73 ± 1.11	116.22 ± 1.25
DL4	187.73 ± 1.44	99.21 ± 1.23	102.03 ± 1.23
DL5	252.12 ± 1.74	190.04 ± 1.24	135.30 ± 1.21

FL: fresh leaves; DL: dried leaves. 1. Ethanolic maceration; 2. Hydroalcoholic (60%) maceration; 3. Decoction; 4. Ethanol Soxhlet extraction; 5. Ethanol ultrasound-assisted extraction. Data are reported to mean ± Standard Deviation (SD) (n = 3). [a]mg chlorogenic acid (CA) equivalents/g extract; QE: [b]mg quercetin (QE) equivalents/g extract; [c]mg aucubin (AU) equivalents/g extract.

Leaves extracts exhibited a higher total phenols content with values in the range 173-376 mg CA equivalents/g extract compared to the fruits with values in the range 34-82 mg CA equivalents/g extract. The same trend was observed for the total flavonoids content (values ranging from 83 to 190 and from 25 to 29 mg QE equivalents/g extract mg for leaves and fruits, respectively), but not for TIC. In fact, values in the range 104.11-195.30 and 102.03-220.14 mg AU equivalents/g extract were found for fruits and leaves, respectively, among being FL5 and DL5 the extracts with the highest content. Both extracts are obtained by ultrasound-assisted extraction by using ethanol as solvent.

Analysing in more detail results of fresh leaves, interesting results were obtained by using decoction (FL3) and ethanol maceration (FL1), with values of 376.01 mg CA equivalents/g of extract and 178.67 mg QE equivalents/g of extract for total phenols and flavonoids content, respectively.

The richest extract in iridoids was obtained by ethanol ultrasound-assisted extraction (220.14 mg AU equivalents/g of extract; FL5). Among extracts from dried leaves, maceration with hydroalcoholic solution (DL2) presented the best content of phenols and iridoids (329.33 and 170.67 mg/g, respectively). The extracts obtained by ethanol ultrasound-assisted extraction (DL5) and Soxhlet (EtOH) extraction (DL4) were rich in flavonoids (190.04 mg/g).

Oliveira et al. (2009) have studied the effects of several solvents (ethanol, methanol, water, and diethyl ether) for the extraction of phenolic compounds from *A. unedo* dried leaves. The highest phenols content was reported for the ethanolic extract (192.66 mg

gallic acid (GAE) equivalents/g), followed by aqueous extract (172.21 mg GAE equivalents/g). Orak et al. (2011a) reported discordant results. In this study, ethanol extract of dried leaves showed a low TPC (119.97 mg/g) compared with methanol (169.05 mg/g) and water (197.16 mg/g) extracts. Hydroalcoholic solution (70% ethanol) is identified as the best solvent for phenolic compounds extraction by Pavlović et al. (2011) that reported high TPC of 204.8 and 286.9 mg catechin equivalents/g, respectively, for *A. unedo* leaves collected in Montenegro and Greece.

7.2. Phytochemicals identification

LC-ESI-QTOF-MS analyses showed the presence of phenolic acids, iridoids, proanthocyanidins, and flavonoids as main constituents of both fruits (Tables 7.4) and leaves (Tables 7.5). Chromatograms for the compounds identification were reported in Appendix (Figures A1-A3). Quinic acid, ferulic acid, gallic acid, caffeic acid, protocatechuic acid, syringic acid, catechin, isoquercitrin, ellagic acid, rutin, geniposide, hyperoside, and kaempferol were confirmed with authentic standards. Other compounds were identified based on UV spectra, and molecular weight (*m*/*z* ion $[M+H]^+$ or $[M+Na]^+$).

The results of chemical characterization of strawberry tree extracts confirm that both extraction solvents and procedures affected the presence of selected classes of compounds. There are several methods to recover phytochemicals from plants, such as maceration, Soxhlet extraction, ultrasound-assisted extraction, and supercritical fluid extraction. Conventional method extraction such as maceration, allow preserving thermolabile compounds while the use of high temperature can allow increasing the extraction yield because of degradation of internal structures. Use of supercritical fluid extraction allow obtaining the solvent free extracts, in particular with use of carbon dioxide. The disadvantage of this method is that the more polar compounds are not extracted because they have a low solubility in carbon dioxide. However, extraction yield and consequently biological effects not only depend on the extraction method but also on the solvent used for extraction. Constituents with different chemical characteristics and polarity may or may not be soluble in a particular solvent. Solvents commonly used for the extraction of bioactive compounds are water and hydroalcoholic solutions. Ethanol has been known as a good solvent for phenols extraction and is acceptable for human consumption (directive 2009/32/EC). In supercritical fluid extraction, using carbon dioxide and ethanol as co-solvent is effective for the isolation of compounds, overcoming limitations linked to use of this extraction method.

Previous studies on *A. unedo* showed that phenols and iridoids are well extracted by using alcoholic solutions, anthocyanins by using methanol solution of HCl and apolar compounds such as saturated fatty acids and carotenoids by employing acetone-petroleum ether mixture (Karikas, 1993; Maleš et al., 2006; Pawlowska et al., 2006).

Table 7.4. Identification of chemical compounds in *A. unedo* fruits using the LC-ESI-QTOF-MS technique

Compound	Rt (min)	Molecular Formula	MH^+/ MNa^+	Error (ppm)	Score (%)	MS fragment (*m/z*)	UV λ (nm)	Fresh fruits					Dried fruits					References
								FF1	FF2	FF3	FF4	FF5	DF1	DF2	DF3	DF4	DF5	
Phenolic acids																		
Anisic acid	1.4	$C_8H_8O_3Na$	175.039	1.2	95		283	√	√	√	√	√	√	√	√	√	√	Ayaz et al., 2000
Ellagic acid 4-*O*-*β*-D-glucopyranoside	10.1	$C_{20}H_{16}O_{13}$	465.066	0.7	100	303.0136	256, 348	√	√	√	√	√	√	√	√	√	√	Yoshida et al., 1994
Ferulic acid°	1.7	$C_{10}H_{10}O_4$	195.065	2.1	100		325	√	√	√	√	√	√	√	√	√	√	Hamad et al., 2011
Gallic acid°	3.2	$C_7H_6O_5$	171.029	0.3	100		217, 271	√	√	√	√	√	√	√	√	√	√	Ayaz et al., 2000
Galloyl quinic acid (3-*O*- or 5-*O*-)	3.9	$C_{14}H_{16}O_{10}$	345.081	0.3	98		Nd	√	√	√	√	√	√	√	√	√	√	Pawlowska et al., 2006
Protocatechuic acid°	6.2	$C_7H_6O_4$	155.035	1.8	100		290	√	√	√			√					Ayaz et al., 2000
Quinic acid°	0.9	$C_7H_{12}O_6$	193.071	0.5	100		-	√	√	√	√	√	√	√	√	√	√	El Shibani, 2017
Shikimic acid gallate (3-*O*- or 5-*O*-)	7.1	$C_{14}H_{14}O_9$	327.071	0.7	100		215, 277	√	√	√	√	√	√	√	√	√	√	Pawlowska et al., 2006
Syringic acid°	10.6	$C_9H_{10}O_5$	199.060	0.1	100		218, 273	√	√							√		Guendouze-Bouchefa et al., 2015
Flavonoids																		
Catechin°	9.4	$C_{15}H_{14}O_6$	291.087	1.2	100		280	√										Pallauf et al., 2008
Hyperoside° (*)	12.8	$C_{21}H_{20}O_{12}$	465.103	0.8	100	303.0499	213,278, 350						√	√	√	√	√	Males et al., 2006
Isoquercitrin° (*)	12.8	$C_{21}H_{20}O_{12}$	465.103	0.8	100	303.0499	213, 253, 353						√	√	√	√	√	Males et al., 2006
Myricetin	12.6	$C_{15}H_{10}O_8$	319.045	0.1	100		220, 255, 375	√	√	√	√	√	√	√	√	√	√	Hamad et al., 2011
Myricetin 3-*O*-rhamnopyranoside (*)	12.7	$C_{21}H_{20}O_{12}$	465.103	0.4	100	319.0389	219, 253, 365						√	√	√	√	√	Sakar et al., 1992
Quercetin 3-*O*-arabinoside (**)	13.4	$C_{20}H_{18}O_{11}$	435.775	0.3	100	303.0499	213, 253, 353	√	√	√	√	√	√	√	√	√	√	Sakar et al., 1992
Quercetin 3-*O*-xyloside (**)	13.5	$C_{20}H_{18}O_{11}$	435.775	0.3	100	303.0499	213, 254, 356	√	√	√	√	√	√	√	√	√	√	Pallauf et al., 2008
Quercitrin	13.6	$C_{21}H_{20}O_{11}$	449.108	0.7	100	303.0499	213, 254, 356	√	√	√	√	√	√	√	√	√	√	Males et al., 2006
Iridoids																		
Asperuloside	1.8	$C_{18}H_{22}O_{11}$	415.121	4.4	84		239									√		Karikas, 1993
Stilbericoside	15.7	$C_{14}H_{20}O_{10}$	349.113	0.1	99		Nd	√					√	√	√	√	√	Davini et al., 1981
Unedide	1.2	$C_{16}H_{24}O_{12}$	409.134	0.4	100		Nd	√	√	√	√	√						Davini et al., 1981

FF: fresh fruits extracts, DF: dried fruits extracts. 1. Ethanolic maceration; 2. Hydroalcoholic (60%) maceration; 3. Decoction; 4. Ethanol Soxhlet extraction; 5. Ethanol ultrasound-assisted extraction. Nd: not detected. ° identified with standard compounds; **in bold** not previously identified in the plant (or plant part); (*) (**) : Interchangeable

Table 7.5. Identification of chemical compounds in *A. unedo* leaves using the LC-ESI-QTOF-MS technique

Compound	Rt (min)	Molecular Formula	MH^+/ MNa^+	Error (ppm)	Score (%)	MS fragment (*m/z*)	UV λ (nm)	Fresh Leaves					Dried Leaves					References
								FL1	FL2	FL3	FL4	FL5	DL1	DL2	DL3	DL4	DL5	
Phenolic acids																		
Anisic acid	1.4	$C_8H_8O_3Na$	175.0389	1.2	95		283	√	√	√	√	√	√	√	√	√	√	Ayaz et al., 2000
Caffeic acid°	5.5	$C_9H_8O_4$	181.0498	0.4	100		238, 322						√	√				El Shibani et al., 2017
Ellagic acid°	12.1	$C_{14}H_6O_8$	303.0136	0.8	100		255, 365	√	√	√	√	√	√	√	√	√	√	Guendouze-Bouchefa et al., 2015
Ellagic acid 4-*O*-*β*-D-glucopyranoside	10.1	$C_{20}H_{16}O_{13}$	465.0659	0.7	100	303.0136	256, 348	√	√	√	√	√	√	√	√	√	√	Yoshida et al., 1994
Ferulic acid°	1.7	$C_{10}H_{10}O_4$	195.0652	2.1	100		325	√	√	√	√	√	√	√	√	√	√	Hamad et al., 2011
Gallic acid°	3.2	$C_7H_6O_5$	171.0287	0.3	100		217, 271	√	√	√	√	√	√	√	√	√	√	Ayaz et al., 2000
Galloyl quinic acid (3-*O*- or 5-*O*-)	3.9	$C_{14}H_{16}O_{10}$	345.0814	0.3	98		Nd	√	√	√	√	√	√	√	√	√	√	Pawloska et al., 2006
Norbergenin	9.2	$C_{13}H_{14}O_9$	315.0710	0.5	100		222, 289						√	√				Taneyama et al., 1983
Quinic acid°	0.9	$C_7H_{12}O_6$	193.0706	0.5	100		-	√	√	√	√	√	√	√	√	√	√	El Shibani, 2017
Shikimic acid gallate (3-*O*- or 5-*O*-)	7.1	$C_{14}H_{14}O_9$	327.0711	0.7	100	174.1350	215, 277	√	√	√	√	√	√	√	√	√	√	Pawlowska et al., 2006
Syringic acid°	10.6	$C_9H_{10}O_5$	199.0601	0.1	100		273	√	√	√	√	√	√	√	√	√	√	Guendouze-Bouchefa et al., 2015
Flavonoids																		
Afzelin	14.4	$C_{21}H_{20}O_{10}$	433.1131	0.1	100	287.0550	265, 301,347	√	√	√	√	√	√	√	√	√	√	Guendouze-Bouchefa et al., 2015
Catechin°	9.4	$C_{15}H_{14}O_6$	291.0866	1.2	100		280	√	√	√	√	√	√	√	√	√	√	Pallauf et al., 2008
Hyperoside°(*)	12.8	$C_{21}H_{20}O_{12}$	465.1031	0.8	100	303.0499	254, 353	√	√	√	√	√	√	√	√	√	√	Males et al., 2006
Isoquercitrin°(*)	12.8	$C_{21}H_{20}O_{12}$	465.1031	0.8	100	303.0499	253, 353	√	√	√	√	√	√	√	√	√	√	Males et al., 2006
Isovitexin 7-*O*-glucoside	12.9	$C_{27}H_{30}O_{15}$	595.1658	0.1	100	432.3768	265,330	√	√	√	√	√	√	√	√	√	√	El Shibani, 2017
Kaempferol°	14.2	$C_{15}H_{10}O_7$	287.0550	0.1	100		254, 365	√	√	√	√	√	√	√	√	√	√	Males et al., 2006
Kaempferol 3-*O*-glucoside	13.2	$C_{21}H_{20}O_{11}$	449.1077	0.3	100	287.0488	264, 347	√	√	√	√	√	√	√	√	√	√	Su, 2012
Myricetin	12.6	$C_{15}H_{10}O_8$	319.0446	0.1	100		255, 375			√	√		√					Hamad et al., 2011

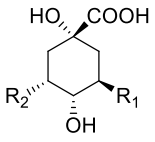
Myricetin 3-*O*-rhamnopyranoside(*)	12.7	$C_{21}H_{20}O_{12}$	465.1031	0.4	100	319.0389	253, 365	√	√	√	√	√	√	√	√	√	√	Sakar et al., 1992
Myricetin 3-*O*-xyloside	12.4	$C_{20}H_{18}O_{12}$	451.0752	0.5	100	319.0435	255, 373	√	√	√	√	√	√	√	√	√	√	Pallauf et al., 2008
Naringenin 7-*O*-glucoside	12.4	$C_{21}H_{22}O_{10}$	435.1257	0.2	100	273.5640	283, 332	√	√	√	√	√	√	√	√	√	√	El Shibani, 2017
Quercetin 3-*O*-arabinoside(**)	13.4	$C_{20}H_{18}O_{11}$	435.7749	0.3	100	303.0499	253, 353	√	√	√	√	√	√	√	√	√	√	Sakar et al., 1992
Quercetin 3-*O*-xyloside(**)	13.5	$C_{20}H_{18}O_{11}$	435.7749	0.3	100	303.0499	254, 356	√	√	√	√	√	√	√	√	√	√	Pallauf et al., 2008
Quercitrin	13.6	$C_{21}H_{20}O_{11}$	449.1079	0.7	100	303.0499	254, 356	√	√	√	√	√	√	√	√	√	√	Males et al., 2006
Rutin°	12.4	$C_{27}H_{30}O_{16}$	611.1612	0.2	100	303.0499	253, 352	√	√	√	√	√	√	√	√	√	√	Pallauf et al., 2008
Proanthocyanidins																		
Epicatechin-4,6-catechin(***)	9.5	$C_{30}H_{26}O_{12}$	579.1495	0.1	100	291.0851	280	√	√	√	√	√	√	√	√	√	√	Pallauf et al., 2008
Epicatechin-4,8-epicatechin(***)	11.8	$C_{30}H_{26}O_{12}$	579.1492	0.3	100	291.0851	280	√	√	√	√	√	√	√	√	√	√	Pallauf et al., 2008
Gallocatechin	6.8	$C_{15}H_{14}O_7$	307.0811	2.9	89		271			√	√		√	√	√			Pallauf et al., 2008
Iridoids																		
Asperuloside	1.8	$C_{18}H_{22}O_{11}$	415.1214	4.4	84		239			√			√	√				Karikas, 1993
Gardenoside	5.9	$C_{17}H_{24}O_{11}$	405.1391	0.5	96		237	√	√	√		√	√	√	√		√	Karikas, 1993
Geniposide°	10.5	$C_{17}H_{24}O_{10}$	389.1447	0.8	98		239						√	√				Karikas et al., 1987
Stilbericoside	15.7	$C_{14}H_{20}O_{10}$	349.1129	0.1	99		Nd								√			Davini et al., 1981
Unedide	1.2	$C_{16}H_{24}O_{12}$	409.1340	0.4	100		Nd			√			√	√	√	√	√	Davini et al., 1981
Unedoside	11.2	$C_{14}H_{20}O_{10}$	333.0819	0.4	95		Nd						√	√				Davini et al., 1981
Phenolic glucosides																		
Arbutin	1.8	$C_{12}H_{16}O_7Na$	295.0793	0.8	96		230, 282						√	√				Guendouze-Bouchefa et al., 2015
p-hydroxybenzoic acid glucuronide	9.8	$C_{13}H_{14}O_9$	315.0712	0.5	94		253		√	√	√	√	√	√	√	√	√	Ayaz et al., 2000

FL: fresh leaves extracts; DL: dried leaves extracts. 1. Ethanolic maceration; 2. Hydroalcoholic (60%) maceration; 3. Decoction; 4. Ethanol Soxhlet extraction; 5. Ethanol ultrasound-assisted extraction.
° identified with standard compounds; **in bold** not previously identified in the plant (or plant part); (*) (**)(***): Interchangeable

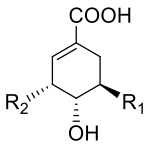
7.2.1. Phenolic acids

Fruits and leaves extracts were characterised by the presence of various phenolic acids (Figure 7.3). The number of phenolic compounds identified in the leaves was higher than in fruits extracts, as confirmed in the literature (Mendes et al., 2011). Quinic acid, anisic acid, ellagic acid 4-*O*-*β*-D-glucopyranoside, shikimic acid gallate (3-*O*- or 5-*O*-), gallic acid, ferulic acid, and galloyl quinic acid (3-*O*- or 5-*O*-) were systematically identified. Caffeic acid, ellagic acid, and *p*-hydroxybenzoic acid glucuronide were identified only in leaves extracts. Moreover, arbutin and caffeic acid were specifically detected in the dried leaves obtained by maceration with ethanol and ethanol/water (6/4), according to the literature (Tenuta et al., 2018).

Acids

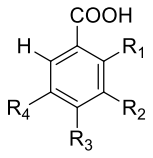


	R_1	R_2
Quinic	OH	OH
3-*O*-Galloyl quinic acid	*O*-Galloyl	OH
5-*O*-Galloyl quinic acid	OH	*O*-Galloyl

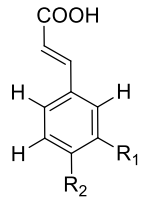


	R_1	R_2
Shikimic acid 3-*O*-gallate	*O*-Galloyl	OH
Shikimic acid 5-*O*-gallate	OH	*O*-Galloyl

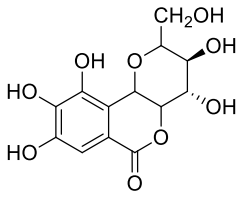
Phenolic acids and glycosides



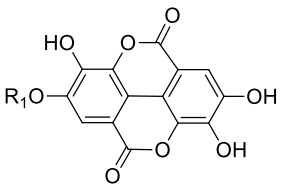
	R_1	R_2	R_3	R_4
Anisic acid	H	OCH_3	H	H
Gallic acid	H	OH	OH	OH
Protocatechuic acid	H	OH	OH	H
Syringic acid	H	OCH_3	OH	OCH_3



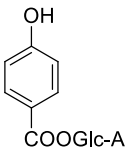
	R_1	R_2
Caffeic acid	OH	OH
Ferulic acid	OCH_3	OH



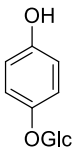
Norbergenin



	R_1
Ellagic acid	H
Ellagic acid 4-*O*-*β*-D-glucopyranoside	*O*-Glc



p-hydroxybenzoic acid glucuronide



Arbutin

Figure 7.3. The main acids, phenolic acids, and phenolic glycosides identified in *A. unedo* extracts

Syringic acid was detected in all leaves extracts and only in the ethanol and hydroalcoholic macerations of fresh fruits and Soxhlet (EtOH) extract of dried fruits. Protocatechuic acid was found only in the fruits extracts (FF1, FF2, FF3 and DF1). The majority of phenolic acids identified in our extracts are previously described (Ayaz et al., 2000; El-Shibani, 2017; Guendouze-Bouchefa et al., 2015; Hamad et al., 2011; Pawlowska et al., 2006; Yoshida et al., 1994).

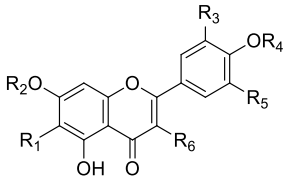
Norbergenin was found only in the dried leaves extracts obtained by maceration with ethanol and ethanol/water.

To the best of our knowledge, quinic, ferulic, caffeic acids, ellagic acid 4-*O*-*β*-D-glucopyranoside and norbergenin were herein found for the first time in *A. unedo*.

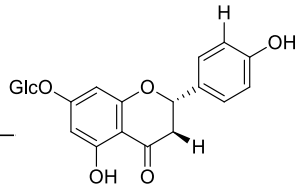
7.2.2. Flavonoids

Flavonoids are identified in all *A. unedo* extracts (Figure 7.4). However, there are difference in dependence of the extraction procedures. Quercetin derivatives (arabinoside, xyloside, and rhamnoside) were detected in all extracts.

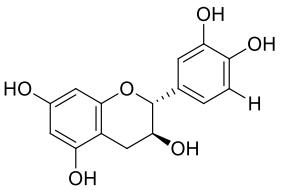
Afzelin, kaempferol 3-*O*-glucoside, naringenin 7-*O*-glucoside, rutin, isovitexin 7-*O*-glucoside, myricetin 3-*O*-xyloside, and kaempferol were found only in the leaves extracts. Isoquercitrin, hyperoside, and myricetin 3-*O*-rhamnopyranoside characterised all leaves extracts and dried fruits extracts; instead, catechin was found in all leaves extracts and ethanol extract obtained after maceration of fresh fruits.



	R_1	R_2	R_3	R_4	R_5	R_6
Afzelin	H	H	H	H	H	*O*-Rha
Hyperoside	H	H	OH	H	H	*O*-Gal
Isoquercitrin	H	H	OH	H	H	*O*-Glc
Isovitexin 7-*O*-glucoside	Glc	Glc	H	H	H	H
Kaempferol	H	H	H	H	H	OH
Kaempferol 3-*O*-glucoside	H	H	H	H	H	*O*-Glc
Myricetin	H	H	OH	H	OH	OH
Myricetin 3-*O*-rhamnopyranoside	H	H	OH	H	OH	*O*-Rha
Myricetin 3-*O*-xyloside	H	H	OH	H	OH	*O*-Xyl
Quercetin 3-*O*-arabinoside	H	H	OH	H	H	*O*-Ara
Quercetin 3-*O*-xyloside	H	H	OH	H	H	*O*-Xyl
Quercitrin	H	H	OH	H	H	*O*-Rha
Rutin	H	H	OH	H	H	*O*-Glc(6←1)Rha



Naringenin 7-O-glucoside



Catechin

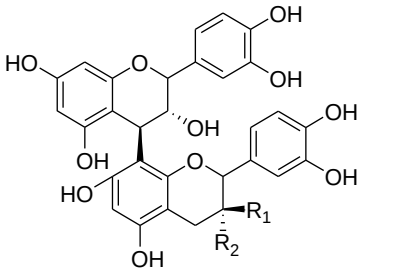
Figure 7.4. Principal flavonoids identified in *A. unedo* extracts

Myricetin was observed in all extracts obtained by fruits and only in the extract obtained by dried leaves ethanolic maceration, decoction and Soxhlet (EtOH) extracts of fresh leaves. Interestingly, for the first time the presence of naringenin 7-*O*-glucoside, kaempferol 3-*O*-glucoside, isovitexin 7-*O*-glucoside, myricetin 3-*O*-rhamnopyranoside, norbergenin, and myricetin were identified in *A. unedo* extracts.

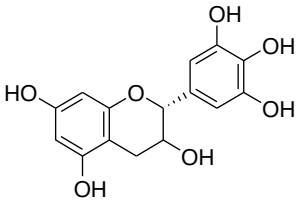
7.2.3. Proanthocyanidins

In contrast to the literature, proanthocyanidins detected in the present study were identified in the leaves, but not in the fruits (Pallauf et al., 2008).

In particular, epicatechin-4,6-catechin and epicatechin-4,8-epicatechin (Figure 7.5) were found in all leaves extracts, while gallocatechin was identified in some extracts such as FL3, FL4, and DL1-3.



	R_1	R_2
Epicatechin-4,8-epicatechin	H	OH
Epicatechin-4,6-catechin	OH	H



Gallocatechin

Figure 7.5. Proanthocyanidins identified in *A. unedo* extracts

7.2.4. Iridoids

Six iridoids (Figure 7.6) were identified in *A. unedo* fruits and leaves extracts. Three of these compounds namely gardenoside, geniposide, and unedoside are specifically produced by the leaves, while other (asperuloside, unedide and stilbericoside) have been found also in the fruits extracts.

Analysing results obtained with fruits extracts, some interesting differences can be highlighted. Stilbericoside (except for FF1) were identified in extracts from dried fruits while unedide in fresh fruits. Interestingly, asperuloside was extracted only from dried fruits by Soxhlet (EtOH) apparatus (DF4).

Geniposide and unedoside were not extracted from fresh leaves but only by ethanol (DL1) and hydroalcoholic maceration (DL2) of dried leaves. Stilbericoside was detected only in the decoction of dried materials (DL3). Unedide was detected in all dried leaves extracts and in a decoction of fresh leaves (FL3).

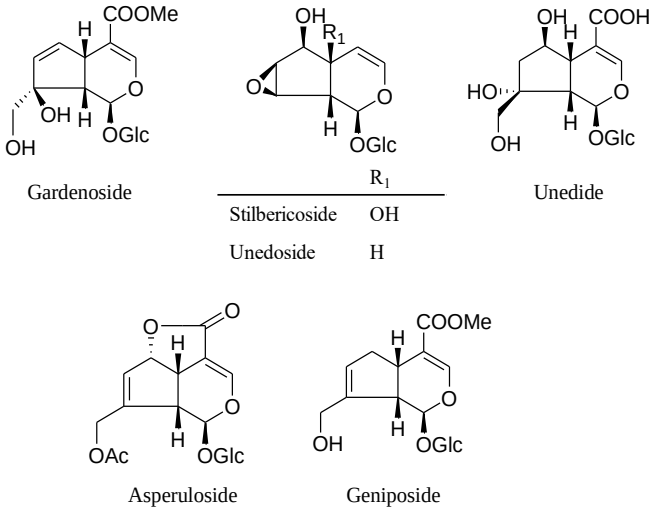


Figure 7.6. Iridoids in the *A. unedo* extracts

Asperuloside and gardenoside were found in fresh and dried leaves extracts. Asperuloside were not only extracted by ethanolic (DL1) and hydroalcoholic (DL2) macerations but also by decoction of the fresh leaves (FL2).

A perusal analysis of the literature revealed the presence of few studies that analysed the presence of iridoids in *A. unedo*. One of these studies is that carried out in 1981 by Davini et al., who isolated unedide and monotropein from the ethanol (90%) extract of the leaves of *A. unedo*. Unedoside is an iridoid rarely biosynthesized in plant kingdom and it is considered as chemotaxonomic marker of *Arbutus* genus (Tenuta et al., 2018).

7.3. *In vitro* biological properties

Oxidative stress occurs when excess reactive oxygen species (ROS) are produced in cells, which could overwhelm the normal antioxidant capacity. When internal defense mechanisms such as antioxidants or enzymes involved in oxygen radical scavenging (e.g. superoxide dismutase, peroxidase, catalase) not controlled the ROS concentration, their over-production result in deleterious effects at DNA, lipids, and proteins.

The World Health Organization classifies several diseases such as diabetes, cardiovascular diseases, arthritis, and allergies, as specific inflammation-mediated diseases. Multiple processes underlie these inflammation-mediated diseases, including unquestionably oxidative stress.

Recent studies have showed that certain natural products have the ability to protect cells from oxidative stress and ameliorate several oxidative stress-related diseases (Pandey et al., 2009). However, still today a large number of natural compounds are unexploited. Understanding the mechanisms of actions of phytochemicals would shed

further light into the application of these compounds in the prevention and treatment of oxidative stress-related diseases in humans.

7.3.1. Antioxidant activity

The increasing interest gained by antioxidants is due to the health benefits provided mainly by natural compounds that consist in preventing the occurrence of oxidative-stress related diseases, caused by the attack of free radicals on key biocomponents like lipids or nucleic acids. Several methods were recently developed for measuring the antioxidant capacity of a sample. These tests vary in the mechanism of generation of different target molecules and/or radicals and in the way end-products are measured.

Considering that different antioxidants may act *in vivo* through different mechanisms of action, to investigate the antioxidant activity choosing adequate assays is critical and no single method can fully evaluate the antioxidant activity of a sample (Pellegrini et al., 2003). Therefore, in the present study, four assays namely ABTS, DPPH, FRAP, and β-carotene bleaching tests were used to investigate and to compare the antioxidant potential of *A. unedo* fruits and leaves extracts. Results are reported in Table 7.6.

ABTS and DPPH assays

A. unedo extracts were tested for their potential free radicals scavenging activity by using ABTS and DPPH assays. ABTS test measures the ability of an antioxidant to scavenge the ABTS radicals that are produced in aqueous phase by the reaction of the ABTS salt with a strong oxidising agent (potassium persulfate or potassium permanganate). The reduction of blue-green ABTS coloured solution by hydrogen-donating antioxidant is measured by the suppression of its characteristic long wave absorption spectrum. DPPH is a stable purple free radical due to the delocalization of the spare electron on the whole molecule. When DPPH radical reacts with a hydrogen donor, the reduced form is generated, accompanied by the disappearance of the violet colour.

All extracts exerted radicals scavenging properties in a concentration-dependent manner. The best results were obtained in ABTS test, particularly with dried leaves that, with IC_{50} values in the range 0.39-1.51 μg/mL, were more active than the positive control ascorbic acid (IC_{50} value of 1.70 μg/mL). Interesting is also the IC_{50} value of FL3 (fresh leaves extracted by decoction) of 1.16 μg/mL.

A. unedo dried fruits were more active than fresh fruits. The best anti-radicals activity was found by extract obtained with Soxhlet (DF4) apparatus (IC_{50} value of 1.16 μg/mL) in ABTS test and by decoction (DF3; IC_{50} value of 32.21 μg/mL) in DPPH test.

Table 7.6. *In vitro* antioxidant activity of *A. unedo* extracts

A. unedo	ABTS test IC_{50} (μg/mL)	DPPH test IC_{50} (μg/mL)	FRAP test IC_{50} (μM Fe (II)/g)	β-Carotene bleaching test IC_{50} (μg/mL)	
				30 min	60 min
Fruits					
FF1	51.30 ± 2.55****	69.07 ± 2.07****	25.70 ± 1.12****	32.68%[a]	31.77%[a]
FF2	38.02 ± 1.30****	47.15 ± 1.04 ****	35.76 ± 2.13****	41.13%[a]	45.78%[a]
FF3	1.93 ± 0.54	67.19 ± 1.05****	24.74 ± 1.67****	27.08 ± 2.54****	28.39 ± 1.89****
FF4	54.62 ± 4.81****	56.81 ± 1.02****	32.08 ± 2.77****	186.42 ± 10.22****	198.44 ± 15.01****
FF5	50.07 ± 2.52*****	70.94 ± 2.07****	20.55 ± 2.55****	98.76 ± 5.30****	46.45%[a]
DF1	3.90 ± 1.98**	61.93 ± 2.70****	24.04 ± 1.78****	2.54 ± 0.35*	4.81 ± 1.23***
DF2	1.93 ± 0.96	53.06 ± 1.29****	35.31 ± 2.09****	19.29 ± 1.45****	21.92 ± 2.54****
DF3	2.32 ± 1.56*	32.21 ± 2.45****	30.64 ± 2.08****	74.94 ± 3.64****	82.45 ± 3.64****
DF4	1.16 ± 0.35	49.12 ± 1.45****	39.59 ± 3.05****	25.11 ± 2.58****	29.52 ± 2.47****
DF5	4.30 ± 2.36***	60.94 ± 5.23****	24.60 ± 2.27****	39.09 ± 3.65****	48.01 ± 1.36****
Leaves					
FL1	6.82 ± 0.61****	6.89 ± 0.70****	94.20 ± 3.56	63.68 ± 2.06****	8.87 ± 0.28****
FL2	7.01 ± 0.72****	7.88 ± 0.85****	83.03 ± 2.50	13.19 ± 1.01****	32.71 ± 1.03****
FL3	1.16 ± 0.02	14.86 ± 1.15****	17.98 ± 1.77****	31.21 ± 1.34****	46.03 ± 1.04****
FL4	7.50 ± 0.75****	7.88 ± 0.64****	91.82 ± 3.83	27.92 ± 1.02****	12.82 ± 1.01***
FL5	8.22 ± 0.80****	6.89 ± 0.83****	84.42 ± 2.78	41.06 ± 2.04****	7.94 ± 0.71****
DL1	0.39 ± 0.03	3.94 ± 0.04***	17.95 ± 1.96****	1.85 ± 0.02	4.09 ± 0.43***
DL2	0.42 ± 0.04	0.98 ± 0.09	32.78 ± 2.44****	3.21 ± 0.03	4.28 ± 0.61***
DL3	1.51 ± 0.21*	3.94 ± 0.03***	24.88 ± 1.96****	8.22 ± 0.81****	10.75 ± 0.66***
DL4	0.78 ± 0.08	27.83 ± 1.45****	32.49 ± 2.78****	13.38 ± 1.42****	12.63 ± 1.16***
DL5	1.30 ± 0.09*	24.83 ± 1.13****	32.56 ± 3.92****	36.74 ± 1.63****	13.10 ± 0.83****
Positive control					
Ascorbic acid	1.70 ± 0.21	5.03 ± 0.80			
BHT			63.20 ± 4.31		
Propyl gallate				1.01 ± 0.01	1.02 ± 0.01

FF: Fresh fruits; DF: Dried fruits; FL: Fresh leaves; DL: Dried leaves. 1. Ethanolic maceration; 2. Hydroalcoholic (60%) maceration; 3. Decoction; 4. Ethanol Soxhlet extraction; 5. Ethanol ultrasound extraction. [a] percentage of inhibition at a concentration of 100 μg/mL. Data are expressed as means ± S.D. (n= 3). Ascorbic acid, BHT and Propyl gallate were used as positive control in antioxidant tests. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001,**p < 0.01, *p< 0.1 compared with the positive controls.

In ABTS test, the best results were obtained with dried leaves. The most active extracts were obtained by maceration with ethanol (DL1) and the hydroalcoholic solution (DL2) with IC_{50} values of 0.39 and 0.42 μg/mL, respectively. The same trend was observed in DPPH test with IC_{50} values of 3.94 and 0.98 μg/mL, respectively. Instead, different results have been obtained for the extracts of fresh leaves. The extract obtained by decoction (FL3) displayed the high antioxidant activity against ABTS radicals with an IC_{50} value of 1.16 μg/mL, while extracts obtained by both ethanol (FL1) and ultrasound (FL5) maceration were the most active towards DPPH radicals with IC_{50} value of 6.89 μg/mL. The same trend was observed in DPPH test with IC_{50} values of 3.94 and 0.98 μg/mL, respectively for DL1 and DL2. Instead, different results have been obtained for the extracts of fresh leaves. The extract obtained by decoction (FL3) displayed the high antioxidant activity against ABTS radicals with an IC_{50} value of 1.16 μg/mL, while extracts obtained by both ethanol (FL1) and ultrasound (FL5) maceration were the most active towards DPPH radicals with IC_{50} values of 6.89 μg/mL.

Several studies investigated the antioxidant properties of *A. unedo* leaves and fruits are present in literature. Some of these studies assessed the influence of the extraction solvent on the antioxidant activity (Mendes et al., 2011; Oliveira et al., 2009; Orak et al., 2011a).

Leaves

Oliveira et al. (2009) used different solvent (ethanol, methanol, water, and diethyl ether) for the extraction of leaves and evaluated *in vitro* antioxidant activity. In the DPPH test, the extract more active was ethanol extract with IC_{50} value of 63.2 μg/mL, followed by water extract with IC_{50} value of 73.7 μg/mL. In the superoxide anion test, with IC_{50} of 6.9 μg/mL, methanol extract showed high potential. The ethyl ether extract has no antioxidant activity in any antioxidant tests. For Orak et al. (2011a) the aqueous extract has the highest content of total polyphenols content (197.16 mg GAE/g) compared to the methanol and ethanol extracts (169.05 mg GAE/g and 119.97 mg GAE/g, respectively). Contrary to expectations, the ethanol extracts are more active in inhibiting the DPPH radical with an IC_{50} of 0.423 mg/mL.

Previously, Mendes et al. (2011) studied the antioxidant activity of leaves aqueous extract with different *in vitro* assays. Data obtained showed that the antioxidant activity was correlated with high phenolic content (170 mg/g), as demonstrated by DPPH test (0.087 mg/mL). This result was further confirmed by the prevent effect on haemolysis of erythrocyte (IC_{50} of 0.062 mg/mL).

Fruits

Orak et al. (2012) reported the antioxidant activity of *A. unedo* fruits fresh and after drying treatment. Generally, fresh fruits exhibited high antioxidant activity compared to dried fruits. Mendes et al. (2011) reported the phenolic content (16.7 mg/g) and

antioxidant properties of aqueous extract of *A. unedo* fruits. The aqueous extract of *A. unedo* fruits presented IC_{50} value of 0.79 mg/mL in the DPPH test. In addition, the antihemolytic activity and lipid peroxidation inhibitory activity reported values of IC_{50} of 0.43 and 0.73 mg/mL, respectively. A recent study demonstrated the potential antioxidant activity for ethanol extract of fruits with values of IC_{50} of 324.06 and 515.76 μg/mL for respectively DPPH and ABTS tests correlated with high content of polyphenol (204.5 mg GAE/g) and flavonoid (34.18 mg QE/g) total. While, the methanol extract showed the lower value of polyphenol and flavonoid (180.75 mg GAE/g and 28.81 mg QE/g, respectively), with IC_{50} values of 379.50 and 523.87 μg/mL for DPPH and ABTS test (Salem et al., 2018). Our results obtained by fruits and leaves were better compared with literature data.

β-Carotene bleaching test

β-Carotene bleaching test was used to investigate the ability of *A. unedo* extracts to inhibit the lipid peroxidation. Maceration with ethanol of dried leaves (DL1) lead to the most active extract as inhibitor of lipid peroxidation with IC_{50} values of 1.85 and 4.09 μg/mL, respectively, after 30 and 60 min of incubation. For the fresh leaves after 30 min of incubation, the hydroalcoholic maceration (FL2) was indicated for the best IC_{50} value of 13.19 μg/mL; while after 60 min the ultrasound (FL5) ethanol extract demonstrated a better inhibition of lipidic peroxidation with IC_{50} value of 7.94 μg/mL. Interesting data were obtained with the maceration in ethanol (DF1) of dried fruits with IC_{50} values of 2.54 and 4.81 μg/mL, respectively after 30 and 60 min of incubation.

Orak et al. (2011a) have tested preventing the lipid peroxidation of leaves extracts with distinct solvents (water, methanol and ethanol). The ethanol extract, with an IC_{50} of 0.666 mg/mL, is more active compared to methanol and aqueous extracts.

Previously, Mendes et al. (2011), showed the ability of the decoction of dried leaves and fruits the prevent lipid peroxidation with IC_{50} values of 0.075 and 0.73 mg/mL, respectively. Our results are in agreement with these data regarding dried fruits decoction. On the contrary, much better values have been obtained in our study in relation to the leaves.

FRAP test

The antioxidant properties of *A. unedo* samples were also assessed by using FRAP test. The used methods have different reaction mechanisms. For instance, ABTS and DPPH tests are based on electron and H atom transfer, while the FRAP test is based on electron transfer reaction. Except for FL3, extracts obtained from fresh leaves exhibited the highest activity with values in the range 83.03-94.20 μM Fe (II)/g.

These results, better than obtained by the positive control BHT (63.20 μM Fe (II)/g) highlight the greater capacity of fresh leaves to reduce iron. All other extracts showed low values ranging from 17.95 to 39.59 μM Fe (II)/g.

Oliveira et al. (2009) used different solvents (ethanol, methanol, water and diethyl ether) for the extraction of leaves. The corresponding extracts were studied for their *in vitro* antioxidant activity. The more active extract was obtained by using ethanol with an IC_{50} of 232.7 μg/mL, followed by water extract (IC_{50} of 287.7 μg/mL). Interesting results were previously founded by Mendes et al. (2011) for the leaves and fruits aqueous extracts with IC_{50} value of 0.318 and 2.89 mg/mL, respectively.

GAS and RACI approaches were used to select the extract with the best antioxidant activity (Figures 7.7 and 7.8). Among fruits extracts, FF3 (GAS= 1.40; RACI = −0.44) and DF4 (GAS= 1.19; RACI = −0.58) samples showed the highest antioxidant potential.

Among leaves extracts the highest activity was attributed to DL2 (GAS and RACI values of 0.07 and −0.88, respectively), and DL1 (GAS = 1.11; RACI = −0.93).

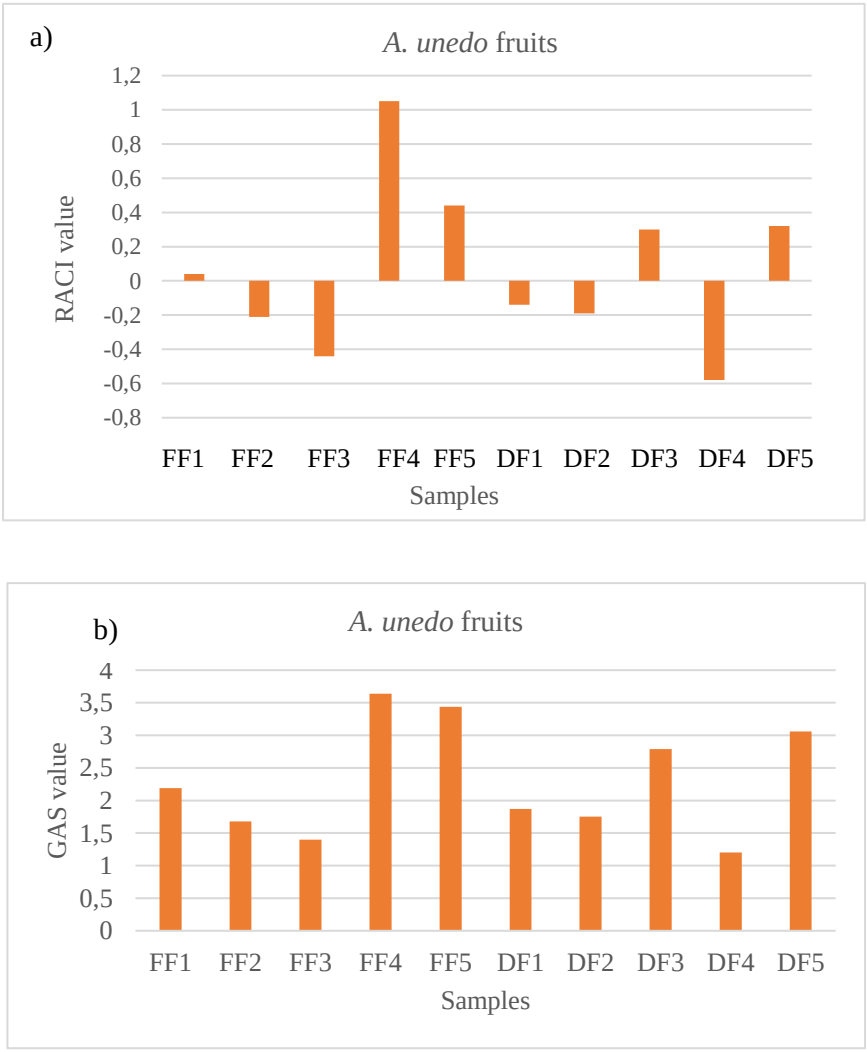


Figure 7.7. Evaluation of total antioxidant activity of fruits through RACI (a) and GAS (b). FF: Fresh fruits; DL: Dried leaves. 1. Ethanolic maceration; 2. Hydroalcoholic (60%) maceration; 3. Decoction; 4. Ethanol Soxhlet extraction; 5. Ethanol ultrasound-assisted extraction.

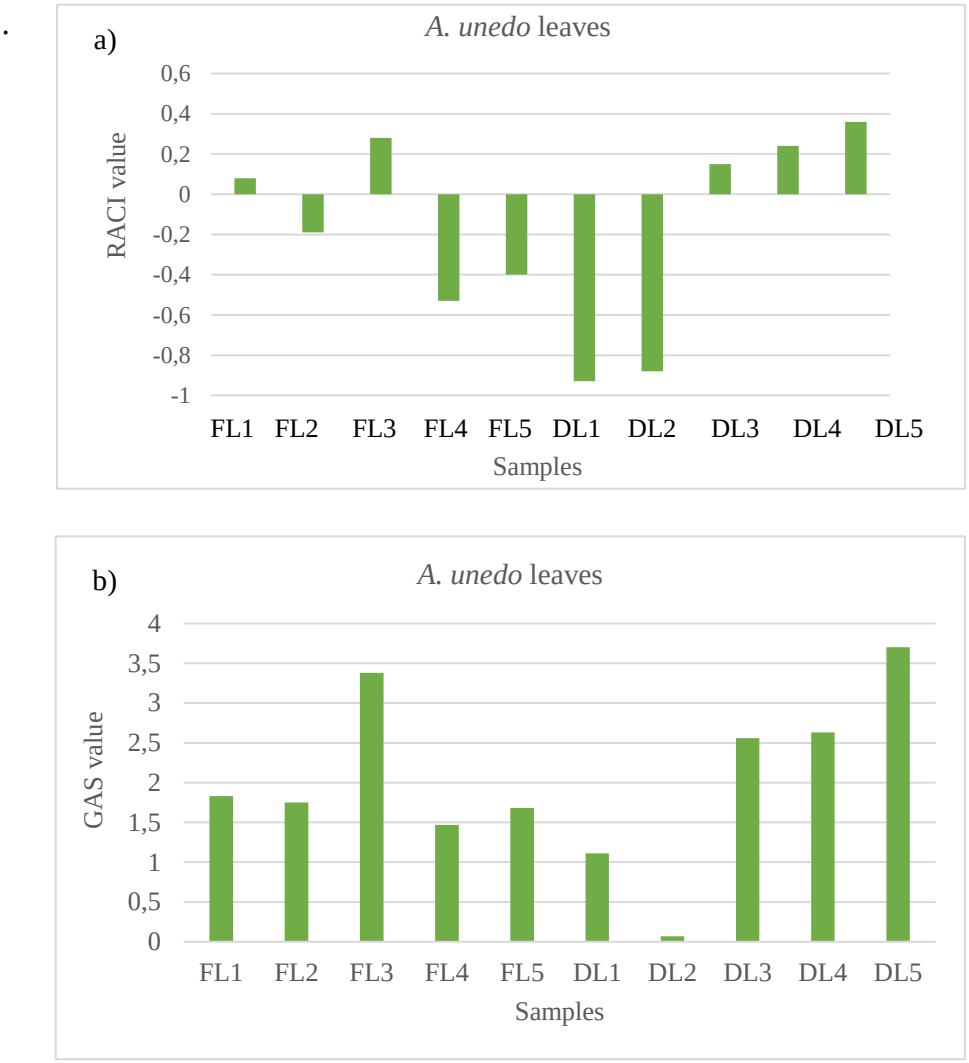


Figure 7.8. Evaluation of total antioxidant activity of leaves through RACI (a) and GAS (b). FL: Fresh leaves; DL: Dried leaves. 1. Ethanolic maceration; 2. Hydroalcoholic (60%) maceration; 3. Decoction; 4. Ethanol Soxhlet extraction; 5. Ethanol ultrasound-assisted extraction.

In conclusion, among fruits extracts, the technique that allow having extracts with a greater antioxidant activity were obtained by ethanol Soxhlet extraction and ethanol maceration of dried fruits. However, generally, except for FRAP test, the most active antioxidant extracts were obtained by ethanol (DL1) and hydroalcoholic maceration (DL2) of dried leaves. The main identified compounds in high quantity in these extracts are quercitrin, kaempferol, quercetin, shikimic acid gallate, syringic acid, quinic acid, and ellagic acid. The principal iridoids are gardenoside and geniposide.

The comparison of these extracts (DL1 and DL2) with the less active one (DL5) revealed interesting differences. Arbutin, caffeic acid, unedoside, geniposide, and norbergenin were identified only in the extracts with high antioxidant activity (DL1 and DL2). Moreover, both extracts are enriched in quercitrin, ellagic acid, and shikimic acid

gallate. Syringic acid, quinic acid, and geniposide was not identified in DL5, while *p*-hydroxybenzoic acid glucuronide and gardenoside were found in high concentration.

Different studies aimed at investigating the antioxidant potential of these compounds are present in literature. In DPPH assay, norbergenin, the *O*-demethylated derivative of bergenin, exhibited an IC_{50} value of 13 μM (Takahashi et al., 2003), 11.2 μg/mL (Tangmouo et al., 2009) and an inhibition of 85% (Zamarrud et al., 2011), respectively. Caffeic acid is one of the hydroxycinnamate and phenylpropanoid metabolites more commonly distributed in medicinal plants and foods. It is known to possess antioxidant activity *in vitro* (Chen et al., 1997; Gülçin, 2006; Masek et al., 2016; Sidoryk et al., 2018).

Recently, in the DPPH test, Sidoryk et al. (2018) showed an IC_{50} value of 32.2 μM. This compound was tested also in ABTS and FRAP tests, with percentage of 29.8 and 26.8%, respectively (Masek et al., 2016). While Gülçin (2006) reported value of 92.9 and 53.2%, for ABTS and FRAP tests, respectively. Arbutin is the monoglucoside of hydroquinone that is known as potent antioxidant compound with two oxidizable hydroxyl groups in its structure. Arbutin is the monoglucoside of hydroquinone that is known as potent antioxidant compound with two oxidizable hydroxyl groups in its structure. Arbutin retains one of these hydroxyl groups.

Arbutin has antioxidant activity but not strong as its aglycone (Bang et al., 2008). Takebayashi et al. (2010) demonstrated that arbutin possessed weak but long-lasting radicals-scavenging effects and strong antioxidant activity comparable or superior to that of its aglycone in two cell-based antioxidant tests using skin fibroblasts and erythrocytes.

As reported in several studies, generally the antioxidant activity of phenolic compounds was linked to hydroxyl groups present in their structure (Masek et al., 2016). The phenylpropanoids act as antioxidant agents by chelating pro-oxidant metal ions especially iron and by eliminating free radicals (Magnani et al., 2014). The hydroxyl groups of these compounds confer antioxidant activity. However, there are other factors in determining the potency of their effects. The presence of a second hydroxyl group in the *ortho* position is known to increase the antioxidant activity due to an additional resonance stabilization and formation of *o*-quinone. This characteristic can be used to explain the antioxidant efficiency of caffeic acid. In the study of Gálvez et al. (2005) the antioxidant activity of rutin, verbascoside, aucubin, and geniposide were evaluated using DPPH test. Rutin and verbascoside showed the highest antioxidant activity with IC_{50} values of 9.5 and 11.52 μM, respectively. Instead, aucubin, and geniposide do not present DPPH radicals scavenging activity.

7.3.2. Hypoglycaemic activity

Diabetes mellitus is a group of chronic disorders characterised by high blood sugar levels either because pancreas do not produce enough insulin or cells do not respond to the produced insulin. Therefore, useful for the treatment of diabetes is to decrease post-prandial hyperglycaemia. One of the most important therapeutic approaches in the management of hyperglycaemia is the reduction of gastrointestinal glucose production

and absorption through the inhibition of carbohydrates-hydrolysing enzymes, α-amylase and α-glucosidase. Herein, we have investigated *A. unedo* extracts as potential inhibitors of both enzymes. Data are summarised in Table 7.7.

Table 7.7. Carbohydrates-hydrolysing enzymes inhibitory activity of *A. unedo* fruits and leaves extracts

A. unedo	IC_{50}, μg/mL		Selectivity	
	α-Amylase	α-Glucosidase	α-Amylase[a]	α-Glucosidase[b]
Fruits				
FF1	20.30% [a]	181.05 ± 9.68****	-	-
FF2	258.13 ± 12.36****	28.42 ± 0.82	0.11	9.08
FF3	35.14% [a]	215.21 ± 6.57****	-	-
FF4	22.83% [a]	423.77 ± 5.34 ****	-	-
FF5	27.18%[a]	40.25 ± 0.79	-	-
DF1	107.51 ± 9.15****	255.50 ± 7.89****	2.38	0.42
DF2	146.51 ± 8.98****	316.81 ± 9.68****	2.16	0.46
DF3	295.14 ± 3.02****	456.23 ± 2.56****	1.55	0.65
DF4	77.51 ± 1.08**	151.27 ± 4.63****	1.95	0.51
DF5	120.58 ± 3.48****	239.73 ± 6.58****	1.99	0.50
Leaves				
FL1	63.43 ± 1.68	232.73 ± 6.49****	3.67	0.27
FL2	191.56 ± 2.58****	31.38 ± 0.24	0.16	6.10
FL3	222.22 ± 3.67****	162.66 ± 5.47****	0.73	1.37
FL4	329.07 ± 4.58****	267.76 ± 6.36****	0.81	1.23
FL5	291.41 ± 10.36****	19.56 ± 0.22	0.07	14.90
DL1	269.51 ± 6.57****	379.00 ± 2.14****	1.41	0.71
DL2	683.80 ± 9.44****	146.89 ± 3.68****	0.21	4.65
DL3	125.87 ± 2.39****	125.00 ± 6.31****	0.99	1.01
DL4	297.54 ± 3.69****	193.31 ± 4.59****	0.65	1.54
DL5	592.71 ± 4.57****	201.20 ± 2.14****	0.34	2.95
Positive control				
Acarbose	50.01 ± 1.43	35.50 ± 1.10	0.71	1.41

FF: Fresh fruits; DF: Dried fruits; FL: Fresh leaves; DL: Dried leaves. 1. Ethanolic maceration; 2. Hydroalcoholic (60%) maceration; 3. Decoction; 4. Ethanol Soxhlet extraction; 5. Ethanol ultrasound-assisted extraction. [a] percentage of inhibition at concentration of 1 mg/mL. Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001, **p < 0.01, *p< 0.1 compared with the positive control. [a] Selectivity for α-amylase is defined as IC_{50} (α-glucosidase)/IC_{50} (α-amylase). [b] Selectivity for α-glucosidase is defined as IC_{50} (α-amylase)/IC_{50} (α-glucosidase).

A. unedo extracts inhibited both carbohydrates-hydrolysing enzymes in a concentration-dependent manner. Against α-amylase, the most active extracts were FL1 that exhibited an IC_{50} of 63.43 μg/mL (with a selectivity for this enzyme of 3.67) and DF4 that showed an IC_{50} value of 77.51 μg/mL. However, both samples are less active than the positive control (IC_{50} value of 50.01 μg/mL). Results that are more interesting have been obtained in the inhibition test of α-glucosidase. The most promising inhibitors of this enzyme are fresh leaves extracts obtained by ethanol ultrasound-assisted extraction (FL5) and hydroalcoholic maceration (FL2) with IC_{50} values of 19.56 and 31.38 μg/mL, respectively. These values are better than to the positive control acarbose (IC_{50} value of 35.50 μg/mL). SI values of 14.90 and 6.10 were found for FL5 and FL2, respectively. The most active extract among *A. unedo* fruits extracts was FF2 with an IC_{50} value of 28.42 μg/mL.

In conclusion, there is not present an extract with high inhibition against both enzymes. Normally the hypoglycaemic drugs reported a high α-glucosidase inhibition and moderate α-amylase, basing to this consideration thus the fresh leaves extracts obtained by hydroalcoholic maceration and ethanol ultrasound-assisted extraction, could be considered a potential antidiabetic agents.

To the best of our knowledge, no previous works analysed strawberry tree fruits and leaves as carbohydrates-hydrolysing enzymes inhibitory agents.

Some studies reported the potential activity of *A. unedo* roots as antidiabetic agents. Bnouham et al. (2010) showed *in vivo* a decrease of the levels of glucose after co-administration of glucose and water extract of *A. unedo* roots in the Oral Glucose Tolerance Test (OGTT) but no in Intravenous Glucose Tolerance Test (IVGTT). More recently, Mrabti et al. (2018) showed a α-glucosidase inhibitory activity of *A. unedo* roots aqueous extract (IC_{50} value of 94.81 μg/mL) more efficient than the positive control (IC_{50} value of 199.53 μg/mL).

7.3.3. Inhibitory effects on nitric oxide (NO) production

Nitric oxide (NO) is recognized as a potent signaling mediator in several cellular processes. It is crucial in the regulation of neurotransmission, vascular tone, host defence mechanisms, and inflammation (Sharma et al., 2007). Therefore, NO inhibitors may represent important therapeutic agents in the management of inflammatory diseases.

In this study, the beneficial effects of *A. unedo* extracts on the inhibition of the production of NO was evaluated in fibroblasts (HFF1 cells). The pre-treatment of HFF1 cells with IL-2β induces NO production, which can be quantified by utilizing the chromogenic Griess reaction, which measures the accumulation of nitrite, a stable metabolite of NO.

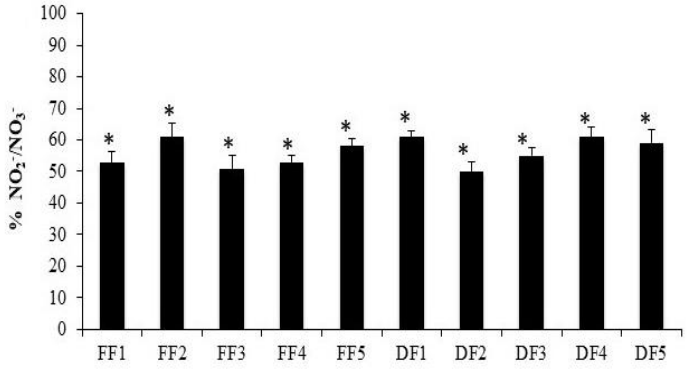


Figure 7.9. NO_2^-/NO_3^- levels in HFF1 cells treated with fruits extracts of *A. unedo* at 12.5 μg/mL and stimulated with IL-2β (10 μg/mL). Values are the mean ± S.D. of four experiments in triplicate. *Significant *vs* IL-2β treated control cells (100% NO_2^-/NO_3^- levels): $p<0.001$.

As shown in Figures 7.9 and 7.10, *A. unedo* extracts were able to reduce the nitrite formation. No significant differences were observed inside the group of fruits or leaves

extracts but all fruits extracts showed a more NO inhibitory activity respect to leaves extracts.

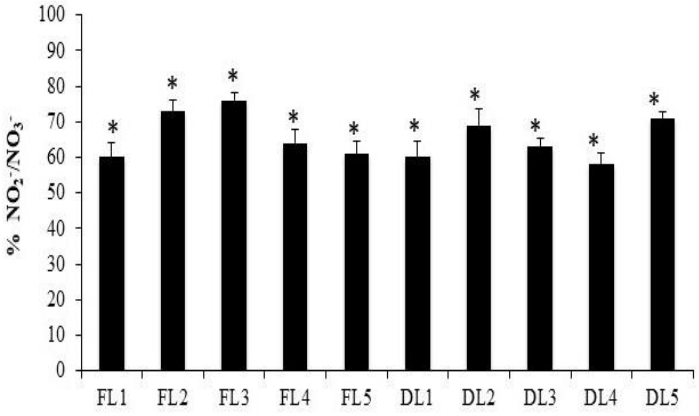


Figure 7.10. NO_2^-/NO_3^- levels in HFF1 cells treated with leaves extracts of *A. unedo* at 12.5 μg/mL and stimulated with IL-2β (10 μg/mL). Values are the mean ± S.D. of four experiments in triplicate. *Significant *vs* IL-2β treated control cells (100% NO_2^-/NO_3^- levels): $p<0.001$.

A. unedo fruits and leaves extracts showed a cytotoxic effect in a concentration dependent manner, decreasing cell viability of HFF1 cells independently of the extraction technique used (Figure 7.11).

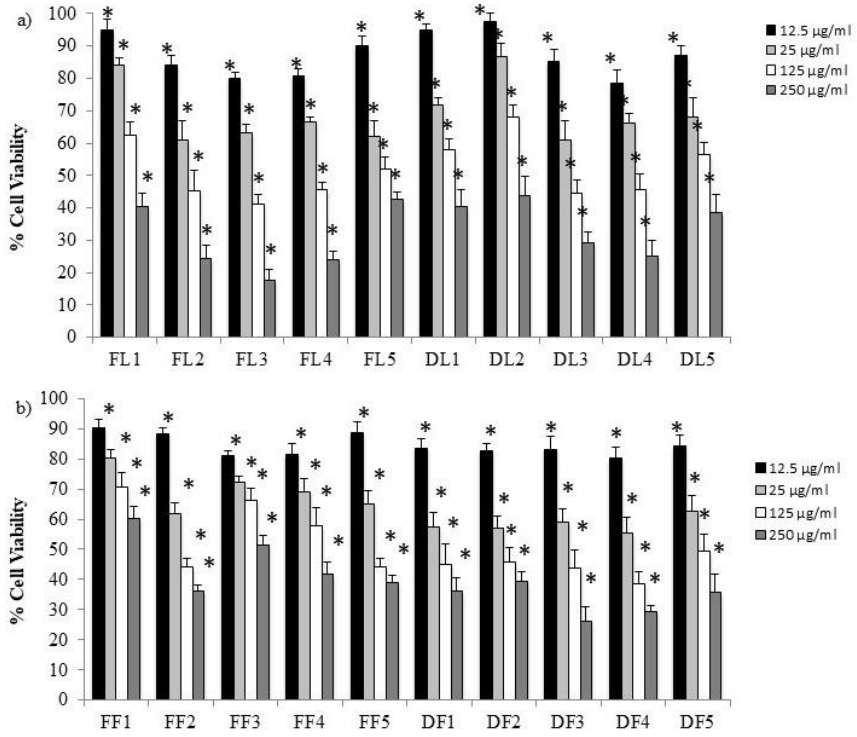


Figure 7.11. Cell viability in HFF1 cells untreated and treated for 24h - a) with leave extracts of *A. unedo* at different concentrations (12.5-250 μg/mL) - b) with fruit extracts of *A. unedo* at different concentrations (12.5-250 μg/mL). Values are the mean ± S.D. of four experiments in triplicate. Control cells were incubated only with medium and considered as 100% of cell viability. *Significant *vs* untreated control cells and *vs* other concentrations of the same extract $p< 0.001$.

7.4. Correlation between biological activity and chemical profile

Pearson's correlation coefficient was used to describe the correlation between the biological activities and the content of components (TPC, TFC, and TIC). The following matrices were considered: fresh fruits, dried fruits, fresh leaves and dried leaves.

Generally, as inferable from the data on the correlation analysis, the antioxidant potential measured by ABTS, DPPH, FRAP, and β-carotene bleaching assays, essentially follow the differences in content of total phenols, that is, the increase of the concentration of phenols corresponds to an increase of the antioxidant activity of the fruits extracts.

However, a good correlation with the antioxidant activity of extracts was also appreciable with respect to total iridoids content. In fact, a strong positive correlation was found between TIC and β-carotene bleaching test after 30 and 60 min of incubation for fresh fruits (r= 0.80 and 0.94, respectively). An r value of 0.73 was found for the total iridoids content and DPPH test. In addition fresh fruit total flavonoids content strongly positively correlated with α-amylase inhibitory activity (r= 0.93).

Moreover, in fresh fruits a positive correlation was observed also for α-glucosidase inhibitory activity and total iridoids content (r= 0.87). Considering fresh leaves extracts, the statistical analysis reveals a good correlation between the antioxidant activity (β-carotene bleaching test at 30 min of incubation) and the flavonoids content (r= 0.88), less relevant for total polyphenols (r= 0.50), whereas no correlation was evidenced for TIC (r= − 0.03). The same trend was observed with dried leaves.

Of interest is also the correlation between the α-amylase inhibitory activity of both fresh and dried leaves *A. unedo* extracts and the total iridoids (r= 0.82 and 0.84, respectively). Overall obtained results showed that the significant difference of antioxidant capacity of *A. unedo* extracts was not related only to the phenols content, but also to other constituents such as flavonoids and iridoids.

7.5. Conclusion

It is well established that consumption of vegetables and fruits has been closely associated with reduced risk of chronic degenerative diseases, and that plant extracts including fruits and berries show interesting antioxidant, anti-inflammatory, and hypoglycaemic activities. This study revealed a notable impact of different solvents and extraction procedures on yield extraction and phytochemicals composition, as well as on the founded bioactivities of *A. unedo* fruits and leaves.

Considering all results, maceration gives extracts that mainly preserve bioactive compounds compared with the other extractive procedures. The extracts obtained from ethanolic and hydroalcoholic macerations of the dried leaves presented the highest antioxidant activity and contained some secondary metabolites (arbutin, caffeic acid, unedoside, geniposide, norbergenin) only found by using those methods. Moreover, a different quantity of compounds among the different extracts had been found, in

particular between the most active and the less active ones. Globally, the biological evaluation demonstrated the implication of phenolic compounds and iridoids.

Leaves extracts showed high antioxidant and hypoglycaemic activities compared with fruits extracts. Surprisingly, in the radicals scavenging and FRAP tests, leaves extracts were more active compared with positive control. Fruit extracts present high anti-inflammatory activity and a moderate toxicity compared with leaves extracts. Moreover, alcoholic and hydroalcoholic maceration of extracts exhibited the highest ability in decreasing mitochondrial dehydrogenases activity.

The LC-ESI-Q-TOF-MS has allow finding compounds that are chemotaxonomic markers of the *Arbutus* genus and for the first time ellagic acid 4-*O*-*β*-D-glucopyranoside, kaempferol 3-*O*-glucoside, and norbergenin.

The observed activities of *A. unedo* provide some basic evidence for the potential health benefits of strawberry tree phytochemicals and suggest that studies of strawberry tree extracts should be carried out in appropriate *in vivo* models of inflammation diabetes, to prospect a potential use as nutraceuticals and/or functional foods.

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Chapter 8

Vaccinium corymbosum: results and discussion

8.1. Extraction yields and total phytochemicals content

V. corymbosum fruits (3.8 kg) and leaves (3.1 kg) were collected. Fresh fruits (1.8 kg) and leaves (1.6 kg) were immediately extracted. Fruits (2 kg) were dried at 50 °C for 7 days. Leaves (1.5 kg) were dried at room temperature for 7 days in the dark. At the end of the drying procedures, 760 and 570 g of dried fruits and dried leaves, respectively, were obtained and subjected to extraction.

The choice of solvent plays a crucial role in the extraction of phytochemicals. Previous studies report the use of polar solvents for effective extraction of phenolic compounds (Bora et al., 2011). Table 8.1 shows the extractions yields of *V. corymbosum* samples.

Excluding decoction, extraction yields from dried fruits were highest than the fresh fruits. The highest yields were obtained by using ethanol with Soxhlet apparatus for the extraction of dried fruits (32.1%), followed by ethanolic and hydroalcoholic maceration (26.2 and 25.1%, respectively). The same trend was found for fresh fruits with yields of 13, 10.1 and 9.9% for Soxhlet apparatus, ethanol and hydroalcoholic maceration, respectively.

Table 8.1. Extractive yield **(%)** of *V. corymbosum*

	Yield (%)[a]			
Extraction procedure	Fruits		Leaves	
	Fresh	Dried	Fresh	Dried
Maceration (EtOH)	10.11 ± 1.14	26.21 ± 2.74	16.20 ± 1.62	17.75 ± 1.83
Maceration (Hydroalcoholic 60%)	9.90 ± 0.95	25.13 ± 2.50	18.70 ± 1.92	24.01 ± 2.42
Decoction (H_2O)	7.84 ± 0.73	6.71 ± 0.65	9.63 ± 0.94	20.50 ± 2.13
Soxhlet apparatus (EtOH)	13.05 ± 1.3	32.15 ± 3.24	24.15 ± 2.40	31.71 ± 3.20
Ultrasound-assisted extraction (EtOH)	7.23 ± 0.70	14.04 ± 1.40	8.71 ± 0.95	17.03 ± 1.70

Data are reported as mean ± standard deviation (n= 3). [a] Expressed as (g dried extract/ g plant materials) × 100

High yield extraction in fresh and dried leaves with Soxhlet (EtOH) use was observed, with 24.1% and 31.7% values, respectively. In addition, hydroalcoholic maceration and ethanol maceration reported good yield extraction with percentage of 18.7% and 16.2% for fresh leaves, and 24% and 17.7% for dried leaves, respectively. Lowest extraction yields were obtained by using the ethanol ultrasound-assisted extraction, except for dried fruits extracts. Results are summarized in the Figures 8.1 and 8.2.

Data reported in this study showed that use of high temperature that allow to having a best extraction yield. An increase in the extraction temperature would improve the solubility of the solute and its diffusion inside the plant matrix, consequently reducing the extraction times. In general, the increase in temperature could cause an increase in the concentration of some phenolic compounds and iridoids due, probably to an increase in

the solubility of some of these compounds, but also to the breakdown of cellular components that would retain the bioactive compounds present (Lim et al. , 2007; Náthia-Neves et al., 2017). Majority flavonols are glycosides and their yields were increased through heating, as reported by Mokrani et al. (2016). Heat could be soften the plant tissue with weaken phenol-polysaccharide and phenol interaction, with consequently migration of flavonols in the solvent.

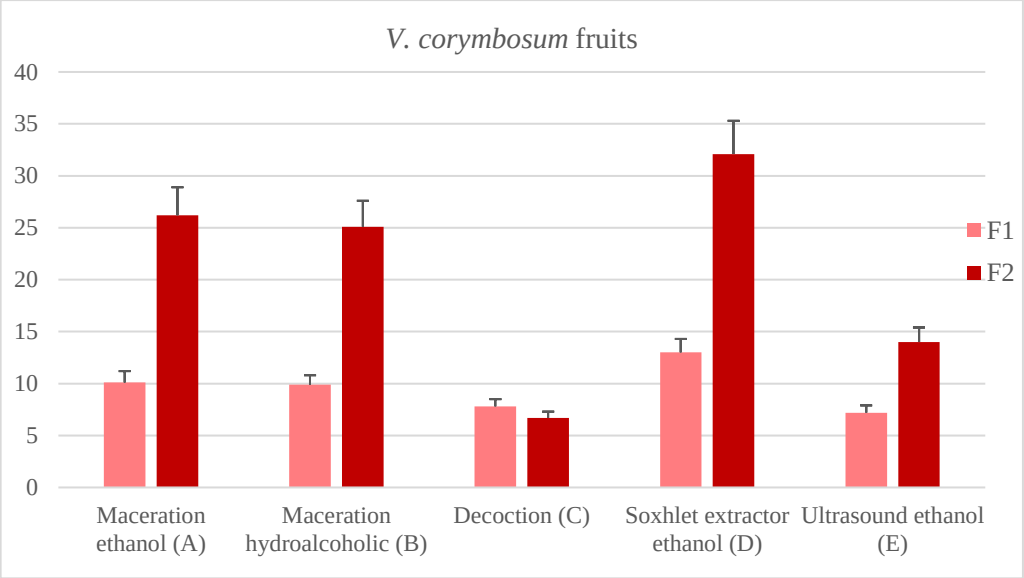


Figure 8.1. Extraction yield (%) of *V. corymbosum* fruits. F1: fresh fruits, F2: dried fruits.

Another important parameters was extraction time because its influence the solubility and transfer of bioactive compounds in the solvent, that are correlated to their molecular weight and structure (Belwal et al., 2016; Vuong et al., 2011).

In addition, it is crucial to economize to best save on extraction cost. Prolonged extraction time could allow degradative processes of bioactive compounds, catechin (Vuong et al., 2011). In the study of Tchabo et al. (2018) showed that extraction time selected to 40 min, for avoid the evaporation of solvent and for increase the extract antioxidant properties. Previous study conducted by Zhang et al. (2014) demonstrated that with extraction time over 50 min there is drastic reduction in bioactive components of *Morus* leave when used water as solvent.

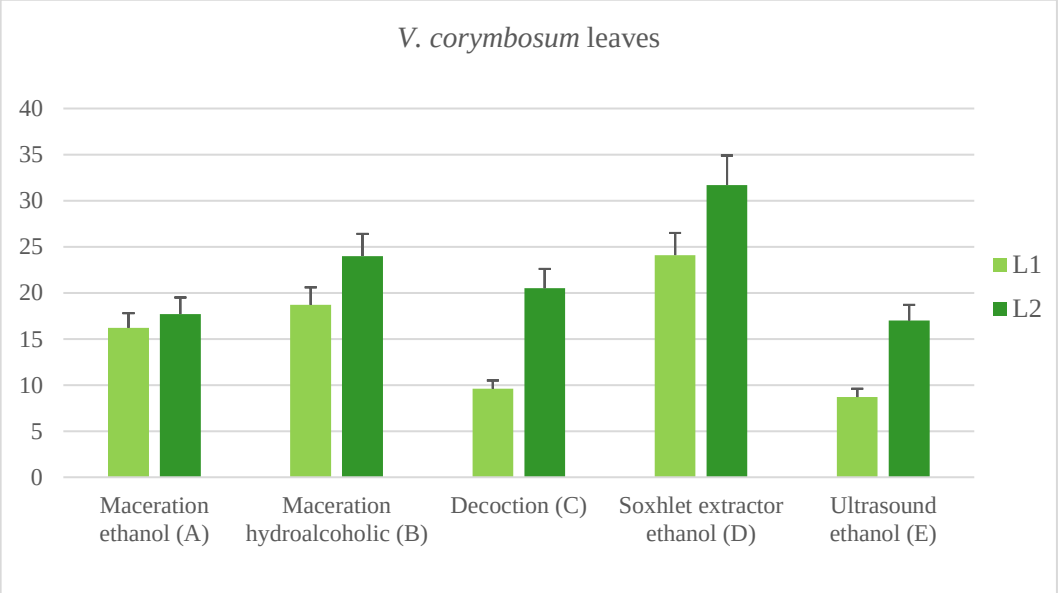


Figure 8.2. Extraction yield (%) of *V. corymbosum* leaves. L1: fresh leaves, L2: dried leaves.

The spectrophotometric determinations of the total phenols, flavonoids, and iridoids contents evidenced a similar content of these phytochemicals in the analysed extracts with some variations according to the extraction technique and the used plants organs, as reported in Table 8.2. The following trend for TPC was found: decoction > hydroalcoholic maceration > Soxhlet apparatus (EtOH) > ethanolic maceration > ultrasound-assisted extraction (EtOH).

The richest extract in flavonoids was obtained by fresh fruits extracted by ethanol Soxhlet apparatus (31.87 mg of quercetin equivalents (QE)/g of extract), while the extract that showed the highest iridoids content (TIC) (110.42 mg aucubin equivalents (AUE)/g of extract) was obtained by the same extraction procedure but from dried fruits.

Variation in total phenols content (TPC) was observed during the maturation (Sun et al., 2018). In green fruit was reported the TPC of 42.35 mg gallic acid equivalents (GAE)/g fresh weight (FW), while in the blue fruit of 26.59 mg GAE/g FW. In particular, was analysed also the TPC present in the skin and in the pulp. TPC of skin (110.48 mg GAE/g FW) is high compared with the content in the pulp (17.32 mg GAE/g FW) but it is four times than in the blue fruit, thus the mainly TPC is present in the skin. Previous studies reported same trend in various cultivar of *V. corymbosum* with halving decrease of TPC from green to blue fruits (Castrejón et al. 2008; Kalt et al., 2001). The total flavonoids content (TFC) followed same tendency of TPC, high in the immature fruits (green colour) that decrease with maturation (blue colour). As TPC, majority of TFC was present in the skin (Nogata et al., 2006; Sun et al., 2018). In agreement with previous reports, the values in this study indicated similar TPC in blue fruits.

The entire data collection allows founding that dried fruits extracts showed a lower total phytochemicals content than fresh fruits extracts. This could be attributed to the fact

that, during drying, the peel, which is the part richer in polyphenols, is the first component to be affected by degradative effects (Ehlenfeldt et al., 2015).

Table 8.2. Total phytochemicals content of *V. corymbosum* extracts

V. corymbosum	Total Phenols Content (TPC)[a]	Total Flavonoids Content (TFC)[b]	Total Iridoids Content (TIC)[c]
Fruits			
F1A	32.81 ± 0.32	30.87 ± 0.25	104.04 ± 0.71
F1B	34.93 ± 0.21	29.33 ± 0.22	71.33 ± 0.63
F1C	35.47 ± 1.23	28.53 ± 0.44	104.71 ± 0.77
F1D	34.87 ± 0.85	31.87 ± 0.37	92.22 ± 0.55
F1E	32.22 ± 0.53	30.53 ± 0.12	106.70 ± 0.86
F2A	31.61 ± 0.32	28.53 ± 0.25	91.33 ± 0.33
F2B	31.87 ± 0.58	28.65 ± 0.35	98.61 ± 1.32
F2C	32.82 ± 1.06	27.87 ± 0.44	80.58 ± 0.21
F2D	29.40 ± 0.67	27.27 ± 0.52	110.42 ± 1.74
F2E	29.27 ± 0.45	26.47 ± 0.27	100.73 ± 2.57
Leaves			
L1A	470.27 ± 3.32	250.70 ± 2.07	126.11 ± 1.62
L1B	469.33 ± 2.41	230.13 ± 1.79	104.03 ± 1.21
L1C	394.60 ± 2.37	274.41 ± 2.66	89.33 ± 1.18
L1D	442.44 ± 2.66	189.87 ± 1.83	86.24 ± 1.02
L1E	464.45 ± 3.63	232.23 ± 1.72	124.69 ± 1.44
L2A	337.33 ± 2.27	199.07 ± 2.54	137.32 ± 1.87
L2B	375.47 ± 2.72	261.32 ± 3.01	107.11 ± 2.69
L2C	444.13 ± 3.90	273.31 ± 2.74	83.34 ± 1.22
L2D	325.30 ± 2.54	265.26 ± 2.78	104.02 ± 1.54
L2E	382.66 ± 3.16	138.13 ± 1.86	100.64 ± 1.96

F1: fresh fruits, F2: dried fruits; L1: fresh leaves; L2: dried leaves. A. Ethanolic maceration; B. Hydroalcoholic (60%) maceration; C. Decoction; D. Ethanol Soxhlet extraction; E. Ethanol ultrasound-assisted extraction. Data are reported to mean ± Standard Deviation (SD) (n = 3). [a]mg chlorogenic acid (CA) equivalents/g extract; QE: [b]mg quercetin (QE) equivalents/g extract; [c]mg aucubin (AU) equivalents/g extract.

Generally, leaves extracts are richer in phenols and flavonoids than fruits extracts. Interesting data were obtained with the ethanol maceration of fresh leaves that showed a TPC and TIC of 470.27 mg of chlorogenic acid equivalents (CAE)/g of extract and 126.11 mg of AUE/g of extract, respectively. The richest extract in flavonoids was given by decoction (274.41 mg of QE/g of extract). Among dried leaves extracts, the highest TPC (444.13mg of chlorogenic acid equivalents/g of extract) and TFC (273.31mg of quercetin equivalents/g of extract) were observed in decoction, whereas the best TIC (137.32mg of aucubin equivalents/g of extract) was found after ethanolic maceration.

A perusal analysis of the literature data showed the presence of few studies on the total phytochemicals content of *V. corymbosum* leaves and none reported the presence of iridoids. However, according with these data, Ehlenfeldt et al. (2001) compared different varieties of *V. corymbosum* and found a higher total phenols content in the leaves (23.58 mg GAE/g ~ 66.29 mg GAE/g) compared to the fruits (0.20 mg GAE/g ~ 1.75 mg

GAE/g). Use of binary solvents was better compared with mono solvents for the extraction of phenolic compounds (Thoo et al., 2010).

Furthermore, Jayaprakasha et al. (2001) have indicated a low yield of phenolic compounds when were used alone solvent as methanol. Moreover, aqueous solution with acetone, methanol and ethanol demonstrate to be more efficient compared with mono solvent solution with the *Vitis rotundifolia* seeds (Yilmaz et al., 2006). The possible explanation could be that majority of phenolic compounds are glycosides and sugar portion is more soluble in water (Ignat et al., 2011). In according with previously data, our results confirmed that hydroalcoholic solution to be the most efficient solvent for polyphenols extraction compared to other solvents.

Moreover, TPC is variable in according with collection time, as confirmed by Routray et al. (2014) that analysed two leaves varieties of *V. corymbosum*. Leaves were collected in different period (May, July, September and October). Authors have exhibited TPC of dried leaves was high in the May (106.9 mg GAE/g and 123.7 mg GAE/g, respectively for Nelson and Elliot varieties); with reduction in July (86.4 mg GAE/g and 106.1 mg GAE/g respectively for Nelson and Elliot varieties), for increase in September, October (152.3 mg GAE/g and 155.8 mg GAE/g respectively for Nelson and Elliot varieties).

8.2. LC-ESI-Q-TOF-MS phytochemical profile

Anthocyanins are the main compounds described in literature in *V. corymbosum* fruits. Their extraction need of an acidified alcoholic solution and for this reason, herein they were not detected. Compounds were identified based on UV spectra, molecular weight (*m/z* ion $[M+H]^+$), and chemotaxonomic significance. Moreover, the presence of caffeic acid, chlorogenic acid, myricetin 3-*O*-glucoside, hyperoside, isoquercitrin, quercetin, rutin, geniposide, and were confirmed by using standards.

The principal classes detected in *V. corymbosum* fruits and leaves extracts are phenolic acids, flavonoids, and iridoids (Tables 8.3 and 8.4.). Chromatograms used for the chemical identification were reported in Appendix (Figures A4-A5). Leaves showed greater chemical diversity than fruits and only some of identified constituents are biosynthesized by both fruits and leaves. Five phenolic acids, seven flavonoids, and two iridoids were reported in fruits extracts, while seven phenolic acids, thirteen flavonoids, and four iridoids characterised leaves extracts.

Table 8.3. Identification of chemical compounds in *V. corymbosum* leaves extracts using the LC-ESI-QTOF-MS technique

Compounds	Rt (min)	Molecular Formule	MH+	Error (ppm)	Score (%)	MS fragment	UV λ (nm)	Fresh leaves					Dried leaves					Reference
								L1A	L1B	L1C	L1D	L1E	L2A	L2B	L2C	L2D	L2E	
Phenolic acids																		
Caffeic acid°	5.5	$C_9H_8O_4$	181.0500	0.4	100		295, 322	√										Ștefănescu et al., 2019
4-*O*-Caffeoylquinic acid methyl ester (*)	8.9	$C_{17}H_{20}O_9$	369.1185	0.3	99		294, 328	√	√	√	√	√	√	√		√	√	Ștefănescu et al., 2019
5-*O*-Caffeoylquinic acid methyl ester (*)	10.2	$C_{17}H_{20}O_9$	369.1185	0.9	89		294, 328	√	√	√	√	√	√	√		√	√	Ștefănescu et al., 2019
3-*O*-Caffeoylquinic acid methyl ester (*)	11.1	$C_{17}H_{20}O_9$	369.1185	0.7	91		294, 328	√	√	√	√	√	√	√	√	√	√	Ștefănescu et al., 2019
3,5-di-*O*-Caffeoylquinic acid methyl ester	14.3	$C_{26}H_{26}O_{12}$	531.1502	0.3	100		244, 330	√			√							Ștefănescu et al., 2019
Caffeoyl coumaroylquinic acid	14.3	$C_{25}H_{24}O_{11}$	501.1396	0.2	98		240, 336	√			√							Ștefănescu et al., 2019
Chlorogenic acid°	9.8	$C_{16}H_{18}O_9$	355.1029	0.3	100		242, 325	√	√	√	√	√	√	√	√	√	√	Ștefănescu et al., 2019
Flavonoids																		
Hyperoside° (**)	12.8	$C_{21}H_{20}O_{12}$	465.1031	0.2	100	303.0499	217, 278, 350	√	√	√	√	√	√	√	√	√	√	Su, 2012
Isoquercitrin° (**)	12.8	$C_{21}H_{20}O_{12}$	465.1031	0.2	100	303.0499	215, 253, 353	√	√	√	√	√	√	√	√	√	√	Su, 2012
Isorhamnetin malonylglycoside	14.0	$C_{25}H_{24}O_{15}$	565.1193	0.1	100	316.267	212, 254, 355	√	√	√	√	√	√	√	√	√	√	Wald et al., 1989
Isorhamnetin 3-*β*-D-galactopyranoside	13.3	$C_{22}H_{22}O_{12}$	479.1189	0.1	99	316.267	212, 255, 368	√	√	√	√	√	√	√	√	√	√	Yan et al., 2002
Kaempferol 3-*O*-glucoside	13.2	$C_{21}H_{20}O_{11}$	449.1077	0.2	100	287.2287	210, 265, 346	√	√	√	√	√	√	√	√	√	√	Su, 2012
Myricetin 3-*O*-α-L-arabinofuranoside (***)	12.6	$C_{20}H_{18}O_{12}$	451.0876	0.2	100	319.0389	219, 253, 365	√	√	√	√	√	√	√	√	√	√	Yan et al., 2002
Myricetin 3-*O*-glucoside°	12.0	$C_{21}H_{20}O_{13}$	481.0982	0.8	100	319.0389	212, 266, 364	√	√	√	√	√	√	√	√	√	√	Su, 2012
Quercetin°	16.8	$C_{15}H_{10}O_7$	303.0504	0.1	100		213, 255, 353	√	√	√	√	√	√	√	√	√	√	Su, 2012

Quercetin acetylglycoside	13.9	$C_{23}H_{22}O_{13}$	507.1138	0.2	98	303.0499	213, 252, 354	√	√	√	√	√	√	√	√	√	√	Bhattacharya et al., 2013
Quercetin 3-*O*-arabinoside(***)	13.4	$C_{20}H_{18}O_{11}$	435.7749	0.5	100	303.0499	213, 253, 353	√	√	√	√	√	√	√	√	√	√	Su, 2012
Quercitrin	13.6	$C_{21}H_{20}O_{11}$	449.1079	0.3	100	303.0499	213, 254, 356	√	√	√	√	√	√	√	√	√	√	Su, 2012
Rutin°	12.4	$C_{27}H_{30}O_{16}$	611.1612	0.6	100	303.0499	213, 253, 352	√	√	√	√	√	√	√	√	√	√	Su, 2012
Syringetin 3-*O*-glucoside	13.1	$C_{23}H_{24}O_{13}$	509.1295	0.7	100	347.2967	219, 346	√	√	√	√	√	√	√	√	√	√	Su, 2012
Iridoids																		
Dihydromonotropein	12.1	$C_{16}H_{24}O_{11}$	393.1396	0.2	95		237	√		√	√	√			√			Heffels et al., 2017
Geniposide°	10.6	$C_{17}H_{24}O_{10}$	389.1447	0.1	100		239	√		√	√	√	√					Karikas et al., 1987
Scandoside	10.4	$C_{16}H_{22}O_{11}$	391.1240	0.2	100		241	√		√	√	√	√					Heffels et al., 2017
Vaccinoside	15.2	$C_{25}H_{28}O_{13}$	537.1608	0.3	97		246	√	√		√	√	√					Sakakibara et al., 1973

L1: fresh leaves; L2: dried leaves. A. Ethanolic maceration; B. Hydroalcoholic (60%) maceration; C. Decoction; D. Ethanol Soxhlet extraction; E. Ethanol ultrasound-assisted extraction. ° identified with standard compounds; **in bold** not previously identified in the plant (or plant part); (*) interchangeable; (**) 2 peaks; (***) or isomer.

Table 8.4. Identification of chemical compounds in *V. corymbosum* fruits extracts using the LC-ESI-QTOF-MS technique

Compounds	Rt (min)	Molecular Formule	MH+	Error (ppm)	Score (%)	MS fragment	UV λ (nm)	Fresh Fruits					Dried Fruits					Reference
								F1A	F1B	F1C	F1D	F1E	F2A	F2B	F2C	F2D	F2E	
Phenolic acids																		
Caffeic acid°	5.5	$C_9H_8O_4$	181.0500	0.4	100		295, 322	√		√								Ștefănescu et al., 2019
3,5-di-*O*-Caffeoylquinic acid	10.3	$C_{25}H_{24}O_{12}$	517.1346	0.3	100		244, 330	√	√	√	√	√						Ștefănescu et al., 2019
Chlorogenic acid°	9.8	$C_{16}H_{18}O_9$	355.1029	0.3	100		242, 325	√	√	√	√	√	√	√	√	√	√	Ștefănescu et al., 2019
Syringic acid	10.6	$C_9H_{10}O_5$	199.0606	0.6	100		218, 274	√	√	√	√	√	√	√	√	√	√	Zadernowski et al., 2005
2,3,4 Trihydroxybenzoic acid	2.1	$C_7H_6O_5$	171.0293	0.1	100		255, 292	√	√	√	√	√	√	√	√	√	√	Zadernowski et al., 2005
Flavonoids																		
Hyperoside °(*)	12.8	$C_{21}H_{20}O_{12}$	465.1031	0.2	100	303.0499	217, 278, 350	√	√	√	√	√	√	√	√	√	√	Su, 2012
Isoquercitrin °(*)	12.8	$C_{21}H_{20}O_{12}$	465.1031	0.2	100	303.0499	215, 253, 353	√	√	√	√	√	√	√	√	√	√	Su, 2012
Myricetin 3-*O*-glucoside°	12.0	$C_{21}H_{20}O_{13}$	481.0982	0.8	100	319.0389	212, 266, 364	√	√	√	√	√	√	√	√	√	√	Su, 2012
Quercetin°	16.8	$C_{15}H_{10}O_7$	303.0504	0.1	100		213, 255, 353	√						√				Su, 2012
Quercetin 3-*O*-arabinoside	13.4	$C_{20}H_{18}O_{11}$	435.7749	0.5	100	303.0499	213, 253, 353	√	√	√	√	√	√	√	√	√	√	Su, 2012
Rutin°	12.4	$C_{27}H_{30}O_{16}$	611.1612	0.6	100	303.0499	213, 253, 352	√						√				Su, 2012
Syringetin 3-*O*-glucoside	13.1	$C_{23}H_{24}O_{13}$	509.1295	0.7	100	347.2967	219, 346	√	√	√	√	√	√	√	√	√	√	Su, 2012
Iridoids																		
Dihydromonotropein	12.1	$C_{16}H_{24}O_{11}$	393.1396	0.2	95		237	√										Heffels et al., 2017
Geniposide°	10.6	$C_{17}H_{24}O_{10}$	389.1447	0.1	100		239	√		√								Karikas et al., 1987
Scandoside	10.4	$C_{16}H_{22}O_{11}$	391.1240	0.2	100		241	√										Heffels et al., 2017

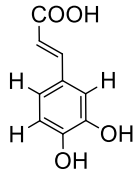
F1: fresh fruits, F2: dried fruits. A. Ethanolic maceration; B. Hydroalcoholic (60%) maceration; C. Decoction; D. Ethanol Soxhlet extraction; E. Ethanol ultrasound extraction. ° identified with standard compounds; **in bold** not previously identified in the plant; (*) 2 peaks.

8.2.1. Phenolic acids

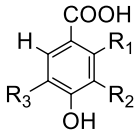
Chlorogenic acid, 2,3,4-trihydroxybenzoic acid and syringic acid were reported in all fruits extracts. Other metabolites are present but related to the extraction procedure or nature of material. Only ethanol maceration and decoction of fresh fruits has allow to extract caffeic acid, while 3,5-di-*O*-caffeoylquinic acid was found only in all fresh fruits extracts. Leaves biosynthesize chlorogenic acid, caffeic acid and in particular caffeoylquinic acid derivatives. Presence of caffeoylquinic acid methyl ester isomers was found in all leaves extracts. Other caffeoylquinic derivatives, 3,5-di-*O*-caffeoylquinic acid methyl ester and caffeoyl coumaroylquinic acid, have been highlighted respectively after extraction of fresh leaves using ethanol by maceration and Soxhlet apparatus.

As previously described in the *Vaccinium* genus or Ericaceae family, fruits and leaves extracts showed the presence of caffeic, caffeoyl quinic, chlorogenic and syringic acids (Olennikov et al., 2010; Ştefănescu et al., 2019; Tenuta et al., 2018; Zadernowski et al., 2005).

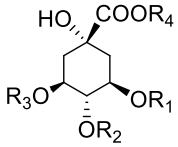
Phenolic acids



Caffeic acid



	R_1	R_2	R_3
Syringic acid	H	OCH_3	OCH_3
2,3,4-trihydroxybenzoic acid	OH	OH	H



	R_1	R_2	R_3	R_4
Chlorogenic acid	Caffeoyl	H	H	H
3-*O*-caffeoylquinic acid methyl ester	Caffeoyl	H	H	CH_3
4-*O*-caffeoylquinic acid methyl ester	H	Caffeoyl	H	CH_3
5-*O*-caffeoylquinic acid methyl ester	H	H	Caffeoyl	CH_3
3,5-di-*O*-caffeoylquinic acid	Caffeoyl	H	Caffeoyl	H
3,5-di-*O*-caffeoylquinic acid methyl ester	Caffeoyl	H	Caffeoyl	CH_3

Figure 8.2. The dominant constituents of *V. corymbosum* extracts

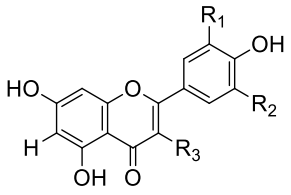
8.2.2. Flavonoids

Some flavonoids, namely myricetin 3-*O*-glucoside, syringetin 3-*O*-glucoside and quercetin derivatives (arabinofuranoside, galactoside (hyperoside) and glucoside

(isoquercitrin)) were found in all fruits and leaves extracts (Figure 8.2). Rutin and quercetin were extracted with ethanol maceration of fresh fruits and hydroalcoholic maceration of dried fruits. Other flavonoids, isorhamnetin 3-*O*-*β*-*D*-galactopyranoside, isorhamnetin malonylglycopyranoside, kaempferol 3-*O*-glucoside, quercetin acetylglucopyranoside, myricetin 3-*O*-*α*-L-arabinofuranoside, quercetin, quercitrin and rutin characterised all leaves extracts.

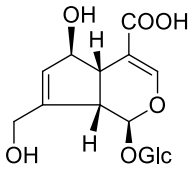
The majority of identified flavonoids are characteristic of the genus and are classically found in Ericaceae family (Bhattacharya et al., 2013; Olennikov et al., 2010; Su, 2012; Tenuta et al. 2018; Yan et al., 2002). This study revealed the presence of isorhamnetin malonylglycoside and quercetin acetylglucopyranoside. These compounds are rarely described in the plant kingdom.

Flavonoids

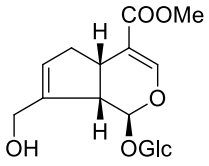


	R_1	R_2	R_3
Hyperoside	OH	H	*O*-Gal
Isoquercitrin	OH	H	*O*-Glc
Isorhamnetin 3-*O*-β-D-galactopyranoside	OCH_3	H	*O*-Galpyranoside
Isorhamnetin malonylglycoside	OCH_3	H	*O*-MalonylGlc
Kaempferol 3-*O*-glucoside	H	H	*O*-Glc
Myricetin 3-*O*-*α*–L-arabinofuranoside	OH	OH	*O*-Ara (f)
Myricetin 3-*O*-glucoside	OH	OH	*O*-Glc
Quercetin	OH	H	OH
Quercetin acetylglicoside	OH	H	*O*-Acetylglicoside
Quercetin 3-*O*-arabinoside	OH	H	*O*-Ara
Quercitrin	OH	H	*O*-Rha
Rutin	OH	H	*O*-Glc(6←1)Rha
Syringetin 3-*O*-glucoside	OCH_3	OCH_3	*O*-Glc

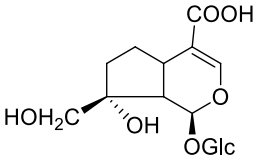
Iridoids



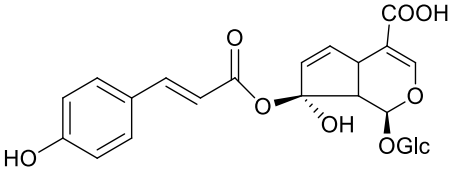
Scandoside



Geniposide



Dihydromonotropein



Vaccinoside

Figure 8.2. The dominant constituents of *V. corymbosum* extracts (*continued*)

The identification of the phytochemical composition is in agreement with the previously published data. In the *Vaccinium* genus, hydroxycinnamic acids derivatives were abundant and they were described both in leaves and fruits. Determination of phenolic compounds in blueberry fresh leaves methanol extract was studied by Riihinen et al. (2008). The authors not have showed difference in TPC in green and red leaves, yet the qualitatively interesting differences were reported, in fact anthocyanins were present only in red leaves, in addition were revealed flavonols as kaempferol and quercetin,

hydroxycinnamic acid as ferulic, caffeic and *p*-coumaric acids and proanthocyanidins: prodelphinidins and procyanidins. Green leaves present similar composition but with lower concentration, except for proanthocyanidins that are present in high concentration.

This composition was confirmed in the study of Wang et al. (2015a) that investigated 104 methanolic dried leaves extracts of various blueberry cultivars.

In literature, phytochemical compounds identified are anthocyanins, hydroxycinnamic acids, flavonols and proanthocyanidin. The most abundant compounds in *V. corymbosum* leaves were chlorogenic acid together with esters of quinic and caffeic acid (Riihinen et al., 2008; Ştefănescu et al., 2019). Same trend was observed in this study with abundance in chlorogenic acid, caffeoylquinic acid methyl ester isomers, hyperoside and isoquercitrin.

8.2.3. Iridoids

Four iridoids namely scandoside, geniposide, vaccinoside, and dihydromonotropein were identified in *V. corymbosum* extracts. Expect for vaccinoside only detected in the leaves, iridoids are present in leaves and fruits. Their extraction is sensitive to the procedures used. Except for the hydroalcoholic maceration, scandoside, and dihydromonotropein were found in all fresh leaves extracts, and in the ethanolic maceration of fresh fruits. In addition, dihydromonotropein was detected also in the decoction of dried leaves. Except for hydroalcoholic maceration, geniposide was extracted in fresh leaves extracts, in the ethanol maceration of dried leaves and fresh fruits and in the decoction of fresh fruits. Vaccinoside was detected in all extracts from fresh leaves except in decoction and only in the ethanol maceration of dried leaves.

The assignment of compounds was confirmed with UV-*vis* and with data from literature. These iridoids are previously described in the *Vaccinium* genus (Heffels et al., 2017; Sakakibara et al., 1973) or Ericaceae family (Karikas et al., 1987; Heffels et al., 2017; Leissner et al., 2017).

8.3. Antioxidant activity of *V. corymbosum* extracts

Several tests are available to investigate the antioxidant activity of foods and their derived products (Puchau et al., 2009; Floegel et al., 2011). In this study, DPPH, ABTS, FRAP, and β-carotene bleaching tests were used. ABTS, DPPH and FRAP tests are based on electron transfer mechanism, whereas β-carotene bleaching investigate the counteract effect of sample on radicals induced by oxidation of fatty acid (Floegel et al., 2011; Huang et al., 2005). DPPH assay was based on the theory that a hydrogen donor is an antioxidant. The antioxidant effect of natural compounds is proportional to the disappearance of DPPH•. In the ABTS assay, the antioxidant activity of the natural products is determined by the decolourisation of the ABTS. While, in the FRAP test, under acidic conditions, when Fe^{3+}-TPTZ complex is reduced to the Fe^{2+}-TPTZ complex by an antioxidant, an intense blue colour develops. The formation of Fe^{2+}-TPTZ complex is proportional to antioxidant effect of natural compounds.

Antioxidant activity of fruits extracts

Fresh fruits were more active than dried fruits in all tests except in FRAP test. In ABTS assay, extract obtained by hydroalcoholic maceration of fresh fruits (F1B) resulted the most active with an IC_{50} value of 1.14 μg/mL. This result is not confirmed in DPPH test where decoction of fresh fruits (F1C) showed an IC_{50} value of 50.64 μg/mL.

Among dried fruits extracts, decoction and ethanol Soxhlet extracts reported high activity for DPPH (F2C) and ABTS (F2D) test, with IC_{50} values of 54.52 and 3.14 μg/mL, respectively.

Decoction of *V. corymbosum* fresh fruits (F1C) showed high activity ferric reducing power compared with other extracts (11.42 μM Fe(II)/g), while in the dried fruits decoction (F2C) resulted low active (0.80 μM Fe(II)/g). It is interesting to note that both fresh and dried fruits extracted by decoction did not inhibit lipid peroxidation. In fact, F1A and F1E showed the best performance after 30 min incubation with IC_{50} values of 14.13 and 14.97 μg/mL, respectively.

The radical scavenging activity of *V. corymbosum* fresh and dried fruits was previously investigated. Namiesnik et al. (2014) evidenced a DPPH radical scavenging activity of 108.09 μM Trolox equivalents (TE)/g DW for *V. corymbosum* dried fruits aqueous extract, in this case, the aqueous extract resulted low active in comparison with ethyl acetate and diethyl ether extracts. Podsedek et al. (2014) investigated the antioxidant activity of *V. corymbosum cv* Toro fruit extract. Extraction from fresh fruits was done primarily with 70% acetone and consequently water. The last extract showed an ABTS radical scavenging activity of 27.09 μM TE/g fruit and a FRAP value of 16.86 μM Fe(II)/g. These data are in line with our results. Contrary, in literature aqueous extracts of dried fruits showed a high percentage of inhibition of lipid peroxidation (80.1%), compared with other extracts obtained by use of diethylether and ethyl acetate with percentages of 6.79 and 8.13% (Namiesnik et al., 2014). Previously, Rodrigues et al. (2011) investigated the antioxidant potential of fresh fruits of *V. corymbosum cv* Bluecrop. The ultrasonic methanol extract showed radical scavenging potential with values of 1253.90 and 1244.13 μM/100 g FW for ABTS and DPPH, respectively. A value of 699.78 μM/100 g FW was found in FRAP assay.

Antioxidant activity of leaves

Most of *V. corymbosum* leaves have a greater antioxidant power than the fruits and their IC_{50} values are lower than that reported for the positive control ascorbic acid (IC_{50} value of 1.70 μg/mL) (Table 8.5). Dried leaves extract obtained by decoction resulted the most active as DPPH radical scavenging agent with an IC_{50} value of 12.77 μg/mL.

In addition, decoction from fresh leaves showed a similar activity as DPPH radicals scavenging agent (L1C, IC_{50} of 15.75 μg/mL). In addition, radical scavenging activity of dried leaves decoction extract (L2C) was confirmed in the ABTS test, exhibiting also a highest radical scavenging activity with IC_{50} value of 0.77 μg/mL. The same value of

ABTS radical scavenging activity was found in dried leaves Soxhlet (EtOH) extract (L2D). Promising results were obtained also with L1E, L1C and L1D in the ABTS test with IC_{50} values of 1.06, 1.09 and 1.10 μg/mL. *V. corymbosum* leaves evidenced greater FRAP activity than fruit extracts. The ethanolic maceration of the dried leaves (L2A) showed the highest activity with FRAP value of 26.34 μM Fe(II)/g. However this value is 2.4 time lower than the positive control BHT (63.20 μM Fe(II)/g). In the FRAP test, not significant difference was reported in all extracts. Moreover, great variability in protection of lipid peroxidation was observed. Hydroalcoholic maceration of fresh leaves extract (IC_{50} value of 8.97 μg/mL) showed the highest activity followed by L2A extract (IC_{50} value of 9.72 μg/mL). The same trend was observed also after 60 min of sample incubation however with higher IC_{50} values.

In conclusion, the use of decoction for the extraction of dried leaves and maceration for fresh fruits allow to obtain the more active extracts as radicals scavenging agents.

Conversely, extracts obtained by maceration of fresh fruits and leaves showed high ability in the inhibition of lipids peroxidation.

Some studies that investigated the antioxidant activity of *V. corymbosum* leaves are present in literature. Pervin et al. (2013) reported IC_{50} values of 0.12 and 0.049 mg/mL for dried leaves hydroalcoholic extract in DPPH and ABTS test, respectively. Routray et al. (2014) studied the antioxidant activity variation through commonly test used (DPPH and FRAP tests) of *V. corymbosum* dried leaves (Nelson and Elliot varieties) collected in different time. DPPH inhibition activity of both varieties was high in May, but reduce in July and increase in September, October. Same trend was observed in the FRAP test.

These results follow the fluctuation of TPC values observed in the same study. According to the results of this study, it was indicated that *V. corymbosum* leave extracts had significant antioxidant activity against various antioxidant systems *in vitro*.

V. corymbosum leaves decoction, resulted rich in hydroxycinnamic acids and quercetin glycosides, present antioxidant effects on the brain and liver of neonatal rats in which oxidative stress is induced with toxic dose of selenium (Ferlemi et al., 2015).

It is widely believed that the antioxidant capacity of plant extract resides mainly in phenolic ability to donate hydrogen. Anthocyanins as mainly phenolic compounds present in blueberries, as reported by Zheng et al. (2003), indicating that anthocyanins represented 55.4% of the total phenol and contributed to 56.3% of the antioxidant activity presented by the fruit. In our study, it was demonstrated that the antioxidant activity was correlated with other compounds present in the phytocomplex, such as phenolic acids (in particular chlorogenic acid), iridoids, and flavonoids, in particular quercetin derivatives.

Chlorogenic acid is one of most abundant phenolic acids found in plant kingdom, in alone form or together its esters with quinic acid, including coumaroyl quinic acids, feruloyl-, caffeoyl- and dicaffeoyl. Chlorogenic acid and its derivatives have the ability of scavenging superoxide anions or hydroxyl radicals. Chlorogenic acid and its derivatives directly scavenge hydroxyl radical, but at the same time neutralise hydroxyl radicals, as superoxide anion is a precursor of much of the •OH generated *in vivo*.

Table 8.5. *In vitro* antioxidant activity of *V. corymbosum* extracts

V. corymbosum	ABTS test IC_{50} (μg/mL)	DPPH test IC_{50} (μg/mL)	FRAP test IC_{50} (μM Fe (II)/g)	β-Carotene bleaching test IC_{50} (μg/mL)	
				30 min	60 min
Fruits					
F1A	3.80 ± 0.44***	89.71 ± 2.32****	5.54 ± 0.34****	14.13 ± 1.20****	54.29 ± 2.40****
F1B	1.14 ± 0.12	53.55 ± 1.29****	2.19 ± 2.28****	40.40 ± 4.83****	53.07 ± 2.30****
F1C	5.31 ± 0.56****	50.64 ± 1.21****	11.42 ± 2.32****	4.65%[a]	13.74%[a]
F1D	5.32 ± 0.51****	51.61 ± 1.23****	5.42 ± 0.97****	39.60 ± 3.01****	62.65 ± 1.63****
F1E	3.41 ± 0.35**	52.58 ± 1.25****	1.64 ± 0.35****	14.97 ± 1.63****	88.17 ± 2.82****
F2A	34.31 ± 1.48****	71.88 ± 2.56****	16.47 ± 2.46****	40.59 ± 4.22****	57.67 ± 5.21****
F2B	18.22 ± 1.22****	195.75 ± 1.92****	5.88 ± 0.39****	52.51 ± 5.32****	45.64%[a]
F2C	8.83 ± 0.57****	54.52 ± 1.55****	0.80 ± 0.09****	4.65%[a]	20.27%[a]
F2D	3.40 ± 0.33**	74.69 ± 1.81****	7.36 ± 0.21****	25.48 ± 2.30****	87.89 ± 8.90****
F2E	3.81 ± 0.21***	144.14 ± 3.56****	12.62 ± 2.15****	41.25%[a]	27.85%[a]
Leaves					
L1A	4.62 ± 0.51****	36.09 ± 0.45****	23.83 ± 1.25****	8.97 ± 0.26***	13.19 ± 0.16****
L1B	3.43 ± 0.03**	26.46 ± 0.28****	24.56 ± 1.67****	19.01 ± 0.25****	26.42 ± 0.21****
L1C	1.09 ± 0.01	15.75 ± 0.12****	20.28 ± 1.89****	34.0%[a]	34.91%[a]
L1D	1.10 ± 0.02	28.43 ± 0.32****	25.63 ± 1.56****	47.35 ± 0.48****	40.78 ± 1.35****
L1E	1.06 ± 0.03	26.46 ± 0.25****	23.94 ± 1.67****	53.73 ± 0.59****	45.20 ± 0.43****
L2A	11.05 ± 0.11****	36.31 ± 1.33****	26.34 ± 1.71****	9.72 ± 0.09***	33.27 ± 1.36****
L2B	1.90 ± 0.20	29.42 ± 1.29****	22.92 ± 1.98****	64.80 ± 0.85****	89.20 ± 2.82****
L2C	0.77 ± 0.06	12.77 ± 0.82****	22.12 ± 1.45****	25.58%[a]	44.82%[a]
L2D	0.77 ± 0.07	34.34 ± 1.36****	24.35 ± 1.64****	49.23 ± 0.52****	43.41 ± 0.86****
L2E	1.50 ± 0.01	28.43 ± 1.25****	23.37 ± 1.56****	48.43%[a]	84.42 ± 0.92****
Positive control					
Ascorbic acid	1.70 ± 0.21	5.03 ± 0.80			
BHT			63.20 ± 4.31		
Propyl gallate				1.01 ± 0.01	1.02 ± 0.01

F1: fresh fruits, F2: dried fruits; L1: fresh leaves; L2: dried leaves. A. Ethanolic maceration; B. Hydroalcoholic (60%) maceration; C. Decoction; D. Ethanol Soxhlet extraction; E. Ethanol ultrasound-assisted extraction. [a] percentage of inhibition at a concentration of 100 μg/mL. Data are expressed as means ± S.D. (n= 3). Ascorbic acid, BHT and Propyl gallate were used as positive control in antioxidant tests. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001,**p < 0.01, *p< 0.1 compared with the positive controls.

Chlorogenic acid has high reactivity with peroxyl radicals as compared with Trolox, the water-soluble analogue of tocopherol (Laranjinha et al., 1994). However, products of chlorogenic acid formed by reaction with free radicals are rapidly broken down further to products that are not capable to generate any free radicals. This is the beneficial nature of antioxidants (Kono et al., 1997). In DPPH test, high antioxidant activity was evidenced with IC_{50} value of 9.10 μg/mL (Oboh et al., 2015). In addition at the concentration of 10 mg/kg chlorogenic acid significantly reduced total and LDL-cholesterol and increased HDL cholesterol by up regulating the gene expression of PPAR-α in hypercholesterolemic rats (Huang et al., 2015; Wan et al., 2013).

Quercetin derivatives content was higher compared with other flavonols in *V. corymbosum* (Wang et al., 2008), as reported also in this study. Quercetin derivatives are effective antioxidants, with high radical scavenging activity (Bors et al., 1990). Quercetin has five hydroxyl groups in its chemical structure. Quercetin (aglycone) was a more effective antioxidant in DPPH test, compared with its derivatives. In fact, the presence of a sugar residue reduced the antioxidant ability, as reported for rutin and hyperoside.

The same trend was observed in FRAP test. Discordant data were reported in the superoxide anion scavenging activity, where strong scavenging activity on superoxide anion was attributed to hyperoside (Zou et al., 2004). In addition, hyperoside showed also the protective effects against oxidative stress by hydrogen peroxide, *via* stimulation of antioxidant enzyme heme oxigenase-1 (HO-1). Through increased of antioxidant enzymes, hyperoside protect against cytotoxicity (Park et al., 2016). Isoquercitrin showed scavenging activity against ROS and RNS, including superoxide anion radicals, hydroxyl radicals, peroxyl radicals, and peroxynitrite (Valentová et al., 2014). Isoquercitrin showed anti-oxidative inhibition of lipid peroxidation through interference with xanthine oxidase (enzyme activity), increasing absorption of vitamin C, chelation of redox-active metals, and direct scavenging of ROS (Appleton, 2010; Heim et al., 2002; Liang et al., 2010). The lipid peroxidation inhibition and radicals-scavenging activities of flavonols were studied *in vitro* (Masuda et al., 2001; de Araújo et al., 2013).

Geniposide plays a crucial role in the prevention of oxidative stress inducted cell injury *via* up-regulation endogenous antioxidative enzymes, as glutathione (GSH), hepatic lipid peroxidase (LPO), glutathione peroxidase (GPx), glutathione-*S*-transferase (GST), glutathione (GSH), and copper- and zinc-containing superoxide dismutase (CuZn-SOD) (Wang et al., 2015b).

RACI and GAS were calculated for have the extract with high antioxidant activity. Both evaluation were produced statistically integrate the antioxidant properties obtained from various *in vitro* methods. Based on RACI and GAS value among fresh fruits hydroalcoholic extract indicated the best value (GAS = 0.8; RACI = −0.32), while F2C presented the highest antioxidant activities among dried fruits samples (GAS =1.07; RACI = −0.85). The sample that reported best values in both evaluation the greater antioxidant capacity was decoction for fresh and dried leaves.

L1C reported GAS and RACI values of 0.81 and −1.26, respectively, while L2C showed the highest antioxidant potential among dried leaves with GAS and RACI values of 0.69 and −1.09, respectively. All data were reported in Figure 8.2 and 8.3.

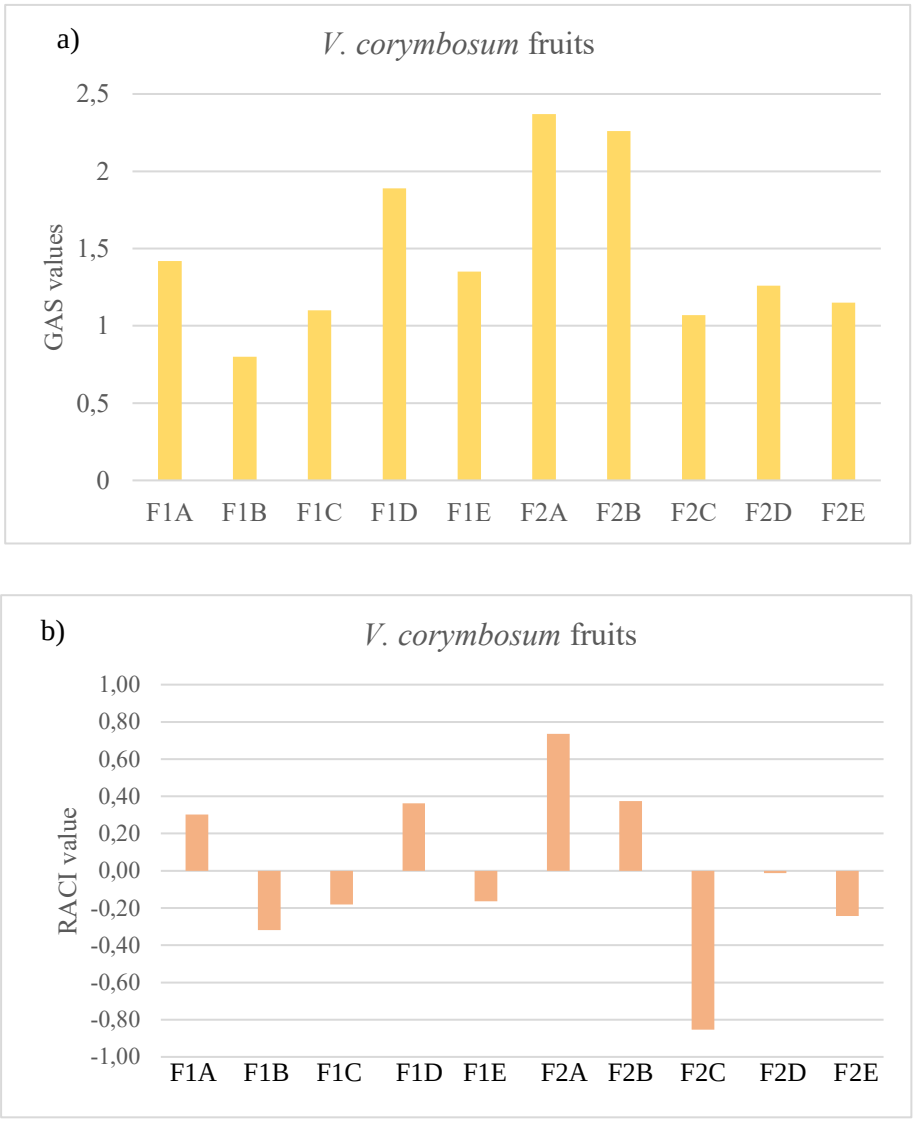


Figure 8.2. Evaluation of total antioxidant activity of fruits through GAS (a) and RACI (b). F1: Fresh fruits; F2: Dried fruits. A. Ethanolic maceration; B. Hydroalcoholic (60%) maceration; C. Decoction; D. Ethanol Soxhlet extraction; E. Ethanol ultrasound-assisted extraction.

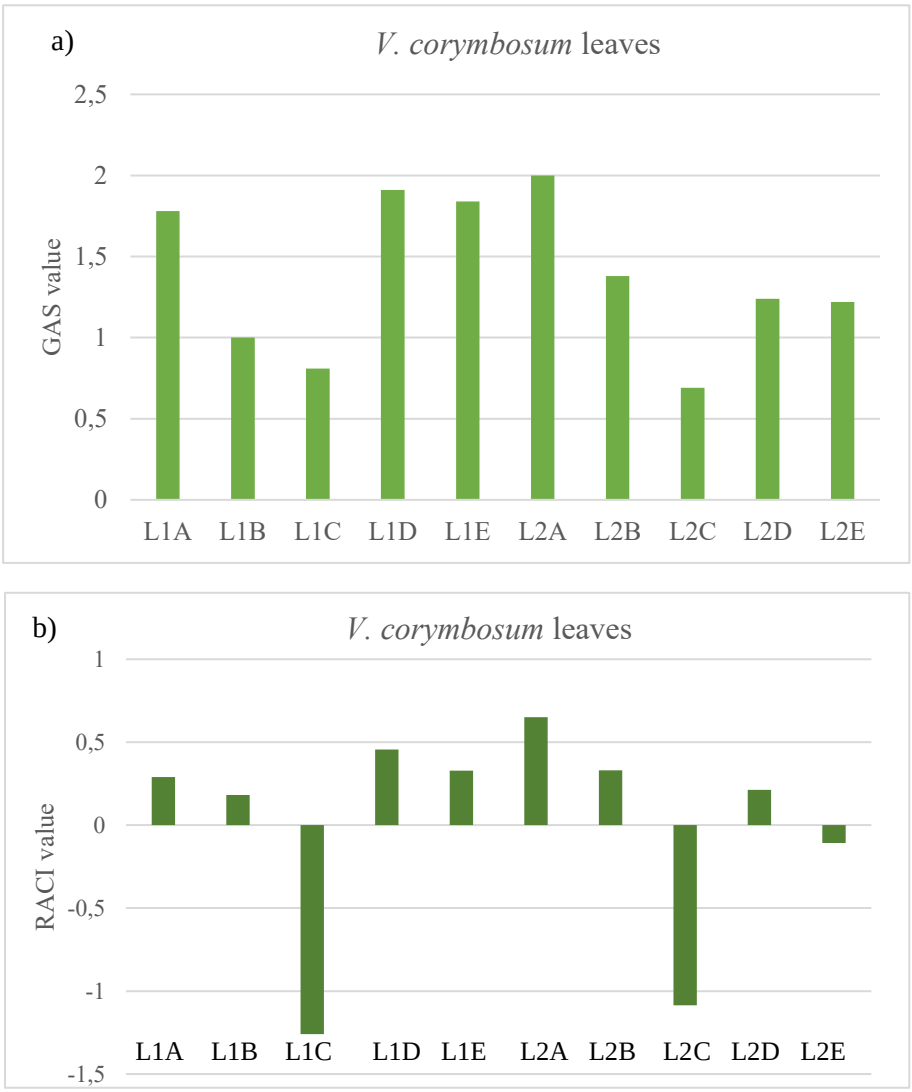


Figure 8.3. Evaluation of total antioxidant activity of leaves through GAS (a) and RACI (b). L1: Fresh leaves; L2: Dried leaves. A. Ethanolic maceration; B. Hydroalcoholic (60%) maceration; C. Decoction; D. Ethanol Soxhlet extraction; E. Ethanol ultrasound-assisted extraction

8.4. Inhibition of nitric oxide, critical mediators in inflammation

V. corymbosum fruits and leaves did not modify viability of HFF1 cells independently to the extraction technique (Figures 8.3 and 8.4).

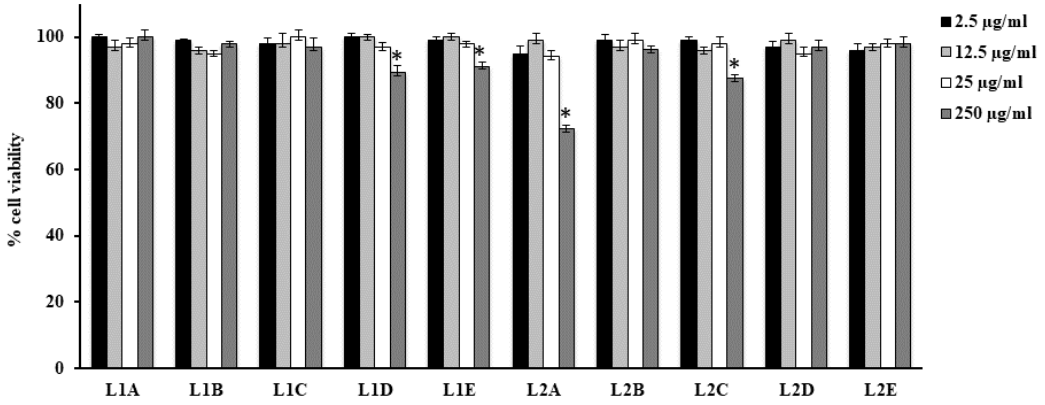


Figure 8.3. Cell viability in HFF1 cells treated for 24 h with *V. corymbosum* leaves extracts at different concentrations (2.5-250 μg/mL). Values are the mean ± SD of four experiments in triplicate. Control cells were incubated only with medium and considered as 100% of cell viability.*Significant *vs* untreated control cells: $p<0.001$.

Only F1B extract showed a slight toxicity respect to dried fruits and fresh/dried leaves extracts. In particular, at 12.5 μg/mL it is able to reduce cell viability nearly to 80% compared to control.

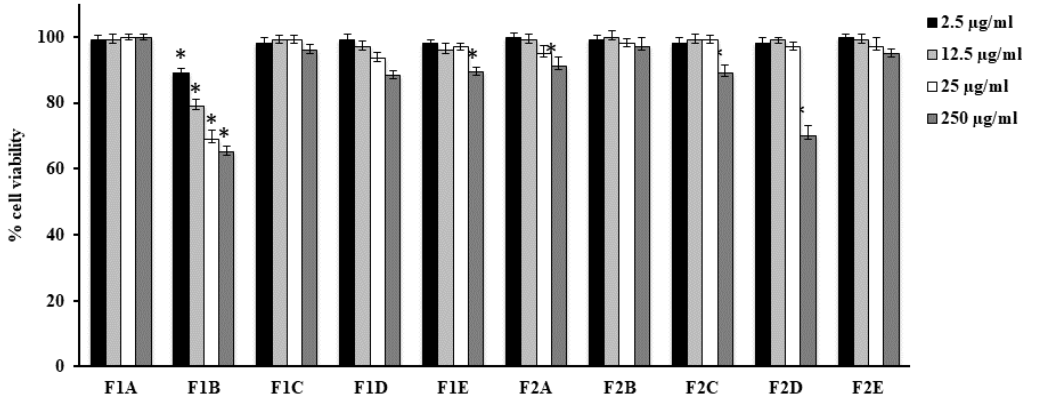


Figure 8.4. Cell viability in HFF1 cells treated for 24 h with *V. corymbosum* fruits extracts at different concentrations (2.5-250 μg/mL). Values are the mean ± SD of four experiments in triplicate. Control cells were incubated only with medium and considered as 100% of cell viability. *Significant *vs* untreated control cells: $p<0.001$.

A large body of literature has demonstrated that chronic inflammation is one key factors in the development of many disorders and diseases, strictly related to condition of oxidative stress and bad eating habits (Norris et al., 2017). It is well known that vegetables and fruits exert anti-inflammatory and anti-oxidative activities due to the phytochemical compounds present (Bonesi et al., 2017).

In our study, all extracts tested showed anti-inflammatory activity; among them fruits extracts elicited an anti-inflammatory effect higher respect to leaves extract (Table 8.6). This data is may be correlated to the presence of the phytochemicals present only in fruit extract (Table 8.4.). In particular, F1C (IC_{50} value of 19.51 μg/mL) and F2C (IC_{50} value of 18.93 μg/mL), obtained by the decoction, presented a more significant activity in inhibiting NO production responsible for the early inflammatory response.

The extract of berries from *V. corymbosum* displayed antinociceptive and anti-inflammatory activity, as reported by Torri et al. (2007). The higher dose of the *V. corymbosum* hydroalcoholic extract of fresh fruits (300mg/kg) inhibited carrageenan and histamine oedema models, indicating anti-inflammatory and antihistaminic effects (Torri et al., 2007). Moreover, in the myeloperoxidase (MPO) assay, the administration of *V. corymbosum* extract produced a significant inhibition of the MPO activity, 42.8 and 46.2%, at 6 and 24 h after injection of carrageenan, respectively. Inflammation is a process that repair damaged tissue by different factors.

Table 8.6. Inhibitory effects of *V. corymbosum* extracts on NO production in HFF1 cells

V. corymbosum	IC_{50} (μg/mL)
Fruits	
F1A	25.72 ± 1.65
F1B	25.30 ± 0.86
F1C	19.51 ± 1.11
F1D	25.12 ± 1.12
F1E	25.30 ± 0.99
F2A	21.55 ± 0.99
F2B	21.26 ±1.32
F2C	18.93 ± 1.61
F2D	26.04 ± 1.24
F2E	20.16 ± 0.79
Leaves	
L1A	27.17 ± 1.21
L1B	28.41 ± 1.11
L1C	27.17 ± 0.65
L1D	28.41 ± 1.08
L1E	24.03 ± 0.81
L2A	26.48 ±1.23
L2B	24.41 ± 1.54
L2C	28.52 ± 1.36
L2D	34.71 ± 1.34
L2E	29.76 ± 0.87

F1: fresh fruits, F2: dried fruits; L1: fresh leaves; L2: dried leaves. A. Ethanolic maceration; B. Hydroalcoholic (60%) maceration; C. Decoction; D. Ethanol Soxhlet extraction; E. Ethanol ultrasound-assisted extraction. Data are expressed as means ± S.D. (n= 4). Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001 compared with the negative control (0 μg/mL).

Inflammation is linked with the production of free radicals by inflammatory cells, thus compounds with radical scavengers activities have some anti-inflammatory capabilities. The use of some plant-derived natural compounds, as phenolic compounds for the treatment of inflammation could be an alternative to synthetic drugs. Protective activities of isoquercitrin (0.125-12.5 μmol/kg) against cadmium-induced lipid

peroxidation and protein oxidative damage were studied in mice. Isoquercitrin was demonstrate to chelate cadmium and to reduce its toxic effects, through reduction of superoxide dismutase (SOD) and catalase activities; reduce lipid peroxidation and production of nitric oxide (Li et al., 2011). In addition, if isoquercetin was locally applied at dose of 10 mg/kg showed preventive effects against inflammation in rats (Morikawa et al., 2003).

Hyperoside suppressed LPS-induced production of TNF-α (20 μM) with reduction of 38% and IL-1β at concentration of 5, 10 and 20 μM with percentage values of 26.4, 39.4, and 55.2%, respectively. In addition, hyperoside decrease in NO production with inhibition of 20.6, 35.0, and 43.7%, respectively at doses of 5, 10 and 20 μM (Fan et al., 2017). Iridoid, geniposide showed of possess potent anti-inflammatory action on paw edema induced by carrageenan. The administration of geniposide (100 mg/kg) reduced paw oedema in rats of 31.7% (Koo et al., 2006).

In addition, geniposide reduced *in vivo* and *in vitro* the production of inflammatory cytokines (Il-6, IL-1β and TNF-α), various protein kinases involved in the initiation and progression of inflammation (Fu et al., 2012; Xiaofeng et al., 2012), levels of NO and prostaglandin E2 (PGE2) (Shi et al., 2014).

Histamine release is a first reaction of allergy and inflammation. Majority of drugs, through inhibition of histamine, reduce the inflammation status in the treatment of asthma and allergy. Chlorogenic acid at dose of 25 μM showed a diminution of 50% the histamine in the rats (Kimura et al., 1985). Moreover, secretion of interleukin-8 (IL-8) and mRNA expression was significantly inhibited by chlorogenic acid (Zhao et al., 2008).

Shin et al. (2017) reported that the anti-inflammatory activities of chlorogenic acid was associated with their catechol groups. Thus, the presence of catechol group in the compounds could be contributed in the prevention of inflammatory diseases.

A recent study showed the anti-inflammatory effects of scandoside by using LPS-induced RAW 264.7 macrophages (He et al., 2018). Scandoside is not a main compound in *V. corymbosum* extracts but, in literature, is known for its anti-inflammatory activities. In the LPS-induced RAW 264.7 macrophages, this iridoid present an important reduction of NO, PGE2, TNF-α and IL-6 after treatment. LPS in the macrophages lead to over-production of NO and PEG2, scandoside reduce the levels of NO and PEG2, through suppression of *i*NOS and COX-2 and inhibited their mRNA expression. Its anti-inflammatory activity was confirmed with molecular docking analysis. In docking experiments on COX-2, His^{90}, Tyr^{355}, Tyr^{385}, and Ser^{530} scandoside formed hydrogen bonds. For the docking experiments on *i*NOS, amino acid residues Trp^{295}, Lys^{296}, Asp^{303}, Glu^{320}, Ile^{321}, Glu^{328} and Lys^{345} formed hydrogen bonds (He et al., 2018).

This analysis showed that scandoside links COX-2 active sites and key amino acids of *i*NOS, as anti-inflammatory compounds (Saravanan et al., 2014; Ramírez-Cisneros et al., 2015).

8.5. *In vitro* hypoglycaemic activity

Herein, the hypoglycaemic capacity of *V. corymbosum* extracts was evaluated through the inhibition of α-amylase and α-glucosidase. The results are reported in Table 8.7. As evident, *V. corymbosum* extracts showed an appreciable activity against α-glucosidase.

Table 8.7. Carbohydrates-hydrolysing enzymes inhibitory activity of *V. corymbosum* fruits and leaves extracts

V. corymbosum	IC_{50}, μg/mL		Selectivity Index (SI)	
	α-Amylase	α-Glucosidase	α-Amylase	α-Glucosidase
Fruits				
F1A	33.79% [a]	189.81 ± 4.15****	-	-
F1B	4.37% [a]	298.42 ± 3.56****	-	-
F1C	36.73% [a]	666.29 ± 7.64****	-	-
F1D	46.08% [a]	195.84 ± 2.65****	-	-
F1E	24.13% [a]	139.88 ± 2.31****	-	-
F2A	4% [a]	188.06 ± 4.56****	-	-
F2B	195.94 ± 4.58****	373.74 ± 5.62****	1.91	0.52
F2C	16.82% [a]	146.89 ± 1.24****	-	-
F2D	NA	139.88 ± 1.47****	-	-
F2E	680.30 ± 8.32****	76.57 ± 0.85****	0.11	8.88
Leaves				
L1A	564.68 ± 3.25****	230.10 ± 4.56****	0.41	2.45
L1B	20.55 ± 0.74****	215.21 ± 3.68****	10.47	0.09
L1C	NA	195.94 ± 1.47****	-	-
L1D	36.92%[a]	134.63 ± 1.57****	-	-
L1E	NA	341.34 ± 3.62****	-	-
L2A	178.42 ± 1.85****	247.62 ± 2.51****	1.39	0.72
L2B	417.54 ± 2.69****	262.51 ± 2.78****	0.63	1.59
L2C	NA	8.80 ± 0.06****	-	-
L2D	16.16 ± 0.01****	259.00 ± 5.69****	16.03	0.06
L2E	325.57 ± 2.35****	176.67 ± 8.45****	0.54	1.84
Positive control				
Acarbose	50.01 ± 1.43	35.50 ± 1.10	0.71	1.41

F1: Fresh fruits; F2: Dried fruits; L1: Fresh leaves; L2: Dried leaves. A. Ethanolic maceration; B. Hydroalcoholic (60%) maceration; C. Decoction; D. Ethanol Soxhlet extraction; E. Ethanol ultrasound-assisted extraction. [a] percentage of inhibition at a concentration of 1 mg/mL. NA: not active. Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001, **p < 0.01, *p< 0.1 compared with the positive control. [a] Selectivity for α-amylase is defined as IC_{50} (α-glucosidase)/IC_{50} (α-amylase). [b] Selectivity for α-glucosidase is defined as IC_{50} (α-amylase)/IC_{50} (α-glucosidase).

Among fruits extracts, significant results were obtained by hydroalcoholic maceration of fresh leaves (L1B) and Soxhlet (EtOH) apparatus dried leaves (L2D) extracts against α-amylase with IC_{50} values of 20.55 and 16.16 μg/mL, respectively.

The activity of these extracts was 2.4 and 3 times higher than positive control for L1B and L2D, respectively. In addition, selectivity values of 10.47 and 16.03 were found for α-amylase. Decoction of dried leaves (L2C) exhibited the highest α-glucosidase inhibitory activity with an IC_{50} value of 8.80 μg/mL, 4.4 times higher than the positive control.

Interesting results were obtained also by ethanol ultrasound-assisted extraction of dried fruits (F2E) with an IC_{50} value of 76.57 μg/mL. Interesting data were reported by L2C, L1D and L1C. These extracts showed a high inhibition against α-glucosidase, but no activity against α-amylase.

Johnson et al. (2011) have compared inhibition capacity against α-amylase and α-glucosidase of various cultivar of *V. corymbosum* fruits grown at the same location under the same environmental conditions *cv* commercial samples. Different cultivar showed high percentage of inhibition, both α-amylase (91.79~103.32%) than α-glucosidase (103.22~193.61%), respect to commercial *V. corymbosum* sample (86.80 and 75.54% for α-amylase and α-glucosidase, respectively).

Cheplick et al. (2015) investigated phenolic-linked bioactive functionality of *V. corymbosum* in type 2 diabetes management during fruit maturation. The mature fruit showed higher α-amylase, α-glucosidase inhibitory activity, and significant potential to improve glucose metabolism, compared to immature fruits.

Previously, McDougall et al. (2005) tested the polyphenol-rich extracts from different berries extracts for their ability to inhibit α-amylase and α-glucosidase. *V. corymbosum* fruits extracts were more effective α-amylase inhibitors than blackcurrant and red cabbage extracts. α-Glucosidase was more readily inhibited by blueberry and blackcurrant extracts.

Chlorogenic acid and its derivatives are known inhibitors of α-amylase. In the mature fruits, its levels were high compared with immature fruits. Blueberry ripening results in a shift of phenolics toward anthocyanin synthesis with a decrease in other phenolic compounds, including hydroxycinnamic acids and flavonols (Kalt et al. 2003; Castrejon et al. 2008). In this case, α-amylase inhibitory activity in ripe fruit could be due to proanthocyanidins and anthocyanins, while in green fruit was due to a phenolic profile.

In the study of Oboh et al. (2015) was studied the inhibitory effects of various phenolic acid on α-amylase and α-glucosidase activities. Chlorogenic acid showed high inhibition against these enzymes with IC_{50} values of 9.10 and 9.24 μg/mL, respectively for α-amylase and α-glucosidase.

In α-glucosidase test, discordant results was found. Inhibition was higher in ethanol extracts than in water extracts, and surprisingly the green fruits reported higher inhibition followed by ripe and green/pink fruit extracts. Phenolic content in ethanol extracts is more significant in the inhibition of α-glucosidase than in water extracts. Glycosylation of flavonoids results in their increased water solubility and, therefore, may make them more available in water extracts than other compounds (Hostel 1981). Anthocyanins and procyanidins are well-known inhibitors of α-glucosidase, and their content increase as fruit develops from green to ripe. In addition, ellagic acid and catechin were identified as active inhibitors of α-glucosidase in raspberry fruits (Zhang et al. 2010).

In a clinical study, 45 g of *V. corymbosum* powder as smoothie were administered to 32 volunteers (adults, obese, and insulin resistant) for breakfast and dinner, for six weeks

(Stull et al., 2010). At the end of this period, the participants showed improved insulin sensitivity. In conclusion, studies confirmed that *V. corymbosum* berries exhibited anti-diabetic properties and protection of pancreatic *β*-cells from glucose-induced oxidative stress (Karcheva-Bahchevanska et al., 2017). Discordant results were reported by Ştefănescu et al. (2018) that studied the effects of *V. corymbosum* dried leaves against diabetes. None positive effects were observed after administration of hydroalcoholic *V. corymbosum* leaves extracts in diabetic rats, compared with the control (non-diabetic rats). A great majority of studies have focused on the effect of *V. myrtillus* leaves and fruits extracts in diabetes (Bljajić et al., 2017; Ştefănescu et al., 2018).

8.6. Correlation between phytochemical content and bioactivity

Pearson correlation analyses were done to predict the relationship between the phytochemicals compounds found in extracts and bioactivities.

Analysing extracts from fresh fruits, TPC showed a strong significant positive correlation (*r* value of 0.89) with FRAP test, while moderate correlation was found with TFC and *β*-carotene bleaching test after 60 min of incubation (*r* 0.69). Same moderate correlation (*r* 0.68) was reported for the TIC and ABTS test. Moreover, in dried fruits the radical scavenging activity against ABTS was positive correlated with TFC (*r* value of 0.79). A positive correlation was found between TFC of fresh fruits and TIC of dried fruits and anti-inflammatory activity with values of *r*= 0.71 and 0.81, respectively.

Analysing fresh leaves, positive correlations were found between TPC and DPPH test and FRAP test with *r* values of 0.83 and 0.76, respectively. This implies the ability of TPC to act as reducing agents and hydrogen donors in neutralising free radical. On the other hand, antioxidant activity of dried leaves may be related to the TIC. In fact, very strong correlations between this phytochemical class and ABTS, DPPH and FRAP tests with *r* values of 0.91, 0.82 and 0.93, respectively, were indicated. Considering high correlation found in this study with other phytochemical compounds as flavonoids and iridoids can be conclude that the bioactivities reported not depending only to anthocyanins content, main phytochemical compounds present in *Vaccinium* genus, but also to other compounds, as confirmed in this study.

8.7. Conclusion

In summary, a phytochemical screening by LC-ESI-Q-TOF-MS, four different *in vitro* antioxidant assays, *α*-amylase and *α*-glucosidase inhibitory activity tests, and the anti-inflammatory activity test by means of the effects on the NO production were executed in order to compare and to correlate the chemical composition of fruits and leaves of *V. corymbosum* with their biological activities.

V. corymbosum extracts are characterised by the presence of flavonoids, phenolic acids, and iridoids as dominant classes of constituents. These phytochemical classes are associated with bioactivities reported for the different extracts. Generally, it is not possible to detect particular differences in the chemical composition of the extracts

obtained from fruits and leaves. For this reason no more active extract was obtained than the others. . Globally, the biological evaluation demonstrated the implication of phenolic compounds and iridoids. To the best of our knowledge, this is the first study that revealed the presence of iridoids in *V. corymbosum*. A promising inhibition of the mediator of inflammation, NO, was found. Fruits extracted by decoction showed the highest activity in counteracting NO production. Considering that *V. corymbosum* fruits are edible, their consumption may be helpful for the treatment of inflammatory disorders.

Overall, our results indicated that extracts of *V. corymbosum* devoid of anthocyanins and rich in flavonoids and iridoids might be considered a source of natural anti-inflammatory and antioxidant agents.

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Chapter 9

Cornus mas and *C. sanguinea*: results and discussion

9.1. *Cornus mas*

9.1.1. Impact of extraction procedures on phytochemicals contents

C. mas fruits (3.1 kg) and leaves (1.6 kg) were collected. Of these, 2.0 kg of fruits and 600 g of leaves were immediately extracted, 1.1 kg of fruits were dried at 50 °C for 7 days in hot air oven obtaining 360 g of dried matrix, and 1.0 kg of leaves were dried at room temperature for 7 days in the dark obtaining 620 g of dried materials. After filtration, removal of the solvent under reduced pressure yielded crude extracts.

Extraction yields (%) are reported in Table 9.1 and Figures 9.1 and 9.2. With the only exception of the hydroalcoholic maceration, the yield of extraction of dried samples (19.30-60.24 and 7.41-25.73% for fruits and leaves, respectively) is higher than this reported for fresh samples (11.40-21.52 and 3.35-17.70% for fruits and leaves, respectively).

Table 9.1. Extractive yield **(%)** of *C. mas* extracts

	Yield (%)[a]			
Extraction techniques	Fruits		Leaves	
	Fresh	Dried	Fresh	Dried
Maceration (EtOH)	15.82 ± 1.63	51.72 ± 5.10	11.84 ± 1.23	16.13 ± 1.65
Maceration (Hydroalcoholic 60%)	15.64 ± 1.62	57.94 ± 5.83	17.70 ± 1.83	16.20 ± 1.61
Decoction (H_2O)	11.40 ± 1.15	19.30 ± 1.93	17.35 ± 1.71	23.35 ± 2.36
Soxhlet apparatus (EtOH)	21.52 ± 2.12	60.24 ± 6.20	12.34 ± 1.21	25.73 ± 2.60
Ultrasound-assisted extraction (EtOH)	14.21 ± 1.47	28.04 ± 2.82	3.35 ± 0.32	7.41 ± 0.71

Data are reported as mean ± standard deviation (n= 3). [a] Expressed as (g dried extract/ g plant materials) × 100

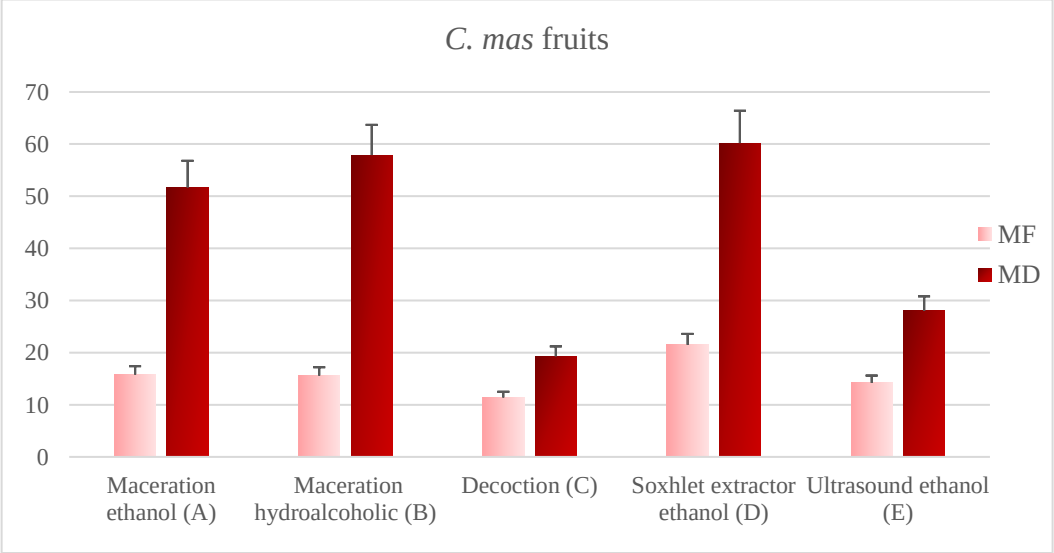


Figure 9.1. Extraction yield (%) of *C. mas* fruits. MF: fresh fruits, MD: dried fruits.

The use of ethanol Soxhlet apparatus allowed obtaining the best yield with values of 21.5 and 60.2% for fresh (MFD) and dried fruits (MDD), respectively. Interesting is also the percentage of 25.7% obtained with dried leaves. Except for dried fruits, lower extraction yields were obtained by using ethanol ultrasound-assisted extraction.

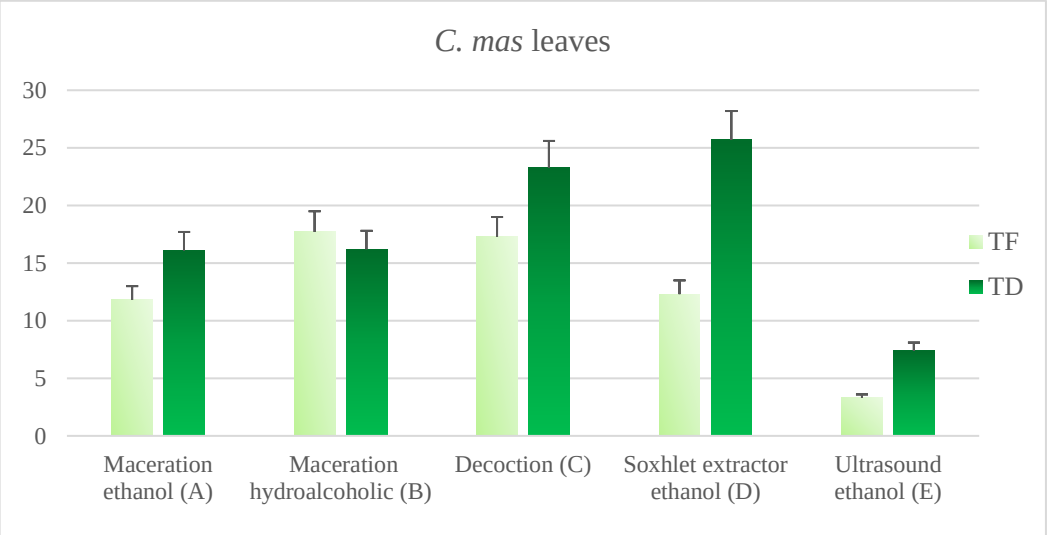


Figure 9.2. Extraction yield (%) of *C. mas* leaves. TF: fresh leaves, TD: dried leaves.

One of the most important parameters for maximizing the extraction yield of bioactive compounds is the temperature. An increase in the extraction temperature would improve the solubility of the solute and its diffusion inside the plant matrix, consequently reducing the extraction times. In general, the increase of temperature could determine an increase in the concentration of some phenolic compounds and iridoids due, probably to a better solubility of some of these compounds, but also to the breakdown of cellular components that would retain the bioactive compounds present (Lim et al. , 2007; Náthia-Neves et al., 2017). Náthia-Neves et al. (2017) observed that a positive effect of temperature on global yield, total phenols content and antioxidant activity of *Genipa americana* ethanol extraction.

Significant differences in extraction yields were found also depending on the drying process used and the chemical-physical characteristics of extracted phenolics.

In the bibliographic part, a high variety of heterosides compounds (flavonoids and iridoids) was found in the leaves and fruits. The water contained in fresh vegetables and enzymes, with the application of heat, could facilitate the hydrolysis and degradation of certain glycosides. These reactions could also partially explain our results.

Usually, phenolic compounds are relatively preserved (Slatnar et al., 2011), but the intensity and duration of heating can influence their stability. Aaby et al. (2007) observed a reduction of 20 and 17%, respectively, in ellagitanninic derivatives and in ellagic acid during heat treatment. Lohachoompol et al. (2004) found in the methanol extract of *V.*

corymbosum that the content in bioactive compounds decreases starting from fresh fruits and after drying of 41%. This result is to be attributed most probably due to the breaking of the vegetable matrix and the consequent loss of compounds of considerable interest.

Furthermore, Mejía-Meza et al. (2008) have shown that in the methanol extract of *V. corymbosum* the phenols content decreases in extracts from dried matrix compared to extracts from fresh matrix if treated with high temperatures and prolonged heat treatments.

Plant-based diets have long been associated with increased life expectancy and a reduced risk of chronic and degenerative diseases. Many berry fruits are considered of interest for their high content in bioactive phytochemicals. Among phytochemicals, phenols (particularly flavonoids) and iridoids attract a huge scientific interest.

The total phenols content (TPC) of *C. mas* extracts, as measured by the Folin-Ciocalteu method and expressed as mg chlorogenic acid (CA) equivalents/g of dry extract, is reported in Table 9.2.

TPC ranged from 33.73 to 81.33 mg CA equivalents/g extract for fresh fruits, and from 33.87 to 97.33 mg CA equivalents/g extract for dried fruits, with the highest TPC being found in the extracts obtained by decoction (MFC and MDC). The extracts with the lowest TPC were obtained by ethanol ultrasound-assisted extraction. The same trend was observed for the total content of flavonoids, expressed as quercetin (QE) equivalents. TFC varied from 24.47 to 38.66 mg QE equivalent/g extract for fresh fruits and from 24.53 to 37.53 mg QE equivalent/g extract for dried fruits. The MFC and MDC showed the highest amount of flavonoid contents. The extracts obtained by ethanol ultrasound-assisted extraction of fresh fruits (MFE) and the hydroalcoholic maceration of dried fruits (MDB) are richest in iridoids, with values of 172.67 mg aucubin (AU) equivalents/g and 184.67 mg AU/g, respectively. An interesting TIC was found also in MDA sample (152.23 mg AU equivalents/g extract).

Table 9.2. Total phytochemicals content of extracts from *C. mas* fruits.

C. mas	Total Phenols Content (TPC)[a]	Total Flavonoids Content (TFC)[b]	Total Iridoids Content (TIC)[c]
Fresh fruits			
MFA	79.73 ± 2.13	26.07 ± 0.61	103.12 ± 2.76
MFB	34.60 ± 1.67	27.07 ± 0.87	131.34 ± 3.62
MFC	81.33 ± 2.65	38.66 ± 0.54	97.33 ± 3.21
MFD	45.21 ± 1.24	27.60 ± 0.36	111.33 ± 2.21
MFE	33.73 ± 1.25	24.47 ± 0.87	172.67 ± 3.32
Dried fruits			
MDA	51.13 ± 0.46	26.33 ± 0.54	152.23 ± 4.44
MDB	36.26 ± 0.56	25.67 ± 0.93	184.67 ± 3.39
MDC	97.33 ± 1.58	37.53 ± 1.13	106.67 ± 4.76
MDD	39.02 ± 0.76	27.11 ± 1.54	80.66 ± 2.56
MDE	33.87 ± 1.11	24.53 ± 0.36	84.59 ± 3.14

MF: fresh fruits, MD: dried fruits; A. Ethanolic maceration; B. Hydroalcoholic (60%) maceration; C. Decoction; D. Ethanol Soxhlet extraction; E. Ethanol ultrasound-assisted extraction. Data are reported to mean ± Standard Deviation (SD) (n = 3). [a] mg chlorogenic acid (CA) equivalents/g extract; [b] mg quercetin (QE) equivalents/g extract; [c] mg of aucubin (AU) equivalents/g extract.

As evident in Table 9.3, results show that extracts prepared starting from the leaves have a phenols content four to five times (or more) higher than the corresponding extracts obtained from the fruits. The TPC ranged from 233.02 to 479.62 mg CA equivalents/g extract for fresh leaves, and from 263.72 to 490.13 mg CA equivalents/g extract for dried leaves, with the highest TPC being found in the extracts obtained by ethanolic maceration of fresh leaves (TFA) and ethanol Soxhlet apparatus of dried leaves (TDD). The extracts with the lowest total phenols content were obtained by ethanol ultrasound-assisted extraction of both matrix (fresh and dried).

Table 9.3 also reports data regarding flavonoid contents in the selected *C. mas* extracts. Comparing the same extraction procedure, similarly to what already observed for TPC, the flavonoids content was always higher in leaves extracts of *C. mas* with respect to fruits extracts. The total flavonoids content ranged from 79.87 to 247.60 mg QE equivalent/g extract for fresh leaves, and from 75.73 to 296.08 mg QE equivalent/g extract for dried leaves. Decoction (with values of 296.08 and 247.6 mg QE/g respectively for dried and fresh materials) represents the technique that allowed obtaining the highest flavonoids content.

Table 9.3. Total phytochemicals content of extracts from *C. mas* leaves.

C. mas	Total Phenols Content (TPC)[a]	Total Flavonoids Content (TFC)[b]	Total Iridoids Content (TIC)[c]
Fresh leaves			
TFA	479.62 ± 2.20	226.67 ± 1.63	117.33 ± 1.94
TFB	462.27 ± 3.33	92.65 ± 0.85	126.13 ± 2.81
TFC	437.33 ± 3.61	247.60 ± 2.24	96.67 ± 0.74
TFD	240.81 ± 2.40	79.87 ± 0.61	122.64 ± 2.71
TFE	233.02 ± 1.72	126.41 ± 1.45	130.11 ± 2.75
Dried leaves			
TDA	263.72 ± 2.23	89.33 ± 1.43	152.55 ± 2.87
TDB	339.73 ± 2.64	75.73 ± 1.24	113.33 ± 3.32
TDC	410.66 ± 3.15	296.08 ± 4.82	126.70 ± 2.41
TDD	490.13 ± 3.46	187.47 ± 3.71	121.33 ± 2.52
TDE	238.15 ± 2.21	126.03 ± 2.81	174.02 ± 3.51

TF: fresh leaves; TD: dried leaves. A. Ethanolic maceration; B. Hydroalcoholic (60%) maceration; C. Decoction; D. Ethanol Soxhlet extraction; E. Ethanol ultrasound-assisted extraction. Data are reported to mean ± Standard Deviation (SD) (n = 3). [a] mg chlorogenic acid (CA) equivalents/g extract; [b] mg quercetin (QE) equivalents/g extract; [c] mg of aucubin (AU) equivalents/g extract.

The total iridoids content (TIC) ranged from 96.67 to 130.11 mg AU equivalent/g extract for fresh leaves, and from 113.33 to 174.02 mg AU equivalent/g extract for dried leaves. The highest TIC was obtained by ethanol ultrasound-assisted extraction with values of 174.02 and 130.11 mg AU/g for dried and fresh leaves, respectively.

Cosmulescu et al. (2017) evaluated the total phenols and flavonoids content of hydroalcoholic ultrasounds-assisted extraction of *C. mas* fresh fruits, reporting values of 184.69 mg gallic acid equivalents (GAE)/g and 17.27 mg QE/g, respectively.

In a previous study, the following trend for TPC was observed for the fruits extracted by different solvents: ethyl acetate, acetone, methanol, petroleum ether, and water (Stanković et al., 2014). The highest content (179.05 mg GAE/g) was obtained by ethyl acetate. The same trend was observed for TFC with values ranged from 41.49 and 3.53 mg rutin (RU) equivalents/g. In agreement with our results, leaves was richest in phenols than fruits. In this work, the aqueous extract showed the high TPC (341.09 mg GAE/g), followed by acetone, methanol, petroleum ether and ethyl acetate extracts.

Our results are in agreement also with these reported by Celep et al. (2013) that found total phenols and flavonoids contents of 342.6 mg GAE/g and 72.83 mg QE/g in the hydroalcoholic maceration of dried leaves of cornelian cherry.

9.1.2. LC-ESI-Q-TOF-MS analyses

Tables 9.4 and 9.5 present the chemical composition of *C. mas* fruits and leaves extracts, respectively.

Compounds were identified based on UV spectra, molecular weight (*m/z* ion $[M+H]^+$), and chemotaxonomic significance (Dinda et al., 2016; Karikas, 1993; Maleš et al., 2006). The presence of quinic acid, ferulic acid, gallic acid, chlorogenic acid, ellagic acid, catechin, isoquercitrin, quercetin, rutin, hyperoside, loganin and cornuside were verified with authentic standards. Chromatograms obtained by LC-ESI-Q-TOF-MS were reported in Appendix (Figures A6-A8). Eleven phenolic acids, fourteen flavonoids, six iridoids, and one tannin were identified.

Phenolic and organic acids

Eleven phenolic acids were identified (Figure 9.3) in leaves extracts. Of these only gallic acid and shikimic acid characterised all fruits extracts. Among the phenolic acids present in the leaves, five of them are extracted whatever the procedures used, in fact ellagic acid 4-*O*-rutinoside, ferulic acid, gallic acid, shikimic acid, and salidroside were identified in all leaves extracts. Moreover, two extracts of the fresh leaves (TFC, TFD) and two extracts of the dried leaves (TDC and TDE) contain only these phenolic acids (Table 9.5).

Extraction of all other compounds are sensitive to the procedures used and have been detected only in a few extracts. Gallic acid 4-*O*-glucopyranoside was found in the extracts obtained by ethanolic maceration and ultrasound-assisted extraction of fresh leaves. Interesting results were obtained also analysing ethyl caffeate, 3-(3,4,5-trimethoxyphenyl) propanoic acid, and chlorogenic acid. In fact, chlorogenic acid was extracted only by using hydroalcoholic maceration of both fresh and dried leaves; 3-(3,4,5-trimethoxyphenyl) propanoic acid was identified only in ethanol maceration of both fresh and dried leaves.

Table 9.4. Identification of chemical compounds in *C. mas* fruits extract using LC-ESI-QTOF-MS technique

Compounds	Tr	Molecular Formule	MH^+/ MNa^+	Error (ppm)	Score (%)	MS fragment (*m/z*)	UV λ (nm)	Fresh fruits					Dried fruits					Reference
								MFA	MFB	MFC	MFD	MFE	MDA	MDB	MDC	MDD	MDE	
Phenols																		
Gallic acid°	3.2	$C_7H_6O_5$	171.0287	1.1	100		217, 271	√	√	√	√	√	√	√	√	√	√	Deng et al., 2013
Shikimic acid	8.1	$C_7H_{10}O_5Na$	197.0425	1.1	100		215	√	√	√	√	√	√	√	√	√	√	Drkenka et al., 2014
Flavonoids																		
Hyperoside °(*)	12.8	$C_{21}H_{20}O_{12}$	465.1031	1.5	100	303.0499	217, 278, 350						√					Pawlowska et al., 2010
Isoquercitrin °(*)	12.8	$C_{21}H_{20}O_{12}$	465.1031	1.5	100	303.0499	215, 253, 353						√					Pawlowska et al., 2010
Kaempferol 3-*O*-galactoside (**)	13.2	$C_{21}H_{20}O_{11}$	449.1077	1.5	100	287.2287	210, 263, 344	√	√	√	√	√	√	√	√	√	√	Pawlowska et al., 2010
Kaempferol 3-*O*-glucoside (**)	13.2	$C_{21}H_{20}O_{11}$	449.1077	1.5	100	287.2287	210, 265, 346	√	√	√	√	√	√	√	√	√	√	Pawloska et al., 2010
Kaempferol 3-*O*-glucuronide	13.7	$C_{21}H_{18}O_{12}$	463.0876	1.1	100	287.2287	210, 264, 345	√	√	√	√	√	√	√	√	√		Badalica-Petrescu et al., 2014
Quercetin 3-*O*-galactosyl 7-*O*-rhamnoside (****)	12.4	$C_{27}H_{30}O_{16}$	611.1612	1.3	100	303.0499	212, 253, 356						√	√	√	√	√	Badalica-Petrescu et al., 2014
Quercetin 3-*O*-glucopyranoside (***)	12.3	$C_{21}H_{18}O_{13}$	479.0825	1.1	100	303.0499	215, 247, 354	√	√	√	√	√	√	√	√	√	√	Pawlowska et al., 2010
Quercetin 3-*O*-glucuronide (***)	13.0	$C_{21}H_{18}O_{13}$	479.0825	1.8	100	303.0499	216, 244, 354	√	√	√	√	√		√	√	√	√	Pawlowska et al., 2010
Quercetin 3-*O*-β-D-glucuronide-6"-methyl ester	13.1	$C_{22}H_{20}O_{13}$	493.0982	1.5	97	303.0499							√					Pawlowska et al., 2010
Rutin° (****)	12.4	$C_{27}H_{30}O_{16}$	611.1612	0.7	100	303.0499	213, 253, 352						√	√	√	√	√	Pawlowska et al., 2010

Iridoids																		
Cornuside°	12.0	$C_{24}H_{30}O_{14}$	543.1713	0.2	100	218, 273	√	√	√	√	√	√	√	√	√	√	Szummy et al., 2015
Loganic acid	6.4	$C_{16}H_{24}O_{10}$	377.1447	1.1	100	238	√	√	√	√	√	√	√	√	√	√	Deng et al., 2013
Loganin°	8.4	$C_{17}H_{26}O_{10}$	391.1604	1.3	100	240		√		√	√	√	√	√	√	√	Deng et al., 2013
Sweroside	8.0	$C_{16}H_{22}O_9$	389.1447	1.2	100	245	√	√	√	√	√	√	√	√	√	√	Deng et al., 2013

MF: Fresh fruits; MD: Dried fruits. A. Ethanolic maceration; B. Hydroalcoholic (60%) maceration; C. Decoction; D. Ethanol Soxhlet apparatus; E. Ethanol ultrasound-assisted extraction. ° identified with standard compounds; **in bold** were not previously identified in the plant (or plant part); (*)(**) (***)(****) interchangeable or 2 peaks.

Table 9.5. Identification of chemical compounds in *C. mas* leaves extract using LC-ESI-QTOF-MS technique

Compounds	Rt (min)	Molecular Formule	MH^+/ MNa^+	Error (ppm)	Score (%)	MS fragment (*m/z*)	UV λ (nm)	Fresh leaves					Dried leaves					Reference
								TFA	TFB	TFC	TFD	TFE	TDA	TDB	TDC	TDD	TDE	
Phenols																		
Chlorogenic acid°	9.8	$C_{16}H_{18}O_9$	355.1029	1.6	100		242, 325		√					√				Deng et al., 2013
Ellagic acid°	12.8	$C_{14}H_6O_8$	303.0140	0.9	100		255, 365							√				Deng et al., 2013
Ellagic acid 4-*O*-rutinoside	12.3	$C_{26}H_{26}O_{17}$	611.1248	1.2	100	303.0136	274	√	√	√	√	√	√	√	√	√	√	Malhotra et al., 1981
Ethyl caffeate	11.0	$C_{11}H_{12}O_4$	209.0813	2.1	88		298, 322	√					√			√		
Ferulic acid°	1.7	$C_{10}H_{10}O_4$	195.0652	0.9	100		325	√	√	√	√	√	√	√	√	√	√	Krivoruchko, 2014
Gallic acid°	3.2	$C_7H_6O_5$	171.0287	1.1	100		217, 271	√	√	√	√	√	√	√	√	√	√	Deng et al., 2013
Gallic acid 4-*O*-glucopyranoside	3.1	$C_{13}H_{16}O_{10}Na$	355.0641	1.5	99	171.0257	256, 298	√				√						Lee et al., 2011
Quinic acid°	0.9	$C_7H_{12}O_6$	193.0706	0.9	100									√				Drkenka et al., 2014
Salidroside	9.1	$C_{14}H_{20}O_7$	301.1287	1.1	100		280	√	√	√	√	√	√	√	√	√	√	Rosendal, 1973
Shikimic acid	8.1	$C_7H_{10}O_5Na$	197.0425	1.1	100		215	√	√	√	√	√	√	√	√	√	√	Drkenka et al., 2014
3-(3,4,5-trimethoxyphenyl) propanoic acid	11.2	$C_{12}H_{16}O_5$	241.1076	1.8	98			√					√					

Flavonoids																		
Hyperoside °(*)	12.8	$C_{21}H_{20}O_{12}$	465.1031	1.5	100	303.0499	217, 278, 350	√	√	√	√	√	√	√	√	√	√	Pawlowska et al., 2010
Isoquercitrin °(*)	12.8	$C_{21}H_{20}O_{12}$	465.1031	1.5	100	303.0499	215, 253, 353	√	√	√	√	√	√	√	√	√	√	Pawlowska et al., 2010
Kaempferol 3-*O*-galactoside (**)	13.2	$C_{21}H_{20}O_{11}$	449.1077	1.5	100	287.2287	210, 263, 344	√	√	√	√	√	√	√	√	√	√	Pawlowska et al., 2010
Kaempferol 3-*O*-glucoside (**)	13.2	$C_{21}H_{20}O_{11}$	449.1077	1.5	100	287.2287	210, 265, 346	√	√	√	√	√	√	√	√	√	√	Pawloska et al., 2010
Kaempferol 3-*O*-glucuronide	13.7	$C_{21}H_{18}O_{12}$	463.0876	1.1	100	287.2287	210, 264, 345	√	√	√	√	√	√	√	√	√	√	Badalica-Petrescu et al., 2014
Kaempferol 3-*O*-rutinoside	12.9	$C_{27}H_{30}O_{15}$	595.1663	1.2	98	287.2287	210, 265, 342	√				√		√		√		Li et al., 2014
Quercetin°	16.8	$C_{15}H_{10}O_7$	303.0504	1.2	100		213, 255, 353		√					√		√		Sochor et al., 2014
Quercetin 3-*O*-galactosyl 7-*O*-rhamnoside (****)	12.4	$C_{27}H_{30}O_{16}$	611.1612	1.3	100	303.0499	212, 253, 356	√				√	√					Badalica-Petrescu et al., 2014
Quercetin 3-*O*-glucopyranoside (***)	12.3	$C_{21}H_{18}O_{13}$	479.0825	1.1	100	303.0499	215, 247, 354							√	√	√	√	Pawlowska et al., 2010
Quercetin 3-*O*-glucuronide (***)	13.0	$C_{21}H_{18}O_{13}$	479.0825	1.8	100	303.0499	216, 244, 354	√	√	√	√	√	√	√	√	√	√	Pawlowska et al., 2010
Quercetin 3-*O*-β-D-glucuronide-6"-methyl ester	13.1	$C_{22}H_{20}O_{13}$	493.0982	1.5	97	303.0499		√	√	√	√	√	√	√	√	√	√	Pawlowska et al., 2010
Quercetin 3-*O*-xyloside	13.5	$C_{20}H_{18}O_{11}$	435.7749	0.6	100	303.0499	212, 254, 356	√	√	√	√	√	√	√	√	√	√	Pawlowska et al., 2010
Quercitrin	13.6	$C_{21}H_{20}O_{11}$	449.1079	1.5	100	303.0499	213, 254, 356	√	√	√	√	√	√	√	√	√	√	Pawlowska et al., 2010
Rutin °(****)	12.4	$C_{27}H_{30}O_{16}$	611.1612	0.7	100	303.0499	213, 253, 352	√				√	√	√				Pawlowska et al., 2010
Iridoids																		

α-Dihydrocorninic acid	6.3	$C_{16}H_{24}O_{10}$	377.1447	0.9	100		√		√	√	√	√	√	√		√	Tanaka et al., 2001
Cornuside°	12.0	$C_{24}H_{30}O_{14}$	543.1713	0.2	100	218, 273	√	√	√	√	√	√	√	√	√	√	Szummy et al., 2015
Loganic acid	6.4	$C_{16}H_{24}O_{10}$	377.1447	1.1	100	238	√	√	√	√	√	√		√	√	√	Deng et al., 2013
Loganin°	8.4	$C_{17}H_{26}O_{10}$	391.1604	1.3	100	240					√	√			√		Deng et al., 2013
Secologanin	9.0	$C_{17}H_{24}O_{10}$	389.1447	1.1	100	245	√	√	√	√	√	√	√	√	√	√	Deng et al., 2013
Sweroside	8.0	$C_{16}H_{22}O_9$	359.1342	1.2	100	245	√	√	√	√	√	√	√	√	√	√	Deng et al., 2013
Tannin																	
Gemin D	8.8	$C_{27}H_{22}O_{18}$	635.0884	1.1	100	221, 265	√	√	√	√	√	√	√	√	√	√	Hatano et al., 1989

TF: Fresh leaves; TD: Dried leaves. A. Ethanolic maceration; B. Hydroalcoholic (60%) maceration; C. Decoction; D. Ethanol Soxhlet apparatus; E. Ethanol ultrasound-assisted extraction. ° identified with standard compounds; **in bold** not previously identified in the plant (or plant part); (*)(**) (***)(****) interchangeable or 2 peaks

Ethyl caffeate was reported only in both ethanolic maceration and Soxhlet of dried leaves extracts. Ellagic acid and quinic acid were identified only in dried leaves extracted by hydroalcoholic maceration.

Identified phenolic acids are described in the *Cornus* genus (Dinda et al., 2016; Lee et al., 2011; Rosendal et al., 1973) except for ellagic acid 4-*O*-rutinoside, ethyl caffeate, and 3-(3,4,5-trimethoxyphenyl) propanoic acid that were identified herein for the first time. Instead, 5-*O*-caffeoylquinic, vanillic, salicylic, and *p*-coumaric acids previously found in cornelian extracts were not detected in this work.

The richest extracts of phenolic compounds (8/11) are obtained by ethanolic maceration (TFA) of fresh leaves and by hydroalcoholic maceration of dry leaves (TDB), however, their phenols composition is different.

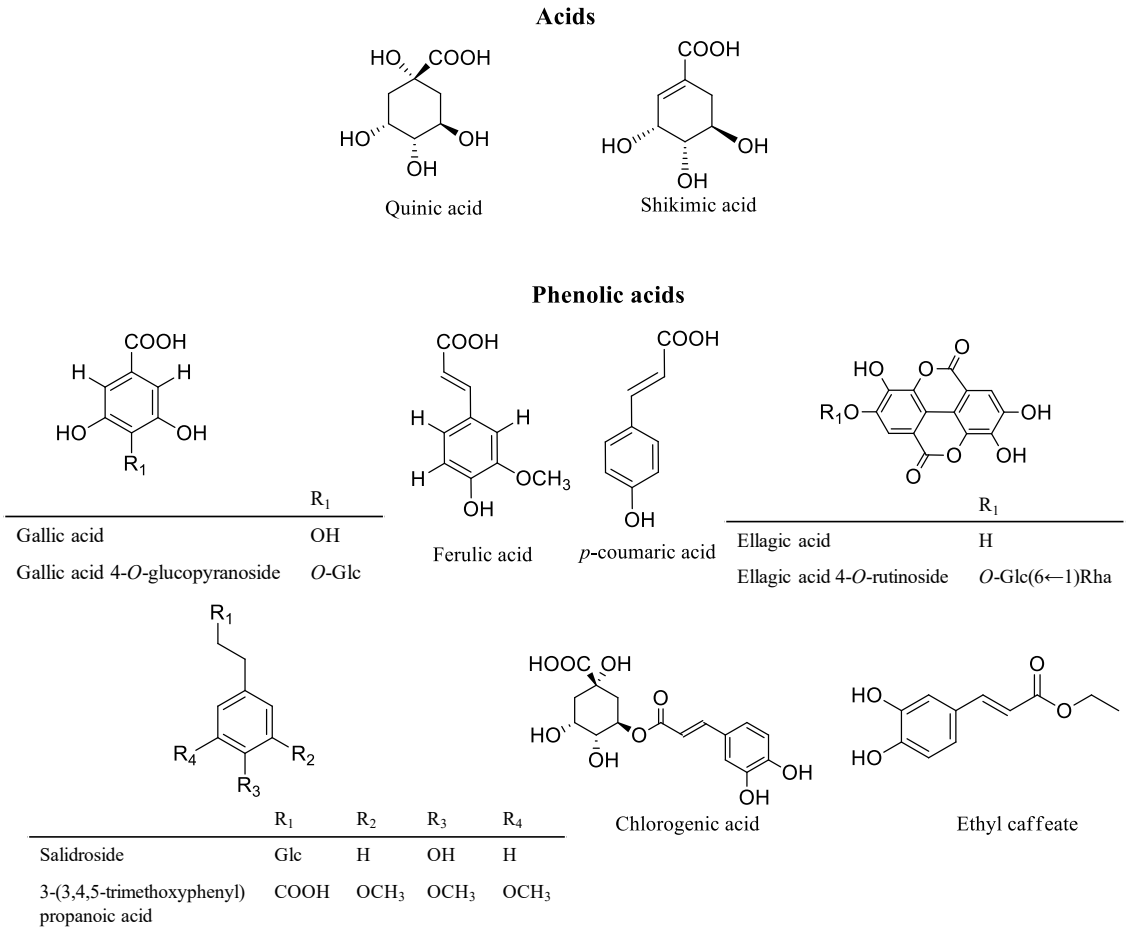
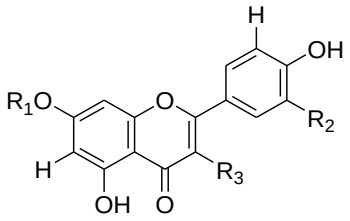


Figure 9.3. Phenols identified in *C. mas* extracts.

Flavonoids

A greater number (14 identified compounds) of flavonoids were identified in the leaves than in the fruits (10 identified compounds). They are kaempferol and quercetin heterosides. The ethanolic maceration of dried fruits (MDA) provided almost all the flavonoids identified (9/10). All fruits extracts showed the presence of kaempferol derivatives (glucoside and galactoside), and quercetin 3-*O*-glucopyranoside. Rutin and quercetin 3-*O*-galactosyl-7-*O*-rhamnoside were detected only in dried fruits. It is interesting that kaempferol 3-*O*-glucuronide was detected in all extracts except to dried fruits extracted by ethanol ultrasound-assisted extraction, while quercetin 3-*O*-glucuronide was not detected only in ethanol maceration of dried fruits.

The presence of hyperoside, isoquercitrin, and quercetin 3-*O*-*β*-D-glucuronide-6"-methyl ester was described only in the ethanol maceration of dried fruits. All leaves extracts were characterised by nine flavonoids. The presence of kaempferol (galactoside, glucoside and glucuronide) and quercetin (galactoside, glucose, glucuronide, rhamnoside, and xyloside) derivatives, and quercetin 3-*O*-*β*-D-glucuronide-6”-methyl ester has been highlighted in all analyses.



	R_1	R_2	R_3
Hyperoside	H	OH	*O*-Gal
Isoquercitrin	H	OH	*O*-Glc
Kaempferol 3-*O*-galactoside	H	H	*O*-Gal
Kaempferol 3-*O*-glucoside	H	H	*O*-Glc
Kaempferol 3-O-glucuronide	H	H	*O*-Glc A
Kaempferol 3-O-rutinoside	H	H	*O*-Glc(6←1)Rha
Quercetin	H	OH	OH
Quercetin 3-*O*-β-D-glucuronide-6’’ methylester	H	OH	*O*-Glc 6’’$COOCH_3$
Quercetin 3-*O*-galactosyl 7-*O*-rhamnoside	Rha	OH	*O*-Gal
Quercetin 3-O-glucuronide	H	OH	*O*-Glc A
Quercetin 3-*O*--rhamnoside	H	OH	*O*-Rha
Quercetin 3-*O*-xyloside	H	OH	*O*-Xyl
Rutin	H	OH	*O*-Glc(6←1)Rha

Figure 9.4. Flavonoids identified in *C. mas* extracts.

Except for ethanol maceration, quercetin 3-*O*-glucopyranoside was identified in all dried leaves. Rutin and quercetin 3-*O*-galactosyl 7-*O*-rhamnoside were identified in the extracts obtained by ethanol ultrasound-assisted extraction of fresh leaves, hydroalcoholic maceration of dried leaves and alcoholic maceration of both fresh and dried leaves.

Kaempferol 3-*O*-rutinoside was found in the ethanol maceration and ethanol ultrasound-assisted extraction of fresh leaves and in dried extracts obtained by ethanol Soxhlet extractor and hydroalcoholic maceration. Quercetin was detected only in the extracts obtained by ethanol Soxhlet extractor of dried leaves and hydroalcoholic maceration of dried and fresh leaves. All flavonoids described in this study were chemotaxonomic markers of the *Cornus* genus (Dinda et al., 2016; Pawlowska et al., 2010) with the exception of kaempferol 3-*O*-glucuronide, kaempferol 3-*O*-rutinoside, and quercetin 3-*O*-β-D-glucuronide-6”-methyl ester that were detected for the first time in the present study in *C. mas*.

Iridoids

Six iridoids namely loganin, loganic acid, sweroside, secologanin, α-dihydrocorninic acid and cornuside were identified in *C. mas* extracts (Figure 9.5). Of these, secologanin and α-dihydrocorninic acid were found only in the leaves extracts and other four are both present in the fruits and leaves. Interestingly, a perusal analysis of the literature revealed the presence of α-dihydrocorninic acid only in *C. capitata* (Tanaka et al., 2001).

Sweroside, cornuside and loganic acid are identified in all fruits extracts. Therefore, it is not possible to identify a selective extraction procedure for these iridoids.

Loganin was not detected in the extracts obtained by ethanol maceration and decoction of fresh fruits. Secologanin, sweroside, and cornuside characterised all leaves extracts while loganic acid was identified in all leaves extracts except for hydroalcoholic maceration of dried matrix. Loganin was found only in the extracts obtained by ethanol ultrasound-assisted extraction of fresh matrix and extracts TDA (ethanol maceration) and TDD (ethanol Soxhlet apparatus) of dried leaves.

Tannin

The tannin gemin D (Figure 9.5), was found in all leaves extracts but not in the fruits. The presence of the tannin was previously described in the aqueous extract of *C. officinalis* fruits (Hatano et al., 1989; Okuda et al., 1984).

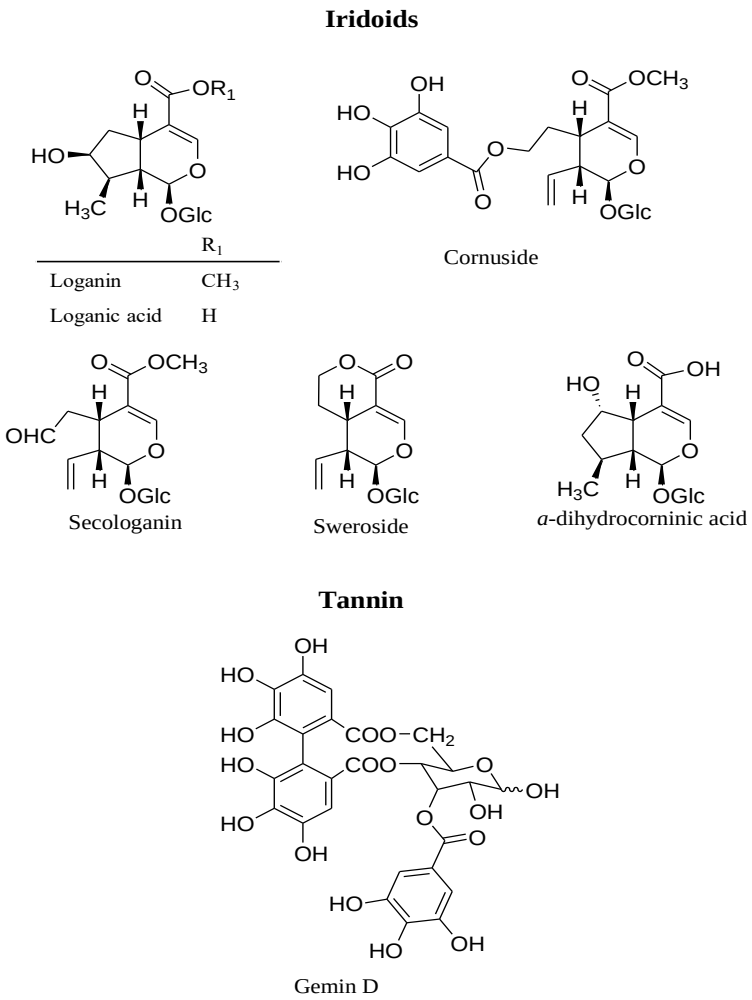


Figure 9.5. Iridoids and tannin in *C. mas* extracts

In conclusion, nearly 55% of the compounds have been systematically detected in all extracts. For fruits, among the 16 compounds, 9 of them have always been identified and for the leaves, the proportion was exactly the same (18 out of 32 compounds).

9.1.3. *In vitro* antioxidant properties

The antioxidant activity of *C. mas* extracts was evaluated by using four *in vitro* assays, namely ABTS, DPPH, FRAP, and β-carotene bleaching tests, each directed towards a specific mechanism. All samples exerted radicals scavenging and antioxidant activity in a concentration-dependent manner. IC_{50} values are reported in Table 9.6.

Antioxidant activity of fruits extracts

Among fresh fruits extracts, the extracts obtained by ethanolic (MFA) and hydroalcoholic (MFB) maceration resulted more active compared with other extracts for the inhibition of radical scavenging with IC_{50} values of 3.88 and 3.94 μg/mL respectively for ABTS and DPPH test.

Table 9.6. *In vitro* antioxidant activity of *C. mas* extracts

C. mas	ABTS test	DPPH test	FRAP test	β-Carotene bleaching test	
	(IC_{50} μg/mL)	(IC_{50} μg/mL)	(μM Fe(II)/g)	(IC_{50} μg/mL)	
Fruits				30 min	60 min
MFA	3.88 ± 0.23	4.89 ± 0.35	14.05 ± 2.21****	85.64 ± 6.37****	29.43%[a]
MFB	5.45 ± 0.11*	3.94 ± 1.24	14.76 ± 2.08****	41.33%[a]	41.13%[a]
MFC	4.27 ± 0.43	13.76 ± 7.30	13.06 ± 2.78****	74.47 ± 7.21****	92.21 ± 8.42****
MFD	4.93 ± 0.57	523.27 ± 9.25****	11.14 ± 2.33****	38%[a]	47%[a]
MFE	5.45 ± 1.23*	8.88 ± 3.65**	15.03 ± 2.87****	35.20%	38.87%[a]
MDA	6.24 ± 2.34**	888.32 ± 8.56****	10.53 ± 2.23****	14.04 ± 2.56**	8.59 ± 2.03
MDB	1.18 ± 1.96	338.40 ± 5.67****	12.81 ± 2.59****	8.12 ± 1.06	9.34 ± 2.56
MDC	1.19 ± 3.55	9.79 ± 2.35	12.17 ± 2.56****	12.72 ± 2.07**	66.31 ± 1.89****
MDD	7.35 ± 2.32****	655.59 ± 5.45****	10.59 ± 2.53****	29.24 ± 6.56****	31.02 ± 4.62****
MDE	5.06 ± 1.47*	633.07 ± 6.35****	9.46 ± 2.00****	17.60 ± 1.98***	19.48 ± 1.34***
Leaves					
TFA	1.16 ± 0.08	2.94 ± 1.25	18.00 ± 2.24****	86.29 ± 5.87****	73.53 ± 4.25****
TFB	1.10 ± 0.05	2.08 ± 1.06	18.25 ± 2.67****	75.31 ± 4.63****	68.46 ± 4.36****
TFC	1.12 ± 0.04	0.49 ± 0.0	16.47 ± 2.76****	13.94 ± 2.03**	11.50 ± 5.61
TFD	1.11 ± 0.03	7.88 ± 0.17	19.78 ± 2.43****	45.41%[a]	65.55 ± 3.87****
TFE	1.93 ± 0.09	136.63 ± 2.30****	15.50 ± 2.75****	15.54 ± 2.14***	15.91 ± 8.96**
TDA	2.32 ± 1.23	146.95 ± 6.89****	14.26 ± 2.87****	1.95 ± 0.08	2.64 ± 2.58
TDB	0.80 ± 0.01	45.18 ± 4.67****	15.88 ± 2.34****	0.49 ± 0.01	0.70 ± 0.06
TDC	1.15 ± 0.02	0.50 ± 0.02	15.56 ± 2.49****	14.88 ± 2.64**	22.20 ± 3.67****
TDD	3.11 ± 1.35	154.46 ± 9.67****	12.49 ± 2.27****	24.26 ± 5.07****	21.64 ± 3.59****
TDE	1.08 ± 0.08	147.89 ± 8.91****	16.06 ± 2.32****	1.65 ± 0.89	2.94 ± 2.08
Positive control					
Ascorbic acid	1.72 ± 0.23	5.04 ± 0.82			
BHT			63.20 ± 4.28		
Propyl gallate				1.02 ± 0.01	1.01 ± 0.01

MF: Fresh fruits; MD: Dried fruits; TF: Fresh leaves; TD: Dried leaves. A. Ethanolic maceration; B. Hydroalcoholic (60%) maceration; C. Decoction; D. Ethanol Soxhlet extraction; E. Ethanol ultrasound-assisted extraction. [a] at a concentration of 100 μg/mL. Data are expressed as means ± S.D. (n= 3). Ascorbic acid, BHT and Propyl gallate were used as positive control in antioxidant tests. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001,**p < 0.01, *p< 0.1 compared with the positive controls.

Interesting is also the activity of the hydroalcoholic maceration (MDB; IC_{50} of 1.18 μg/mL) and decoction (MDC; IC_{50} of 9.79 μg/mL) extracts of dried fruits, in ABTS and DPPH assays, respectively.

In the β-carotene bleaching test, fresh fruits extracts resulted lower activity compared with dried fruits. The most active sample was the hydroalcoholic extract of dried fruits (MDB) with IC_{50} values of 8.12 μg/mL and 9.34 μg/mL, respectively after 30 and 60 min of incubation. Among fresh fruits extracts, only decoction extract (MFC) determined inhibition of lipid peroxidation with IC_{50} values of 74.47 μg/mL and 92.21 μg/mL, respectively after 30 and 60 min of incubation. On the other hand, in the FRAP test, the fresh extract obtained with the ethanol ultrasound technique presented a greater ability to reduce iron (15.03 μM Fe(II)/g); discordant results were observed in dried fruits where the same extract has revealed less active with value of 9.46 μM Fe(II)/g.

There are several studies aimed at evaluating the antioxidant activity of *C. mas* fruits. Cosmulescu et al. (2017) described a value of 0.54 mmol Trolox/100g in the DPPH test of the methanolic extract of fresh fruits. The chemical analysis revealed high concentrations in myricetin (30.54 mg/100 g FW) and caffeic acid (1.26 mg/100 g FW), effective antioxidant compound in various *in vitro* antioxidant tests. A value of 20.41 μmol Fe^{2+}/kg in the FRAP test was reported for the hydroalcoholic extract of the fresh fruits (De Biaggi et al., 2018). De Biaggi et al. (2018) reported the presence of four classes of active compounds such as polyphenols (anthocyanins, catechins, flavonols, cinnamic acids, benzoic acids, and tannins) that represented the prevalent group (37.36%), monoterpenes (26.26%), organic acids (25.91%), and vitamin C (10.47%).

In another study, Moldovan et al. (2016) have compared the antioxidant activity of fresh *C. mas* fruits acetone extract through ABTS and FRAP assays. In both tests, the results were similar in fact submit values of 677.88 μmol Trolox equivalents/100 g and 628.75 μmol Trolox equivalents/100 g, respectively for ABTS and FRAP tests.

The extract showed quercetin 3-*O*-glucuronide, kaempferol 3-*O*-galactoside, and ellagic acid as principal constituents, followed by anthocyanins. In conclusion, as reported in previous studies, the use of different solvents is responsible for the variation of the chemical profile of the investigated extracts.

Popović et al. (2012) indicated ferric reducing antioxidant power in the range 2-65 μmol/mL Fe^{2+} for the hydroalcoholic (80%) dried fruits extract. However, other studies reported high FRAP values in the significant range comprised between 73 and 114 μmol ascorbic acid equivalent per gram of dried weight (Pantelidis et al., 2007; Yilmaz et al. 2009). Instead, Hosu et al. (2016) have studied the variation of antioxidant activity of *C. mas* fruits for 3 weeks of storage at room temperature. In this period, it was noted that the antioxidant activity changed, increasing and decreasing but not significantly reduced. Indeed, with ABTS assay there was a diminution of 6.1% during 3 weeks, whereas in the FRAP test the diminution was minor (3.2%). Thus, in general at the end of experimental the antioxidant ability was decreased only of 0.62%. Tepić Horecki et al. (2018) evaluated

the antioxidant capacity of *C. mas* fruits both fresh and after the use of different drying techniques. In the fresh state, fruits have an IC_{50} value of 16.86 μg/mL in the DPPH test. All the drying procedures used have led to a reduction in antioxidant activity with IC_{50} values in the range 20.70-77.04 μg/mL. Analysing the different solvents (such as methanol, water, ethyl acetate, acetone, and petroleum ether), the ethyl acetate extract of fresh fruits showed, in the DPPH test, the better IC_{50} value of 11.06 μg/mL compared with other extracts that exhibited values between 107.99 and 518.47 μg/mL (Stanković et al., 2014). In a previous study, Serteser et al. (2009) demonstrated that 0.9 mg of hydroalcoholic extract of dried fruits were able to reduce of 50% the radical DPPH. Furthermore, they were calculated the iron chelating and H_2O_2 inhibition activities of hydroalcoholic extract of dried fruits, indicating the percentages of 54.24 and 74.35%, respectively.

Antioxidant activity of leaves

Interesting data were given from leaves on the ability against radical ABTS and DPPH. The radical ABTS was significant locked by hydroalcoholic maceration extracts with IC_{50} values of 1.10 and 0.80 μg/mL respectively for fresh and dried materials. Instead, for the radical DPPH, extracts obtained by fresh and dried decoction resulted more active compared with other extracts with IC_{50} values of 0.49 and 0.50 μg/mL respectively. Ethanolic extraction with Soxhlet and ultrasound were the techniques that produce best extracts with high capacity of reduce iron with IC_{50} values of 19.78 μM Fe(II)/g and 16.06 μM Fe(II)/g, for fresh (TFD) and dried (TFE) leaves respectively. Furthermore, the greater inhibition of lipid peroxidation were demonstrated by decoction of fresh leaves (TFC) with IC_{50} values of 13.94 and 11.50 μg/mL respectively after 30 and 60 min of incubation. While, the hydroalcoholic maceration of dried leaves (TDB) was more active with IC_{50} values of 0.49 and 0.70 μg/mL respectively after 30 and 60 min of incubation. The fresh materials increased the inhibition against lipid peroxidation after 60 min of incubation, while the inhibition of dried materials decrease after 60 min.

In literature, studies conducted on *C. mas* leaves reported their radical scavenging activity low than for fruits, discordant with data reported in this work. Stanković et al. (2014) have studied the antioxidant activity of extracts of dried leaves obtained by different solvents, such as methanol, water, ethyl acetate, acetone, and petroleum ether.

The most interesting data were related to acetone and methanol extracts with IC_{50} values of 32.17 and 39.40 μg/mL, followed by water (59.28 μg/mL), petroleum ether (375.56 μg/mL) and ethyl acetate (381.34 μg/mL). In a previous study, the hydroalcoholic extract (80% methanol) of the dried leaves showed in the DPPH test an IC_{50} value of 165 μg/mL and in the β-carotene bleaching test a percentage of inhibition of lipid peroxidation of 93% at a concentration of 1 mg/mL (Celep et al., 2013). In another study, Serteser et al. (2009) indicated that for decrease the DPPH concentration at 50% its needed 0.7 mg of hydroalcoholic extract (50% methanol) of dried leaves. Moreover, this extract

presented high iron chelating activity and H_2O_2 inhibition activity with percentages of 45.7% and 65.4%, respectively. In conclusion, in this report, the application of conventional extractions (maceration and decoction) allows obtaining the extracts more active in the antioxidant assays compared with other extraction techniques, except for FRAP test. Leaves extracts were more active than fruits extracts. In addition, both fruits and leaves herein investigated showed a higher antioxidant activity than that previously studied. The different mechanisms of action of antioxidant assays and the variety of constituents present in the extracts may contribute to the variability in the antioxidant activity. Due to the circumstances above, it was necessary to get a trend in the data to facilitate the comparison of antioxidant activity between different methods.

For this reason, Global Antioxidant Score (GAS) and Relative Antioxidant Capacity Index (RACI) were used (Figures 9.6 and 9.7). Significant values were reported by hydroalcoholic maceration of dried leaves (TDB) with GAS and RACI values of 0.76 and −0.61, respectively. From total antioxidant evaluation, the most active extract was obtained from hydroalcoholic maceration of dried leaves. Among fresh leaves sample, extract obtained by decoction reported high antioxidant capacity (GAS values = 0.99 and RACI values = − 0.43).

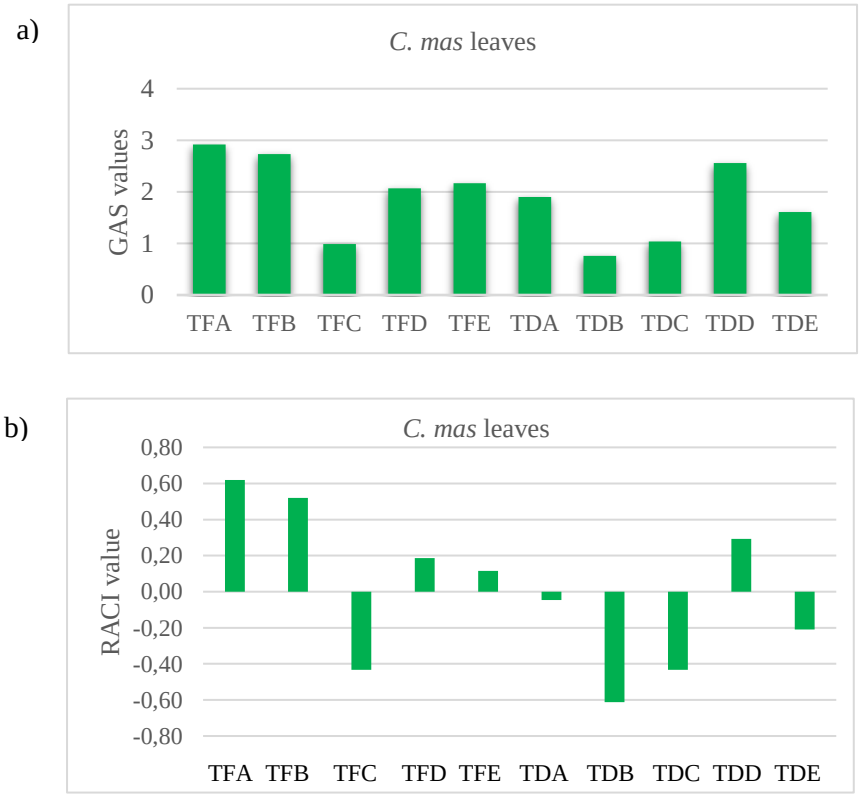


Figure 9.6. Evaluation of total antioxidant activity of leaves through GAS (a) and RACI (b). TF: Fresh leaves; TD: Dried leaves. A. Ethanolic maceration; B. Hydroalcoholic (60%) maceration; C. Decoction; D. Ethanol Soxhlet extraction; E. Ethanol ultrasound-assisted extraction.

Considering fresh fruits samples, MFD extract showed the best values with GAS and RACI values of 1.51 and −0.26 respectively. The hydroalcoholic maceration (MDB) among dried fruit showed high antioxidant activity (GAS values =1.2 and RACI values = −0.45).

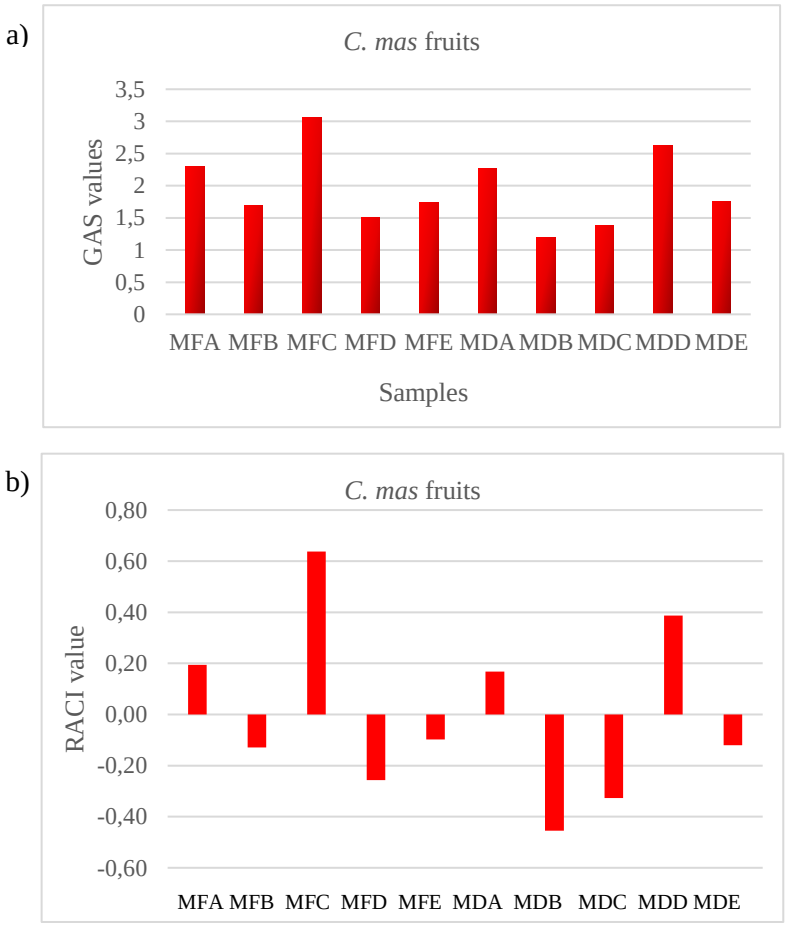


Figure 9.7. Evaluation of total antioxidant activity of fruits through GAS (a) and RACI (b). MF: Fresh fruits; MD: Dried fruits. A. Ethanolic maceration; B. Hydroalcoholic (60%) maceration; C. Decoction; D. Ethanol Soxhlet extraction; E. Ethanol ultrasound-assisted extraction.

A chemical comparison of the most active (TDB) and less active (TFA) extracts allowed us to draw some considerations on the founded biological activities. Some phenolic compounds, such as chlorogenic acid, ellagic acid, quinic acid, and quercetin characterised TDB. These compounds are described in literature as good antioxidant molecules (that exert many other pharmacological activities) and then could contribute to the promising activity described for TDB (Ríos et al., 2018; Sato et al., 2011). In addition, TDB exhibited greater enrichment in sweroside, cornuside, and quercitrin than TFA. Some constituents are identified only in TFA. These compounds are gallic acid 4-*O*-glucopyranoside, ethyl caffeate, 3-(3,4,5-trimethoxyphenyl)propanoic acid, and loganic acid. Instead, quercetin 3-*O*-xyloside and kaempferol 3-*O*-glucuronide are present in both extracts, but TFA has a higher concentration.

9.1.4. *In vitro* hypoglycaemic effects

α-Amylase and α-glucosidase inhibitors are drugs commonly used to reduce the post-prandial plasma levels of glucose in patients affected by diabetes type 2 and obesity. In this work, we have investigated *C. mas* extracts for their potential carbohydrates-hydrolysing enzymes inhibitory activities. A concentration-response relationship was observed for all studied samples. The IC_{50} values are summarised in Table 9.7.

Fruits are more active as α-glucosidase inhibitors than α-amylase inhibitors. Among these samples, MFC and MFD extracts obtained from fresh matrix exhibited the most promising activity against α-glucosidase with IC_{50} values of 16.60 and 30.40 μg/mL, respectively. Both samples are more active than the positive control acarbose (IC_{50} value of 35.50 μg/mL) and displayed a great selectivity towards α-glucosidase than α-amylase (SI of 15.44 and 11.54, respectively).

Table 9.7. Carbohydrates-hydrolysing enzymes inhibitory activity of *C. mas* fruits and leaves extracts

C. mas	IC_{50}, μg/mL		Selectivity Index (SI)	
	α-Amylase	α-Glucosidase	α-Amylase[a]	α-Glucosidase[b]
Fruits				
MFA	208.20 ± 2.58****	134.63 ± 5.56****	0.65	1.55
MFB	312.43 ± 3.16****	141.64 ± 6.32****	0.45	2.21
MFC	256.38 ± 2.84****	16.60 ± 0.03***	0.06	15.44
MFD	350.97 ± 3.65****	30.40 ± 1.56	0.09	11.54
MFE	694.31 ± 4.56****	165.29 ± 2.36****	0.24	4.20
MDA	359.73 ± 2.36****	174.92 ± 5.32****	0.49	2.06
MDB	255.50 ± 1.85****	312.43 ± 2.57****	1.22	0.82
MDC	387.76 ± 2.58****	142.51 ± 3.62****	0.37	2.72
MDD	404.40 ± 4.20****	54.52 ± 2.21***	0.13	7.42
MDE	953.57 ± 6.92****	190.69 ± 4.69****	0.20	5.00
Leaves				
TFA	142.51 ± 2.03****	192.44 ± 8.25****	1.35	0.74
TFB	348.34 ± 3.65****	162.66 ± 8.03****	0.47	2.14
TFC	244.11 ± 2.44****	189.81 ± 7.15****	0.78	1.29
TFD	167.04 ± 1.89****	64.37 ± 2.08****	0.38	2.59
TFE	189.81 ± 2.36****	362.36 ± 4.63****	1.91	0.52
TDA	901.90 ± 6.58****	32.21 ± 1.25**	0.04	28.00
TDB	349.22 ± 3.57****	16.60 ± 0.08***	0.05	21.03
TDC	917.66 ± 2.69****	152.15 ± 4.56****	0.17	6.03
TDD	167.91 ± 1.52****	329.07 ± 5.36****	1.96	0.51
TDE	214.33 ± 2.69****	181.05 ± 1.48****	0.84	1.18
Positive control				
Acarbose	50.01 ± 1.42	35.50 ± 1.12	0.71	1.41

MF: Fresh fruits; MD: Dried fruits; TF: Fresh leaves; TD: Dried leaves. A. Ethanolic maceration; B. Hydroalcoholic (60%) maceration; C. Decoction; D. Ethanol Soxhlet extraction; E. Ethanol ultrasound-assisted extraction. Data are expressed as means ± S.D. (n= 3). Acarbose used as positive control in α-amylase and α-glucosidase tests. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001, **p < 0.01, *p< 0.1 compared with the positive control. [a]SI for α-amylase is defined as IC_{50} (α-glucosidase)/IC_{50} (α-amylase). [b]SI for α-glucosidase is defined as IC_{50} (α-amylase)/IC_{50} (α-glucosidase).

The other extracts showed IC_{50} values in the range 134.63-165.29 μg/mL. The most active extract against α-glucosidase obtained from dried fruits is MDD (ethanol Soxhlet extraction) with an IC_{50} value of 54.52 μg/mL.

A lower activity was found against α-amylase (IC_{50} values in the range 208.20-694.31 μg/mL and 255.50-953.57 μg/mL for fresh and dried fruits, respectively). MFA and MDB were the most active.

The data obtained are interesting if compared with those in the literature. Indeed, Shishehbor et al. (2016) evaluated the hypoglycaemic activity of the hydroalcoholic extract obtained from the fresh fruits reporting IC_{50} values of 6.0 and 6.8 mg/mL respectively for α-amylase and α-glucosidase.

Recently, Świerczewska et al. (2019) evaluated the inhibitory activity of α-amylase of different extracts of *C. mas* dried fruits, reporting IC_{50} values of 134.0, 92.5 and 79.0 μg/mL for aqueous, ethanolic, and methanolic extracts, respectively. In these extracts the presence of flavonoids, anthocyanins and iridoids were confirmed by HPLC analyses. In addition, Świerczewska et al. (2019) have established that responsible to the inhibition of pancreatic enzymes were anthocyanins, followed by iridoids (cornuside and loganic acid). However, a potential synergistic activity of anthocyanins and iridoids in this inhibition was supposed.

In the present report, the hypoglycaemic effects of *C. mas* leaves extracts were evaluated. Leaves extracts inhibited α-amylase with IC_{50} values in the range from 142.51 to 348.34 μg/mL and from 167.91 to 917.66 μg/mL for fresh and dried materials, respectively. A great variability was evidenced against α-glucosidase.

A relevant activity was observed with ethanolic (TDA) and hydroalcoholic maceration (TDB) of dried leaves with IC_{50} values of 32.21 and 16.60 μg/mL, respectively. These extracts exhibited selectivity towards α-glucosidase than α-amylase with SI values of 28.0 and 21.03, respectively.

The differential α-amylase/α-glucosidase inhibitory activities of *C. mas* extracts is closely related to their chemical constituents. α-Amylase catalyses the hydrolysis of α-1,4-glucan bonds in starch and other oligosaccharides. In humans, the digestion of starch begins by the salivary amylase that degrades polymeric substrate into shorter oligomers. Subsequently, this digested material arrives in the gut where it is hydrolysed by the pancreatic α-amylase into smaller oligosaccharides and excreted in the lumen. These oligosaccharides reach the mucous layer of the brush border membrane, where intestinal α-glucosidases hydrolyse them to glucose that then enters the blood (Svensson, 1998).

Thus, this enzyme releases glucose from the non-reducing end of the substrate. Saqib (2008) studied the binding interactions between the competitive acarbose inhibitor and human α-glucosidase.

The active site of human α-glucosidase is represented by a pocket formed mainly by the residues Asp^{398}, Asp^{587}, His^{645} and Arg^{571}. Moreover, the residues Asp^{547}, Asp^{511}, Asp^{398}, Arg^{571}, and His^{645} are important for a strong hydrogen binding interaction.

Some phenolic acids and flavonoids characterised the most active extracts of *C. mas*.

The primary structure of phenols can change the inhibitory effect versus the α-amylase and α-glucosidase enzymes. In a recent work, Rasouli et al. (2017), using molecular docking and virtual screening studies, assessed the α-amylase and α-glucosidase inhibitory activity of 26 polyphenols. It was demonstrated that catechin, silibinin, kaempferol, hesperetin, and pelargonidin significantly inhibit α-amylase. Quercetin, caffeic acid, syringic acid, curcumin, pinoresinol, cyanidin, daidzein, epicatechin, ferulic acid, hesperetin, naringenin, and resveratrol are potent inhibitors of α-glucosidase. Molecular docking results showed that caffeic acid, eriodictyol, cyanidin, curcumin, hesperetin, pinoresinol, resveratrol, epicatechin, and quercetin could directly interact with the Arg^{407} and Arg^{411} residues.

The inhibitory effects of phenolic compounds against α-amylase and α-glucosidase are closely related to their chemical structure. Various factors influence the activity against carbohydrates-hydrolysing enzymes as the presence of the catechol group, methylation in position 4'-OH or 7-OH on B-ring, such as the presence of C2=C3 double bond in C-ring and its planarity, methoxylation, and glycosylation. Moreover, the presence of OH in the position 7 of ring A, 3 of ring C and 4′ and 5′ of ring B play an important role in the inhibitor effects on digestive enzymes (Rasouli et al., 2017).

Methylation and methoxylation of flavonoids, as glycosylation and hydrogenation reduce the inhibitory effects of α-amylase (Lo Piparo et al., 2008). The hydrogenation of the double bond C2=C3 decreases the binding affinity of α-amylase (Figure 9.8) (Tadera et al., 2006).

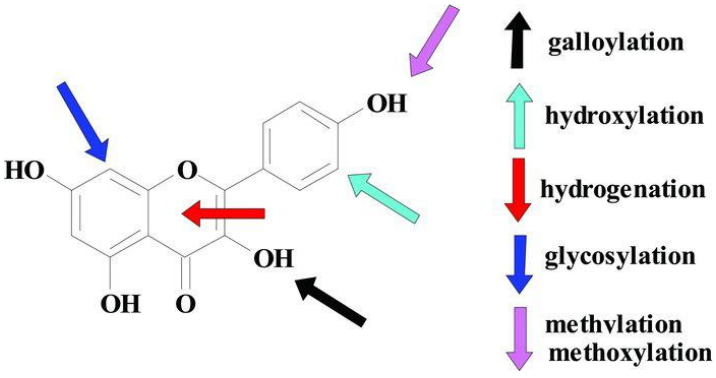


Figure 9.8. The potential sites of flavonoids affecting the inhibitory effect against α-amylase (adapted by Xiao et al., 2013a).

The glycosylation of flavonoids reduce the inhibitory effect on α-amylase in function of the conjugation site and the class of sugar moiety. Probably, the decreasing inhibitory effect on amylase after glycosylation may be due to molecular size increasing and

polarity, and transformation to a nonplanar structure. After glycosylation, it is possible to note weak binding interaction between flavonoids and amylase caused by steric hindrance (Xiao et al., 2013a). The monoglycosides of quercetin are stronger than the polyglycoside form (rutin) as α-amylase inhibitors (Kim et al., 2000). The hydroxyl group plays a very important role in amylase inhibition; in fact, the hydroxylation of flavonoids improved the inhibitory effect on α-amylase (Xiao et al., 2013a). The enzyme α-amylase presents three structural domains: A, B and C. Domain A is the largest domain that houses active site. The active site has three residues: Asp^{197}, Glu^{233}, and Asp^{300}. Few polyphenols showed a high interaction with α-amylase (Rasouli et al., 2017). Among these, kaempferol presents a great interaction potential with a α-amylase enzyme active site, while quercetin and its derivatives do not report direct interaction with residue of active site (Kim et al., 2000). In the study of Lo Piparo et al., (2008) were demonstrated that the inhibitor effects against α-amylase enzyme of phenolic acids (as chlorogenic acid) were associated to the hydrogen bonds formation between the hydroxyl groups of phenolic acids and the binding site of the enzyme. From molecular docking resulted that chlorogenic acid formed hydrogen bonds with His^{305}, Glu^{233}, Asp^{197} and Asp^{300} residues of α-amylase (Xie et al., 2019, Zhang et al., 2018). Other study reported other site of interaction of chlorogenic acid with α-amylase involving hydrogen bonds with residues Lys^{200}, Glu^{240}, Gly^{306}, and Gly^{308} (Pérez-Nájera et al. 2018). Quinic acid showed less activity against α-amylase compared with chlorogenic acid and caffeic acid (Narita et al., 2009). A recent study (Wu et al., 2019) reported the molecular docking analysis for gallic acid and ellagic acid. Main interactions of gallic acid with α-amylase involved conventional hydrogen bonds with residues of Asp^{197}, Arg^{195}, Glu^{233}, His^{299}, and His^{305}, respectively. Ellagic acid formed H-bonds with four amino acid residues with the active site, namely Asp^{197}, Asp^{300}, Arg^{195}, and His^{299}.

The enzymes inhibitory effects of polyphenols were associated with their structure (Figure 9.9). The hydroxylation on 3', 4'-dihydroxyl groups of B ring and 3-OH of C ring of flavonoids improved the inhibition of α-glucosidase, while other hydroxylation on different sites reduce the activity of inhibition. Glycosylation and methylation reduce the effects of flavonoids inhibition on α-glucosidase, as observed for α-amylase (Xiao et al., 2013b). Molecular docking analyses evidenced that polyphenols present a good interaction with α-glucosidase receptor. The biochemical activity of α-glucosidase is strongly associated with the Arg^{407} residue. Other important residues are Asp^{326}, Arg^{197}, Asn^{258}, Asp^{382}, Arg^{411} and Phe^{282} (Jhong et al., 2015).

Quercetin showed high binding affinities for interaction with the α-glucosidase active site; in fact, it could directly interact with the Arg^{407} and Arg^{411} residues (Rasouli et al., 2017). Molecular docking studies of gallic acid on α-glucosidase underline an interaction due to hydrogen bonds (H-bond) with five amino acid residues (Asp^{69}, Arg^{213}, Asp^{215}, Asp^{352}, and His^{351}).

Ellagic acid formed H-bonds with four amino acid residues of active site, namely Asp[197], Asp[300], Arg[195], and His[299] (Wu et al., 2019). Chlorogenic acid formed hydrogen bonds with the residues Ser[161], Phe[165], and Lys[418], and a π-π T-shaped interaction between A ring and Phe[172] was found (Pérez-Nájera et al., 2018). At α-glucosidase binding site, rutin formed five H-bond with the amino acid residues Asp[242], Ser[311], Leu[313], Gln[279] and Glu[411] (Limanto et al., 2019).

Moreover, there is a correlation between molecular weight of compounds and their docking to enzymes active site, in fact polyphenols with medium molecular weight and high solubility in water are very important and could be considered for future development of new drugs based on polyphenols. Polyphenols with a high molecular weight or less water solubility results in poor inhibitory activity against both enzymes.

Another factor linked to molecular weight that influence the docking capacity is the degree of hydroxylation, especially for α-amylase.

In conclusion, polyphenols can decrease or inhibit the α-glucosidase and α-amylase enzyme activity through this type of bind in the active site. Potent inhibitors of both enzymes are polyphenols, while glycosylated phenols are very feeble inhibitors or present not inhibition. So, these compounds or their derivatives could be used as a new class of anti-diabetic drugs.

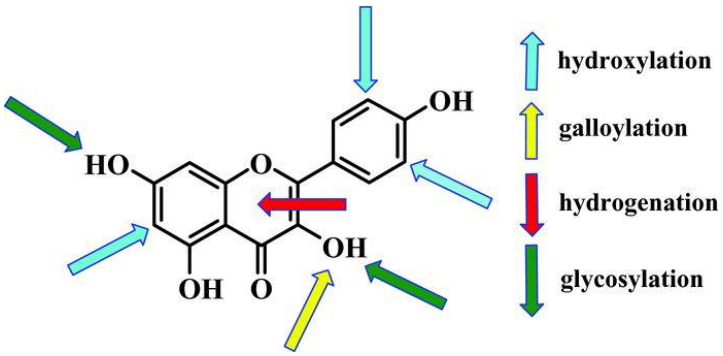


Figure 9.9. The potential sites of flavonoids affecting the inhibitory effect against α-glucosidase (adapted by Xiao et al., 2013b).

The obtained results are of interest from two points of view: a) excessive α-amylase inhibition can give discomfort because a possible accumulation of undigested starch in the colon with stomach distension as result (Puls et al., 1977). Therefore, the extracts that present high α-glucosidase and moderate inhibition can will be considerate election products for treatment of type 2 diabetes; b) this is the first study that investigated the hypoglycaemic potential of *C. mas* leaves.

In our study, a selective activity against α-glucosidase was found; in particular, ethanol and hydroalcoholic maceration of dried leaves and decoction of fresh and dried fruits showed a greater inhibition of α-glucosidase and a moderate inhibition of α-amylase. Taking into account that cornelian cherry leaves are a very rich source of

bioactive compounds and that were considered as agrochemical waste, it is possible to prospect an innovative valorisation of these by-products as nutraceutical-pharmaceutical agents. The only one work present in literature investigated the hypoglycaemic activity of leaves aqueous extracts as aldose reductase inhibitor, reporting an IC_{50} value of 1.30 μg/mL (Miláčková et al. 2017).

9.1.5. Inhibition of NO production in HFF1 cells

Nitric oxide (NO) is a signalling molecule that possesses a crucial role in the pathogenesis of inflammatory disorders. Under normal physiological conditions, NO gives an anti-inflammatory effect. Instead, it is considered as a pro-inflammatory mediator that induces inflammation due to over production in abnormal conditions.

Therefore, NO inhibitors represent important therapeutic advance in the management of inflammatory diseases. *C. mas* extracts showed NO inhibitory activity in a concentration-dependent manner. IC_{50} values are summarised in Table 9.8.

Table 9.8. Inhibition of nitrate/nitrite (IC_{50} μg/mL) of *C. mas* fruits and leaves extracts

C. mas	IC_{50} (μg/mL)
Fruits	
MFA	30.12 ± 2.26
MFB	32.31 ± 2.33
MFC	29.85 ± 1.73
MFD	25.45 ± 2.56
MFE	32.14 ± 1.83
MDA	28.76 ± 2.54
MDB	31.71 ± 1.93
MDC	33.36 ± 1.12
MDD	33.81 ± 1.45
MDE	26.73 ± 1.01
Leaves	
TFA	21.31 ± 1.43
TFB	26.81 ± 2.31
TFC	34.93 ± 2.55
TFD	35.51 ± 2.12
TFE	42.12 ± 1.83
TDA	41.44 ± 1.73
TDB	39.61 ± 2.08
TDC	33.18 ± 1.12
TDD	34.14 ± 2.96
TDE	31.21 ± 0.82

MF: Fresh fruits; MD: Dried fruits; TF: Fresh leaves; TD: Dried leave. A. Ethanolic maceration; B. Hydroalcoholic (60%) maceration; C. Decoction; D. Ethanol Soxhlet extraction; E. Ethanol ultrasound-assisted extraction. Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001 compared with the negative control (0 μg/mL).

No significant difference was reported within leaves and fruits extracts. Among fruits samples, the most active are MFD and MDE with IC_{50} values of 25.45 and 26.73 μg/mL,

respectively. Among leaves extracts, the most active was the ethanol maceration of fresh matrix (TFA) (IC_{50} value of 21.31 μg/mL).

C. mas fruits and leaves extracts showed a cytotoxic effect in a concentration dependent manner, decreasing cell viability of HFF1 cells dependently of the extraction technique used, in particular for ethanol maceration of dried fruits (MDA), decoction of fresh fruits (MFC), ethanol (TFA) and hydroalcoholic maceration (TFB) of fresh leaves and ethanol ultrasound-assisted extraction of dried leaves (TDE). Other extracts showed a cytotoxicity at high concentration of 250 μg/mL (Figures 9.10 and 9.11). In conclusion, both fruits and leaves extracts inhibited the NO production with IC_{50} values ranged from 21.31 to 42.12 μg/mL and showed a low cytotoxic effect at high concentration.

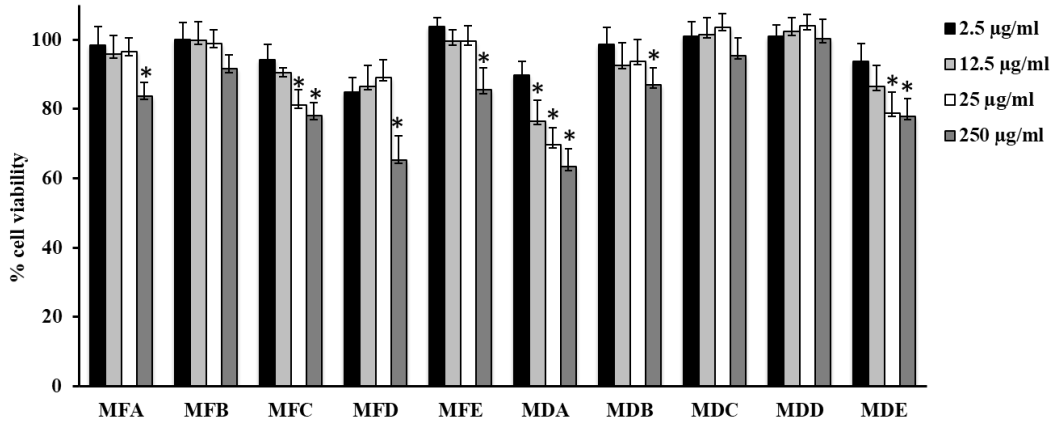


Figure 9.10. Cell viability of HFF1 cells untreated and treated for 24 h with fruits extracts of *C. mas* at different concentrations (12.5-250 μg/mL) evaluated by MTT assay. Values are the mean ± S.D. of four experiments in triplicate. Control cells were incubated only with medium and considered as 100% of cell viability. *Significant *vs* untreated control cells and *vs* other concentrations of the same extract $p< 0.001$.

Previously, leaves of *C. mas* showed significant inhibitory activities against aldose reductase. Over-expression of this enzyme was related to different pathological states, such as inflammation, diabetes, and cancers.

Miláčková et al. (2017) described an aldose reductase inhibitory activity by the aqueous leaves extract of *C. mas* at the concentration of 1 μg/mL. Another study demonstrated that the administration of lyophilized *C. mas* fruits (100 mg/kg), mainly characterised by the presence of anthocyanins and iridoids, had a protective effect on pro-inflammatory cytokines, reducing the concentrations of TNF-α and IL-6 in hypercholesteraemic rabbits (Sozanski et al., 2014).

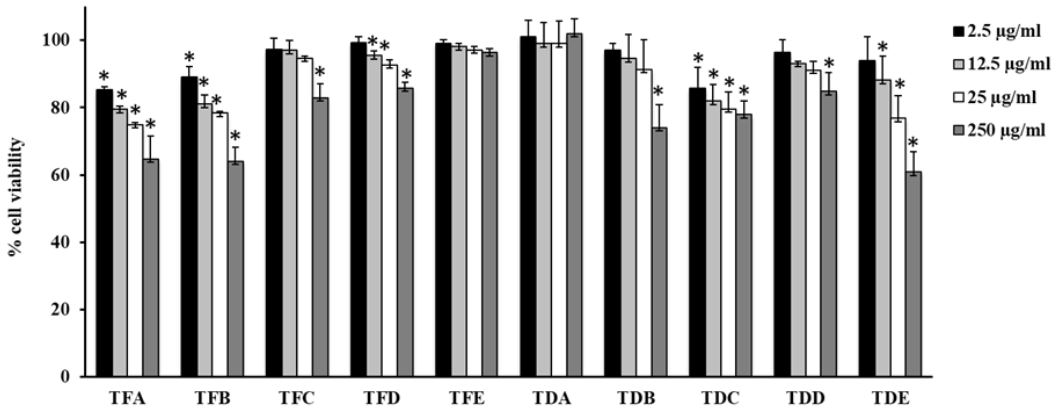


Figure 9.11. Cell viability of HFF1 cells untreated and treated for 24 h with leaves extracts of *C.mas* at different concentrations (12.5-250 μg/mL) evaluated by MTT assay. Values are the mean ± S.D. of four experiments in triplicate. Control cells were incubated only with medium and considered as 100% of cell viability. *Significant *vs* untreated control cells and *vs* other concentrations of the same extract $p < 0.001$.

The administration of 50 g of *C. mas* fruits (for 6 weeks, twice a day after lunch and dinner) showed decreased levels of biomarkers of atherosclerosis (vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1)) in a study conducted on dyslipidemic childrens by Asgary et al. (2013). Overall, these results confirmed that *C. mas* extracts possess significant anti-inflammatory effects. These activities may be of interest from a functional point of view and for the further valorisation of this species that represent an ingredient of the Mediterranean diet.

9.1.6. Correlation between bioactivity and phytochemicals

Pearson's correlation coefficient (r) was applied to evaluate the correlation between the founded bioactivities and the content of phenols, flavonoids, and iridoids. The following matrices were taken into consideration: a) fresh fruits, b) dried fruits, c) fresh leaves, and d) dried leaves. The antioxidant activity of fresh fruits, evaluated by using the β-carotene bleaching test after 30 min incubation, positively correlates with the total phenols content with r value of 0.82. Significant correlations were found also between the flavonoids content and the β-carotene bleaching test after 30 and 60 min incubation with values of $r = 0.79$ and 1.0, respectively.

In addition, a positive correlation results between the total iridoids content and ABTS test ($r = 0.79$) and FRAP test ($r = 0.59$). Regarding dried fruits, polyphenols and flavonoids content was positively correlated with DPPH assay with values of $r = 0.58$ and 0.63, respectively. Moreover, positive correlation was given by the iridoids content and FRAP ($r = 0.54$) and with β-carotene after 60 min incubation ($r = 0.86$). TPC of fresh leaves correlated with β-carotene bleaching test at 30 min of incubation ($r = 0.78$ for TPC).

A *r* value of 0.51 was found for TIC of fresh leaves and DPPH assay. Analysing results obtained with dried leaves, the best correlations were found between TPC and β-carotene bleaching test after 30 and 60 min incubation with *r* values of 0.91 and 0.86, respectively. The same trend was observed between TFC and β-carotene bleaching test after 30 and 60 min of incubation with *r* values of 0.71 and 0.90, respectively.

A positive correlation was found also between the iridoids content and DPPH (r = 0.55). For the hypoglycaemic activity, TIC in the fresh fruits presented a good correlation with α-glucosidase and α-amylase with values of r = 0.70 and 0.94, respectively. There are the positive correlation between α-glucosidase and TPC and TFC with values of r = 0.63 and 0.52, respectively for dried leaves. These correlations demonstrated that flavonoids are responsible to inhibition of lipid peroxidation, reported in fresh fruits and dried leaves.

9.1.7. Selective separation of *C. mas* flavonoids and iridoids-rich fractions

The chemical analysis and the *in vitro* biological investigation of *C. mas* extracts, previously described, allowed us to select some extracts to be subject to bio-fractionation in order to identify bioactive compounds-enriched fractions as potential pharmaceutical/nutraceutical products for the treatment of diseases with a high social impact such as diabetes and closely correlated with oxidative stress.

In the analysis of bioactive compounds (flavonoids and iridoids) from plant-derived foods, a major problem arises owing to the content of sugars and other polar compounds that determine a not well defined separation when are extracted with organic solvents (such as ethanol). The same problem arises with plant extracts that are rich in polar constituents. In recent years, several non-ionic polymeric resins (Amberlite XAD) have been used for the recovery of flavonoids from plant extracts with the elimination of the water-soluble contaminants. Resins can be polar or non-polar polymers with different surface abilities, high absorption velocity, and mechanic strength. Moreover, resins have easy to use and regenerate, present low cost, high efficiency, but factor more important is adapt to industrial scale-up.

Some of these resins are approved of the U.S. Food and Drug Administration, thus extracted compounds obtained could be used as human food supplements. Resins has been applied for the recovery of different phenolic compounds as flavonoids from mulberry leaves, polyphenols from apple juice, hesperidin from *Citrus* peels (Kammerer et al., 2007; 2010; Saleh et al., 2008; Scordino et al., 2003; Wang et al., 2008). HLB is a co-polymeric resin, which provides both lipophilic and hydrophilic retention characteristics.

Herein, we investigated the adsorption/desorption behaviours of polyphenols and iridoids from the more active extracts on different Amberlite resins with the aim to select the resin that allows obtaining the maximum recovery of bioactive compounds.

Two extracts, such as hydroalcoholic maceration of dried fruits (MDB) and dried leaves (TDB) were used. Firstly, TDB was subjected to separation by using HLB, XAD-

4, and XAD-16 (Figure 9.12). The procedure that allowed total recovery of extract was XAD-16 (4.15 g), while with HLB and XAD-4 there is not complete recover of sample, an important loss of ~50% was attributed to HLB use, as reported in Table 9.9.

Table 9.9. TDB: Difference of yield with various separation techniques

	Matrix	H_2O	70% EtOH[a] 80% EtOH[b]	100% EtOH	Recovery
HLB	40 mg	9.1 mg	9.8 mg	4.4 mg	23.3 mg
XAD-4	200 mg	122 mg	43.7 mg	7.3 mg	173 mg
XAD-16	4.15 g	2.5 g	1.15 g	494 mg	4.14 g

[a] for HLB and XAD-4; [b] for XAD-16.

After separation on resins, fractions were analysed by LC-MS. Chromatograms are inserted in Appendix (Figures A14-A19). LC-MS analyses evidenced some difference between the obtained fractions. In particular, HLB not retain all bioactive compounds present in the extract (Appendix, Figure A14). H_2O fraction of HLB was characterised by presence of gallic acid, gemin D, quinic acid hydroxybenzoic acid isomers, benzoyl quinic acid, rutin, and quercetin 3-*O*-glucuronide. Phenolic acids (gallic and ellagic acids), iridoids (sweroside, secologanin, and cornuside), tannin (gemin D) and flavonoids were found in the 70% EtOH fraction of HLB. 100% EtOH has allow the separation of ellagic acid and quercetin.

The use of XAD-4 not resulted more efficient compared with HLB, because compounds such as acids (quinic acid hydroxybenzoic acid isomers, benzoyl quinic acid) and phenolic acids (gallic and ellagic acids) were found in H_2O, 70% and 100% EtOH fractions. In the latter fractions, also iridoids and flavonoids were present thus a clear separation between bioactive compounds there is not obtained (Appendix, Figure A15).

Increasing of particles size and surface area ensured better separation with use of XAD-16. This resin was resulted more selective for separation of interest compounds (flavonoids and iridoids) of this study from all compounds present in the matrix.

Impurities and compounds not retained were washed with distilled water, iridoids were eluted with 80% EtOH, while flavonoids were eluted with 80% and 100% EtOH. Thus, three fractions, TDB (I), TDB (II), and TDB (III), were obtained.

Data are summarised in Table 9.10, and 9.11. Only acids and phenolic acids (particularly gallic acid and ellagic acid) were found in the aqueous fraction (TDB I).

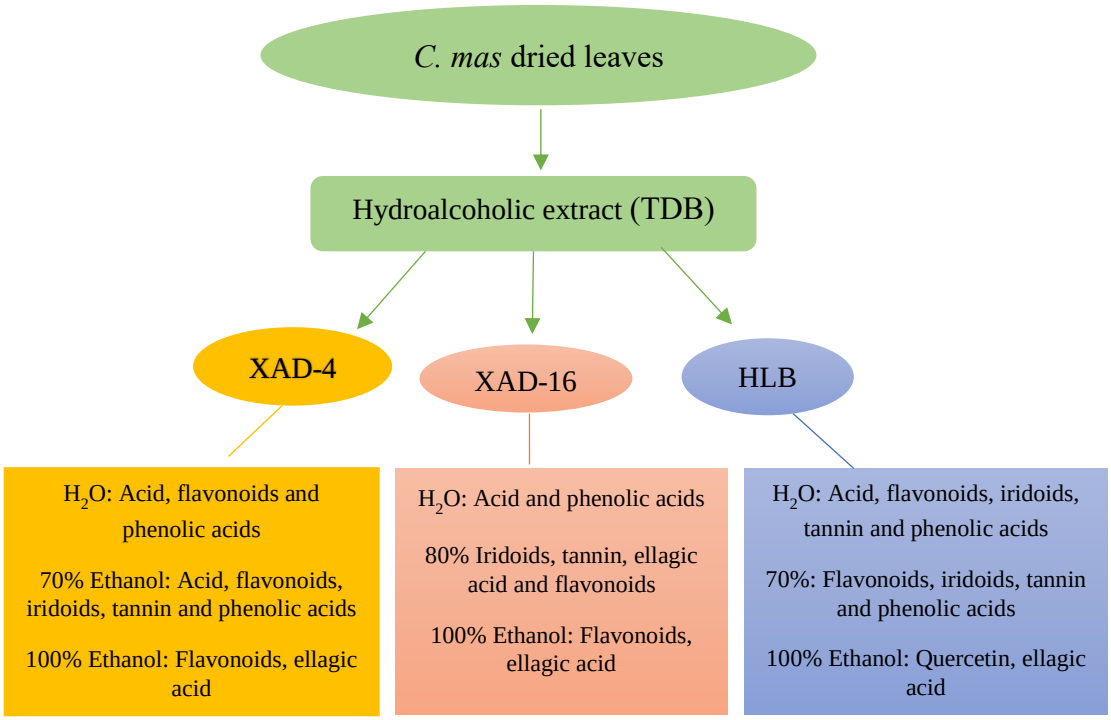


Figure 9.12. The main characteristics of the different separation techniques.

Fraction TDB (II) was characterised by the presence of iridoids, flavonoids, ellagic acid, and gemin D. Iridoids were separated selectively in this fraction. In fact, fraction 100% EtOH (TDB III) showed the presence of flavonoids and ellagic acid.

The use of XAD-16 resin was more specific compared with other separation techniques, because was able to remove all impurities and phenolic acids except ellagic acid and retains iridoids and flavonoids.

Mainly, fractions TDB (II) and TDB (III) were characterised by presence of ellagic acid, followed by kaempferol 3-*O*-rutinoside, quercetin 3-*O*-glucuronide and rutin. In the fraction TDB (II) main iridoids were cornuside and sweroside.

Table 9.10. Chemical compounds of TDB presents in HLB, XAD-4 and XAD-16 fractions

		HLB FRACTIONS			XAD-4 FRACTIONS			XAD-16 FRACTIONS		
Tr (min)	Compounds	H_2O	70% EtOH	100% EtOH	H_2O	70% EtOH	100% EtOH	H_2O	80% EtOH	100% EtOH
2.13	Gallic acid	√	√		√	√		√		
3.20	Gemin D	√	√			√			√	
6.75	Quinic acid hydroxy benzoic acid isomer	√			√	√		√		
7.20	Sweroside		√			√			√	
7.83	Secologanin		√			√			√	
9.14	Quinic acid hydroxy benzoic acid isomer	√			√	√		√		
11.94	Benzoyl quinic acid	√			√	√		√		
15.21	Kaempferol 3-*O*-rutinoside isomer					√			√	√
16.71	Kaempferol 3-*O*-rutinoside isomer					√			√	√
17.58	Cornuside		√			√			√	
18.27	Quercetin 3-*O*-xyloside		√			√			√	√
19.05	Quercitrin		√			√			√	√
19.50	Ellagic acid		√	√	√	√	√	√	√	√
19.86	Rutin	√	√			√	√		√	√
20.15	Ellagic acid 4-*O*-rutinoside		√			√			√	
20.42	Hyperoside		√			√	√		√	√
20.65	Isoquercetin		√			√	√		√	√
21.69	Kaempferol 3-*O*-glucuronide		√			√	√		√	√
21.84	Quercetin 3-*O*-glucuronide	√	√		√	√	√		√	√
26.60	Quercetin			√			√			√

Table 9.11. Chemical compounds identified in TDB fractions by LC-MS.

Compounds	Rt (min)	Molecular Formula	MM	UV λ (nm)	TDB (I)	TDB (II)	TDB (III)	Reference
Gallic acid	2.13	$C_7H_6O_5$	170	216, 270	√			Deng et al., 2013
Gemin D	3.20	$C_{27}H_{22}O_{18}$	634	221, 265		√		Hatano et al., 1989
Sweroside	7.20	$C_{16}H_{22}O_9$	358	245		√		Deng et al., 2013
Secologanin	7.83	$C_{17}H_{20}O_{10}$	388	245		√		Deng et al., 2013
Kaempferol 3-*O*-rutinoside isomer	15.21	$C_{27}H_{30}O_{15}$	594	210, 265, 342		√	√	Li et al., 2014
Kaempferol 3-*O*-rutinoside isomer	16.71	$C_{27}H_{30}O_{15}$	594	210, 265, 342		√	√	Li et al., 2014
Cornuside	17.58	$C_{24}H_{30}O_{14}$	542	218, 273		√		Szumny et al., 2015
Quercetin 3-*O*-xyloside	18.27	$C_{20}H_{18}O_{11}$	434	212, 254, 356		√	√	Pawloska et al., 2010
Quercitrin	19.05	$C_{21}H_{20}O_{11}$	448	213, 254, 356		√	√	Pawloska et al., 2010
Ellagic acid	19.50	$C_{14}H_6O_8$	302	255, 365	√	√	√	Deng et al., 2013
Rutin	19.86	$C_{27}H_{30}O_{16}$	610	213, 253, 352		√	√	Pawloska et al., 2010
Ellagic acid 4-*O*-rutinoside	20.15	$C_{26}H_{26}O_{17}$	610	258, 314		√		Malhotra et al., 1981
Hyperoside(*)	20.42	$C_{21}H_{20}O_{12}$	464	213, 278, 350		√	√	Pawloska et al., 2010
Isoquercetin(*)	20.65	$C_{21}H_{20}O_{12}$	464	213, 253, 353		√	√	Pawloska et al., 2010
Kaempferol 3-*O*-glucuronide(*)	21.69	$C_{21}H_{20}O_{12}$	464	219, 278, 350		√	√	Badalica-Petrescu et al., 2014
Quercetin 3-*O*-glucuronide	21.84	$C_{21}H_{18}O_{13}$	478	205, 258, 356		√	√	Pawloska et al., 2010
Quercetin	26.60	$C_{15}H_{10}O_7$	302	203, 257, 375			√	Sochor et al., 2014

TDB: hydroalcoholic maceration of dried leaves; (I):H2O fraction; (II): 80% Ethanol fraction; (III): 100% Ethanol fraction. (*) interchangeable

Based on the results obtained with TDB extract, MDB extract was subjected only to separation with XAD-16 resin. Three fractions, MDB (I), MDB (II), and MDB (III), were obtained. MDB (I) was characterised by the presence of gallic acid, 1-*O*-galloyl-D-sedoheptulose, cornoside, and loganic acid.

Table 9.12. Chemical compounds identified in MDB fractions by LC-MS.

Compounds	Rt (min)	Molecular Formula	MM	UV λ (nm)	MDB (I)	MDB (II)	MDB (III)	Reference
Unknown	0.7		218	228	√			
Unknown	1.3		308	200-260	√			
Cornoside	1.9	$C_{14}H_{20}O_8$	316	215, 314	√			Nenadis et al., 2005
Unknown	2.1		206	218	√			
Unknown	2.15		320	213		√	√	
Unknown	2.76		332	217-287	√			
Gallic acid	3.7	$C_7H_6O_5$	170	216, 270	√	√		Deng et al., 2013
1-*O*-galloyl-*D*-sedoheptulose	4.7	$C_{14}H_{18}O_{11}$	362	216, 275	√			Lee et al., 1989
Unknown	6.7		300	205, 262, 300	√	√		
Loganic acid	9.2	$C_{16}H_{24}O_{10}$	376	238	√	√	√	Szumny et al., 2015
Cornuside	21	$C_{24}H_{30}O_{14}$	542	220, 277		√	√	Szumny et al., 2015

MDB: hydroalcoholic maceration of dried fruits; (I):H_2O fraction; (II): 80% Ethanol fraction; (III): 100% Ethanol fraction.

MDB (II) is mainly constituted by one phenolic acid (gallic acid) and two iridoids such as loganic acid and cornuside. The last iridoids are found also in MDB (III). Some unknown compounds are present in both fractions.

In conclusion, TDB (II) and TDB (III) from hydroalcoholic maceration of dried leaves, and MDB (II) and MDB (III) from hydroalcoholic maceration of dried fruits, characterised by the presence of iridoids and flavonoids, were subjected to biological evaluation in order to evaluate their contribution to the antioxidant, hypoglycaemic and anti-inflammatory activities of *C. mas*.

Antioxidant activity

The results of the antioxidant studies are summarised in the Table 9.13. Leaves fractions exerted the most promising antioxidant effects in all tests.

Table 9.13. Antioxidant activities of *C. mas* fractions

C. mas	ABTS test	DPPH test	FRAP test	β-Carotene bleaching test	
	(IC_{50} μg/mL)	(IC_{50} μg/mL)	(μM Fe(II)/g)	(IC_{50} μg/mL)	
				30 min	60 min
Leaves fractions					
TDB (II)	0.19 ± 0.01	7.94 ± 0.57	102.53 ± 1.21****	12.07 ± 0.74***	3.22 ± 0.04
TDB (III)	0.30 ± 0.03	16.93 ± 0.61***	101.51 ± 1.08****	10.94 ± 0.47***	4.67 ± 0.06
Fruits fractions					
MDB (II)	0.37 ± 0.07	41.57 ± 0.24****	34.10 ± 0.65****	31.58 ± 0.14****	19.38 ± 0.87****
MDB (III)	0.50 ± 0.04	47.53 ± 0.12****	26.03 ± 0.89****	58.14 ± 0.74****	69.31 ± 0.67****
Positive control					
Ascorbic acid	1.72 ± 0.22	5.01 ± 0.83			
BHT			63.21 ± 4.30		
Propyl gallate				1.01 ± 0.01	1.02 ± 0.01

Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001,**p < 0.01, *p< 0.1 compared with the positive controls.

Leaves fractions resulted more active in the ABTS test than in DPPH assay as show in the Figure 9.13. The leaves fraction characterised by the presence of both iridoids and flavonoids (TDB II) (IC_{50} values of 0.19 and 7.94 μg/mL for ABTS and DPPH assays, respectively) resulted more active than TDB (III) in which iridoids were not identified (IC_{50} values of 0.30 and 16.93 μg/mL for ABTS and DPPH tests, respectively).

TDB (II) showed the highest protection of lipid peroxidation with an IC_{50} value of 3.22 μg/mL after 60 min of incubation followed by TDB (III) with an IC_{50} value of 4.67 μg/mL after 60 min of incubation.

FRAP test demonstrated that both leaves fractions are very active as antioxidants.

Values of 102.5 and 101.5 μM Fe(II)/g for TDB (II) and TDB (III), respectively, better than the positive control (BHT, 63.2 μM Fe(II)/g) were found.

An interesting ABTS radical scavenging activity was reported for MDB (II) (IC_{50} value of 0.37 μg/mL), followed by MDB (III), with an IC_{50} value of 0.50 μg/mL.

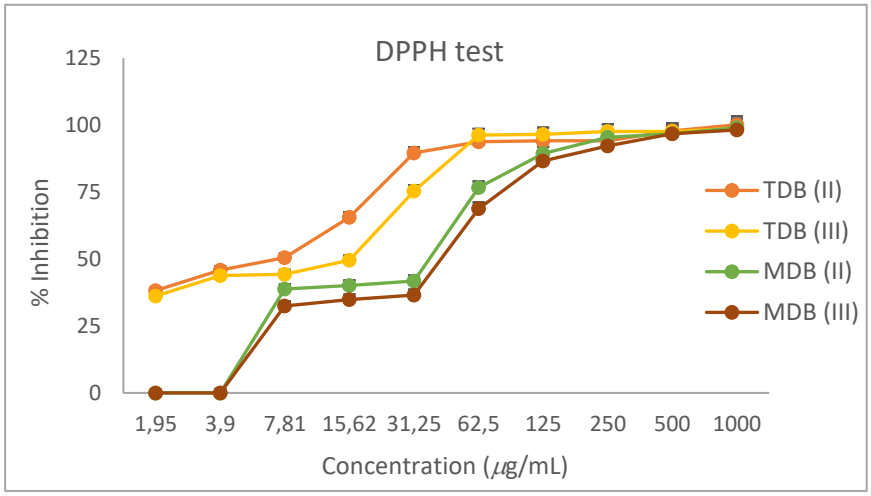


Figure 9.13. DPPH free radical-scavenging activity of fractions TDB (II), TDB (III), MDB (II), and MDB (III) of *C. mas*. Data are mean ± SD (n = 3).

MDB (II) resulted more active than MDB (III) also in the inhibition of lipid peroxidation with IC_{50} values of 31.58 and 19.38 μg/mL after 30 and 60 min of incubation, respectively. Interesting were the data after 60 min of incubation that present major inhibition, if compared after 30 min of incubation.

FRAP test is based to presence of compounds that have the donating a hydrogen atom abilities for break the free radical chain. Fruits fractions reported low FRAP values of 34.10 μM Fe(II)/g and 26.03 μM Fe(II)/g, respectively for MDB (II) and MDB (III). LC-MS analysis of the most active fraction, namely TDB (II) fraction, revealed the presence of both flavonoids and iridoids as dominant compounds.

In conclusion, leaves fractions were more active than fruits fractions. Flavonoids-iridoids-rich fractions, in particular, resulted more active than only flavonoids-rich fractions in all antioxidant assays.

Table 9.14 reports the antioxidant activity of selected compounds that represent the main iridoids and phenolic acid of our fractions, such as cornuside, sweroside, and ellagic acid.

Table 9.14. Antioxidant activities of cornuside, sweroside and ellagic acid.

Standards	ABTS test	DPPH test	FRAP test[#]	β-Carotene bleaching test	
	(IC_{50} μM)	(IC_{50} μM)	(μM Fe(II)/g)	(IC_{50} μM)	
				30 min	60 min
Cornuside	5.62 ± 0.91**	9.16 ± 0.98****	103.04 ± 1.34****	38.34 ± 1.68****	32.40 ± 1.71****
Sweroside	21.06 ± 1.25****	NA	NA	NA	NA
Ellagic acid	10.12 ± 1.02	21.31 ± 1.81**	102.73 ± 1.71****	8.40 ± 0.85*	6.78 ± 0.67
Positive control					
Ascorbic acid	9.65 ± 1.22	28.39 ± 2.83			
BHT			63.20 ± 4.32		
Propyl gallate				4.71 ± 1.42	4.71 ± 1.36

Data are expressed as means ± S.D. (n= 3). NA: not active. [#] at the concentration of 1 mg/mL Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001,**p < 0.01, *p< 0.1 compared with the positive controls.

Cornuside was the most active as radicals scavenging agent, with IC_{50} values of 5.62 and 9.16 μM in ABTS and DPPH test, respectively. The iridoid exerted a major activity than the positive control ascorbic acid in both tests. Sweroside is about four-times less active than cornuside (IC_{50} value of 21.06 μM) in ABTS. In DPPH test, no activity was evidenced at the maximum concentration tested.

The different radicals scavenging activity of the two iridoids was related to the presence of some hydroxyl groups in the chemical structure of cornuside.

Cornuside showed a strong activity also in FRAP test while sweroside is not active. Cornuside in this assay was two-times active than BHT with values of 103.04 *vs* 63.20 μM Fe(II)/g. A comparable activity was reported for ellagic acid such as 102.73 μM Fe(II)/g. Ellagic acid was the most active in the protection of lipid peroxidation with IC_{50} values of 8.40 and 6.78 μM after 30 and 60 min of incubation, respectively. Cornuside showed IC_{50} values of 38.34 and 32.40 μM after 30 and 60 min of incubation, respectively.

In agreement with our results, Wei et al. (2012) evaluated the antioxidant effects of secoiridoids from the roots of *Gentiana straminea* indicated none antioxidant activity of sweroside. Recently, Bozunovic et al. (2018) reported an ABTS and DPPH radicals scavenging activity for sweroside with values of 8.7 mM gallic acid/100 mg fresh plant and 0.7 mM gallic acid/100mg fresh plant. No reports were present in the literature on the radicals scavenger activity of cornuside.

As reported in Table 9.15, other identified compounds in the fractions resulted less active, such as rutin (81.99%) and kaempferol 3-*O*-rutinoside (>100) resulted less active (Loizzo et al., 2016; Plumb et al., 1999).

Table 9.15. Antioxidant activities (IC_{50} μM) of identified compounds from literature.

Compounds	ABTS test	DPPH test	FRAP test	β-Carotene bleaching test	Reference
Rutin	2.83	10.3	21.9	81.99%[a]	Loizzo et al., 2016
Quercetin 3-*O*-glucuronide	115.9	271.2	-	5.1	Chen et al., 2014; Plumb et al., 1999
Kaempferol 3-*O*-rutinoside	> 1[b]	> 100	-	> 100	Plumb et al., 1999; Wang et al., 2018

[a]:at 100 μg/mL. [b]:mM.

In a previous study, ellagic acid inhibited the lipid peroxidation with IC_{50} value of 20 μM (Osawa et al., 1987). These results suggested that ellagic acid was a good lipophilic antioxidant. It demonstrated a major solubility in organic solvents that in water. For this reason, together with high radicals scavenging activity it is a possible candidate this compound for chain-breaking antioxidant, because it can protect vital organs as liver, lungs, and brain preventing insurgence of various illnesses linked to oxidative stress.

The protective effect of ellagic acid against oxidative stress occurs through metal chelation with formation stable complexes and consequently reduction of free radical.

Probably responsible for metal chelation is the presence of a catechol group in the structure of phenolic compounds (Ríos et al., 2018).

In literature, data for FRAP test were reported only for rutin (21.9 μM; Loizzo et al., 2016), sweroside (0.4 mM/100 mg fresh weight; Bozunovic et al., 2018) and ellagic acid.

Ellagic acid at the concentration of 45 μg/mL exhibited a chelation of Fe^{2+} of 48.9%. This value compared with positive control (BHT) and other standard as EDTA, BHA, α-tocopherol, and ascorbic acid were lower because their values were in the range 64.5-96.2%. At the same concentration *p*-coumaric acid showed higher chelation of ferrous ion with percentage of 78.3% (Kiliç et al., 2013), while at the concentration of 15 μg/mL caffeic acid reported a chelating percentage of 53.2% (Gülçin, 2006).

High radical scavenging activity was reported for rutin with values of 2.83 and 10.3 μM, for ABTS and DPPH assays, respectively (Loizzo et al., 2015).

Among tested compounds, a greater antioxidant activity was reported by rutin, cornuside, and ellagic acid. Both rutin and cornuside exhibited the highest radical scavenging activity against ABTS and DPPH radicals, respectively. Ellagic acid has been reported for its excellent inhibition of lipid peroxidation and iron reduction. In FRAP test, also cornuside showed an interesting activity. Several studies were reported about ellagic acid. Ellagic acid is one of main antioxidant compounds and it is known to scavenge radical as reactive oxygen and nitrogen species with similar effects to well-known antioxidants, vitamin C and E (Priyadarsini et al., 2002), were necessary 15 μM for half reduce DPPH radical.

Kiliç et al. (2014) reported an ABTS radical scavenging inhibition at 20 μg/mL of ellagic acid of 93.9%, while a concentration of 30 μg/mL ellagic acid showed DPPH inhibition of 85.6%. This study increase the theory that ellagic acid present high free radical scavenging capacity observed by Zafrilla et al. (2001) in a previous study.

Ellagic acid properties were ascribed to various factors such as DNA binding, protection of DNA from alkylating injury, inhibition of reactive species production and their scavenging (Hassoun et al., 1997). A recent work reviewed on the biological activities of ellagic acid reported its high antioxidant capacity (Ríos et al., 2018). Ellagic acid present a hydrophilic and lipophilic portion for the presence of hydroxyl group, lactones and hydrocarbon rings that imparted its intrinsic capacity to accept electrons and participate in antioxidant redox reactions. Metabolites of ellagic acid preserve its antiradical capacity, and surprisingly this capacity was faster and more efficient in the metabolites that ellagic acid itself. Radical scavenger activity of ellagic acid was high of two or three fold if compared with Trolox, positive control in determination of antioxidant capacity *in vitro* test (TEAC).

Ellagic acid is capable to scavenge a various variety of ROS, as peroxyl radicals, hydroxyl and hydroperoxyl, but also RNS species as peroxynitrite and nitrogen dioxide. Thus, it presents protective capacity against free radical-induced damage, as hyperlipidaemia, hepatic injury and gastric lesions.

Hypoglycaemic activity

Table 9.16 reports the hypoglycaemic effects of fractions obtained by separation on XAD-16 resin in the inhibition of α-amylase and α-glucosidase enzymes.

The MDB (III) fraction resulted the most active against both enzymes with IC_{50} values of 63.43 and 32.21 μg/mL against α-amylase and α-glucosidase, respectively. This fraction displayed a selectivity towards α-glucosidase similar to that of acarbose.

Among leaves fractions, TDB (II) leaves fraction resulted more active in the inhibition of α-glucosidase (42.88 μg/mL) compared with α-amylase (15.26%).

Table 9.16. Hypoglycaemic activity of *C. mas* fruits and leaves.

C. mas	IC_{50}, μg/mL		Selectivity Index (SI)	
	α-Amylase	α-Glucosidase	α-Amylase[a]	α-Glucosidase[b]
Fractions Fruits				
MDB(II)	446.44 ± 1.86****	125.86 ± 1.24****	0.28	3.55
MDB (III)	63.43 ± 0.58****	32.21 ± 0.12*	0.51	1.97
Fractions Leaves				
TDB(II)	250.25 ± 1.58****	472.72 ± 1.68****	1.71	0.53
TDB(III)	15.26%[#]	42.88 ± 0.41****	-	-
Positive control				
Acarbose	50.01 ± 1.43	35.50 ± 1.10	0.71	1.41

Data are expressed as means ± S.D. (n= 3). [#]at concentration of 1000 μg/mL. Data are expressed as means ± S.D. (n= 3). Acarbose used as positive control in α-amylase and α-glucosidase tests. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001 compared with the positive controls. [a]SI for α-amylase is defined as IC_{50} (α-glucosidase)/IC_{50} (α-amylase). [b]SI for α-glucosidase is defined as IC_{50} (α-amylase)/IC_{50} (α-glucosidase).

The analysis of the potential ability of cornuside, sweroside, and ellagic acid to inhibit carbohydrates-hydrolysing enzymes evidenced a strong activity of all compounds if compared to the positive control acarbose (Table 9.17).

Table 9.17. Hypoglycaemic activity of cornuside, sweroside, and ellagic acid.

	IC_{50}, μM		Selectivity Index (SI)	
Compound	α-Amylase	α-Glucosidase	α-Amylase[a]	α-Glucosidase[b]
Cornuside	28.75 ± 1.37	10.76 ± 1.03	0.37	2.67
Sweroside	6.11 ± 0.92	2.28 ± 0.27	0.37	2.68
Ellagic acid	48.90 ± 1.52	8.83 ± 0.94	0.18	5.54
Positive control				
Acarbose	77.45 ± 1.83	54.99 ± 1.32	0.71	1.41

Data are expressed as means ± S.D. (n= 3). Data are expressed as means ± S.D. (n= 3). Acarbose used as positive control in α-amylase and α-glucosidase tests. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, compared with the positive control. [a]SI for α-amylase is defined as IC_{50} (α-glucosidase)/IC_{50} (α-amylase). [b]SI for α-glucosidase is defined as IC_{50} (α-amylase)/IC_{50} (α-glucosidase).

The most active compound is sweroside with a strong inhibition against α-amylase and α-glucosidase with IC_{50} values of 6.11 and 2.28 μM, respectively.

Our data are in agreement with a previous study in which sweroside showed a strong α-glucosidase inhibitory activity (Liu et al., 2015).

A perusal analysis of the literature revealed the presence of several works that investigated the potential hypoglycaemic activity of flavonoids. Among these phytochemicals, there are rutin and quercetin 3-*O*-glucuronide (Ahmed et al., 2019; Loizzo et al., 2015). Rutin showed higher inhibition of α-glucosidase than α-amylase (IC_{50} values of 0.037 and 0.043 μM, respectively). The same trend was observed for quercetin 3-*O*-glucuronide with IC_{50} values of 89.20 and 128.34 μg/mL, respectively.

Kaempferol 3-*O*-rutinoside inhibited α-glucosidase with an IC_{50} value of 19.36 μM (Habtemariam, 2011). Ellagic acid evidenced a high inhibition for both α-glucosidase and α-amylase enzymes with IC_{50} values of 3.3 and 2.0 μM, respectively (De Souza Schmidt Gonçalves et al., 2010).

Inhibition of NO production and NF-κB activation

C. mas fractions showed significant inhibition of NO production in a concentration-dependent manner (Table 9.18), with IC_{50} values ranged from 9.61 μg/mL for TDB (III) to 13.59 μg/mL for MDB (III). Interesting results were obtained testing cornuside and sweroside that exhibited IC_{50} values of 8.44 and 8.35 μg/mL, respectively.

Table 9.18. Inhibition of NO production of *C. mas* fruits and leaves fractions

C. mas	IC_{50}, μg/mL
Fruits fractions	
MDB (II)	11.79 ± 1.01
MDB (III)	13.59 ± 0.76
Leaves fractions	
TDB (II)	11.66 ± 1.17
TDB (III)	9.61 ± 0.68
Pure compounds	
Cornuside	8.44 ± 0.72
Sweroside	8.35 ± 0.59
Ellagic acid	N.T.

Data are mean ± SD (n = 3). N.T.: not tested. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, compared with the negative control (0 μg/mL).

Fractions and pure compounds were preventively tested for their potential cytotoxic activity by using MTT test (Figures 9.14 and 9.15).

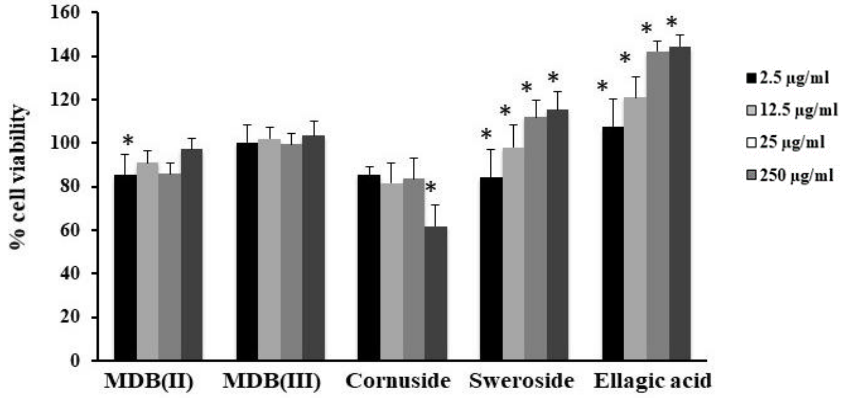


Figure 9.14. Cell viability of HFF1 cells untreated and treated for 24h with fractions from *C. mas* fruits at different concentrations (12.5-250 μg/mL) evaluated by MTT assay. Values are the mean ± S.D. of four experiments in triplicate. Control cells were incubated only with medium and considered as 100% of cell viability. *Significant *vs* untreated control cells and *vs* other concentrations of the same extract $p< 0.001$.

Both MDB (II) and MDB (III) did not affected HFF1 cells viability at the tested concentrations. TDB (II) reduced cell viability in a concentration-dependent manner.

Ellagic acid increased cell viability in a concentration-dependent manner, reaching the most significant effect at the highest concentration of 250 μg/mL. The exposure to sweroside at the concentration of 2.5 μg/mL reduced cell viability by about 15% while at the concentrations of 12.5-25-250 μg/mL, cell viability increased by about 15% compared to the untreated control cells. Cornuside decreased cell viability at all concentrations tested compared to the untreated control. The decrease was approximately 40% at 250 μg/mL.

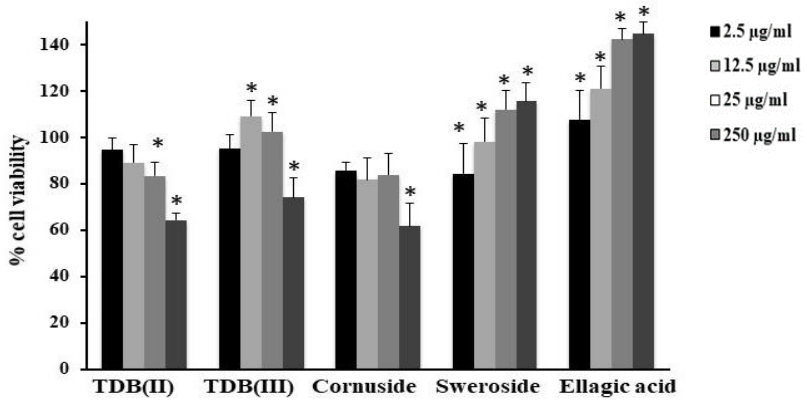


Figure 9.15. Cell viability in HFF1 cells untreated and treated for 24h with fractions from *C. mas* leaves at different concentrations (12.5-250 μg/mL) evaluated by MTT assay. Values are the mean ± S.D. of four experiments in triplicate. Control cells were incubated only with medium and considered as 100% of cell viability. *Significant *vs* untreated control cells and *vs* other concentrations of the same extract $p< 0.001$.

NO is an inflammatory mediator generated by the inducible nitroxide synthase (*i*NOS) enzyme, whose expression is stimulated via different inflammatory stimuli such as bacterial LPS through the toll-like receptor 4 (TLR4).

The induction of *i*NOS expression is mediated by MAPKs activation or via NF-κB that is the main transcription factor involved in inflammatory response.

A global analysis of the data obtained so far on the *C. mas* fractions allowed us to select TDB (II) and MDB (II) to be further tested for their ability to reduce the activation of the transcription factor NF-κB (Figure 9.16).

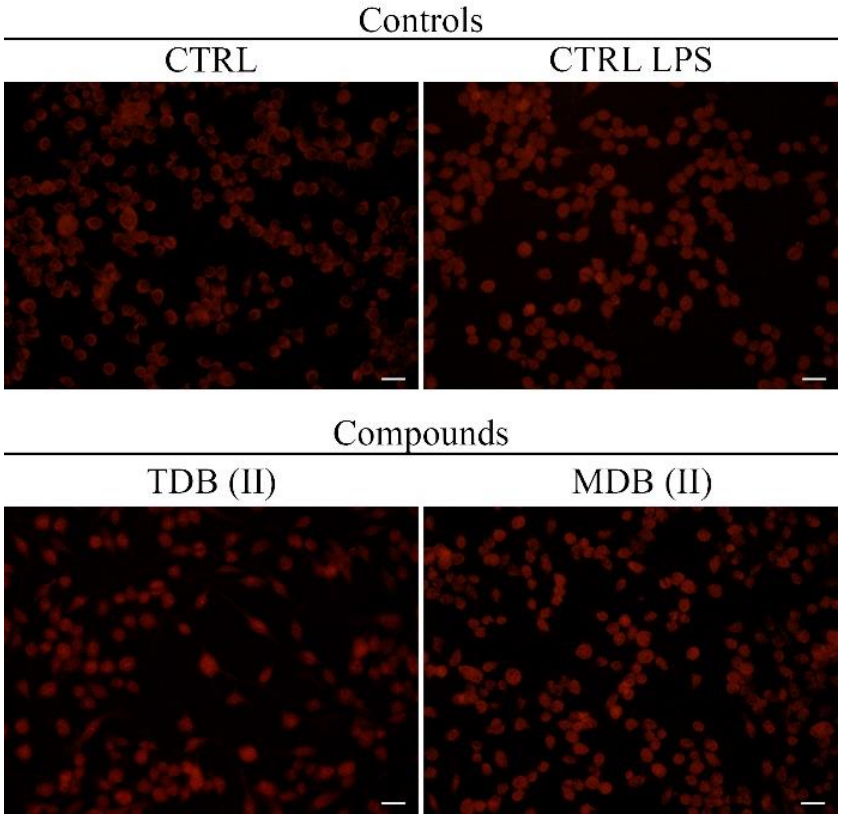


Figure 9.16. Immuno-fluorescent localization of NF-κB in RAW 264.7 cells treated for 1 h with DMSO (CTRL), 1 μg/mL LPS + DMSO (CTRL LPS), 1 μg/mL LPS + TDB (II) at IC_{50} value (TDB (II)), 1 μg/mL LPS + MDB (II) at IC_{50} value (MDB (II)). Scale bar: 25 μm.

In particular, after the treatment of LPS-stimulated RAW 264.7 cells, the translocation of NF-κB into the nucleus was monitored. As shown in figure 9.16 CTRL cells appears with no fluorescence into the nuclei reflecting the NF-kB inactivation. At the same time, CTRL LPS cells appears with cytoplasmic and nuclear fluorescence, confirming the translocation of NF-κB into the nuclei. The results obtained in this study highlighted the ability of MDB (II) to reduce the LPS-induced translocation of NF-κB into the nucleus (Figure 9.16) and at the same time showed that TDB (II) was not able to reduce the translocation suggesting a different mode of action for this extract.

TDB (II) and MDB (II) *C. mas* fractions were subjected to haemolysis assay on human blood to check the toxicity direct on blood cells, in particular, on red blood cells (RBCs). Both fractions did not show a significant release of haemoglobin, resulting non-toxic towards the RBCs. In fact, TDB (II) exhibited a percentage of haemolysis of 1.11 and 4.73% after 1 and 24 h, respectively. Comparable results were obtained with MDB (II) fraction (percentage of haemolysis of 1.25 and 5.54% after 1 and 24 h, respectively).

Other identified compounds in *C. mas* fractions were studied as NO inhibitors. Among them, rutin and quercetin 3-*O*-glucuronide exerted IC_{50} values of 41.5 and 10 μM, respectively (Al-Shalmani et al., 2010; Chen et al., 2001). An IC_{50} value > 100 μM was described for kaempferol 3-*O*-rutinoside (Wang et al., 2018).

Interesting results were reported in literature about iridoids and ellagic acid. Cornuside inhibited inflammatory mediators decreasing the levels of Tumour Necrosis Factor (TNF-α), interleukins (IL-1β and IL-6) and NO production in murine macrophages stimulated by lipopolysaccharide (LPS), ameliorating the inflammation status (Jiang et al., 2009). Choi et al. (2011) confirmed these properties and demonstrate that cornuside was able to decrease the NO production of 67.6% at 30 μM concentration in LPS-stimulated RAW 264.7 cells. In the same study, the cytotoxicity and the effects of cornuside in inhibiting other inflammatory mediators, as prostaglandin (PGE2), TNF-α, IL-6 and IL-1β, were evaluated. Cornuside had not cytotoxic effects on RAW 264.7 cells at dose of 100 μM; in addition, PGE2 production was attenuated in a concentration-dependent manner. The treatment with cornuside (30 μM) inhibited the production of TNF-α and interleukins (IL-6 and IL-1β) with percentages of 50.8, 75.6, and 55.4%, respectively. A perusal analysis of the literature revealed that cornuside possess a good activity in the inhibition of inflammatory mediators. Other iridoid with a high activity against mediators of inflammation was sweroside. Sweroside not presented antioxidant activity but resulted a good anti-inflammatory.

Sweroside at the dose of 120 mg/kg showed hepatoprotective effects, preventing the accumulation in the liver of bile acid and decreasing the transaminases levels and the expression of some pro-inflammatory cytokines, such as TNF-α, IL-6. A previous study showed that sweroside inhibited the production of NO, TNF-α, and IL-6 in LPS-stimulated RAW 246.7 cells (Chen et al., 2004). Recently, the protective effects of sweroside on inflammation in osteoarthritis rats were reported (Zhang et al., 2018). Osteoarthritis is a degenerative inflammation of joint, characterised by release of inflammatory mediators as IL-1β, NO, PGE2. Sweroside at different concentrations (0.1, 1, and 10 μg/mL) decreased the NO and PGE2 production with consequently reduction of IL-1β.

One of the most investigated phenolic acid is ellagic acid. In recent years, many studies were devoted to evaluate the protection afforded by ellagic acid in scavenging several free radicals in aqueous solutions and at physiological pH (Galano et al., 2014). These studies also demonstrated that ellagic acid displayed an efficient protection against

oxidative stress even at low concentrations through regeneration after the scavenging of two radical molecules. Moreover, a large body of evidence in the literature demonstrated the role of ellagic acid in different inflammation processes.

In a recent paper, Ríos et al. (2018) reviewed the most promising studies that investigated the antioxidant, anti-inflammatory, neuroprotective, and anti-atherogenic, effects of ellagic acid. Masamune et al. (2005) showed that ellagic acid at different concentration (1, 5, 10, and 25 μg/mL) locked the IL-1β and TNF-α-induced activation of activator protein 1 (AP-1) and mitogen-activated protein kinase (MAPK), such as extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK), and p38, but not nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). In agreement with us, are the results obtained by González-Sarrías et al. (2010) that demonstrated that ellagic acid at concentration of 10 μM not decreased NF-κB activation.

Cornélio Favarin et al. (2013) have evaluated the potential preventive or therapeutic effects of ellagic acid administration in the development or establishment of acute lung injury. In the mice with inducted acute lung injury, the administration of 10 mg/kg of ellagic acid decreased IL-6 (pro-inflammatory cytokine) increased IL-10 (anti-inflammatory cytokine). Also in this case the NF-κB pathway was not involved.

El-Shitany et al. (2014) have indicated the involved of ellagic acid in modulation of cyclooxygenase-2 (COX-2) mRNA production *via* the inhibition of ROS generation and with indirectly inhibition of NF-κB activation. In addition, ellagic acid showed high affinity with COX-2 active site compared with two anti-inflammatory drugs (diclofenac and meloxicam). This high affinity was attributed to the ellagic acid capacity to make four hydrogen bonds with Arg^{120}, Ser^{530}, Tyr^{355}, and Tyr^{385}. Thus, this study suggested that ellagic acid anti-inflammatory activity involved also the inhibition of COX-2 receptor. More recently, Fikry et al. (2019) have evaluated the effects of ellagic acid administration in rats with rheumatoid arthritis. Ellagic acid decreased gradually the levels of paw oedema, reporting after 21 days the inhibition of 67.6%, same inhibition if compared with anti-inflammatory drugs (64%).

The administration of ellagic acid decreased massive inflammatory cell infiltration, oedema, cartilage destruction, and increase of inflammation proteins. Previously, a study demonstrated that ellagic acid at 1-30 mg/kg inhibited the levels of IL-1β and TNF-α respect that observed in rats with paw oedema (control). Moreover, the increase of dose (30 mg/kg for 5 hours) reduced the pro-inflammatory proteins expression. However, at the same dose none effect on NO levels in oedema paw tissue was evidenced. The association of ellagic acid and the anti-inflammatory drug indomethacin showed a significant reduction of NO production and cellular infiltrates number (Mansouri et al., 2015). BenSaad et al. (2017) conducted *in vitro* tests on RAW 246.7 cells and demonstrated that ellagic acid was able to reduce the NO production, with a maximum of inhibition at the concentration of 200 μg/mL.

9.2. *Cornus sanguinea*

C. sanguinea (common dogwood) is a *Cornus* European species, widely distributed in the temperate regions of Europe, whose fruits are used for the production of jams and juices. Unlike *C. mas*, which has been extensively studied, few studies in the literature have investigated the chemical constituents and the biological properties of *C. sanguinea*.

There are no reports on the iridoids identification and on the hypoglycaemic and anti-inflammatory properties. Only the antioxidant effects are assessed.

The objective of this part of the research project was to investigate the effects of solvents and extraction procedures on the extraction of flavonoids and iridoids from both fruits and leaves of *C. sanguinea* and investigate their potential bioactivities oxidative stress-related by several *in vitro* methods.

9.2.1. Extraction yields and total phytochemicals content

C. sanguinea fruits (3.7 kg) and leaves (3.8 kg) were divided in two parts for the preparation of fresh and dried materials. Fruits (2 kg) were dried in the oven with heated air at 50 °C for 7 days. Leaves (2.1 kg) were dried at room temperature for 7 days in the dark. After these procedures, plant materials (1.7 kg of fresh fruits, 1.6 kg of dried fruits, 1.7 kg of fresh leaves, and 652 g of dried leaves) were subjected to extraction.

The hydroalcoholic maceration resulted the more efficient technique for the extraction of bioactive compounds for dried fruits (10.31%) and leaves (9.20 and 35.61% for fresh and dried leaves, respectively) of *C. sanguinea* (Table 9.19).

Table 9.19. Extractive yield **(%)** of *C. sanguinea* extracts

	Extractive yield (%)[a]			
Extractive techniques	Fruits		Leaves	
	Fresh	Dried	Fresh	Dried
Maceration (EtOH)	7.53 ± 0.81	8.91 ± 0.92	8.42 ± 0.82	11.71 ± 1.22
Maceration (Hydroalcoholic 60%)	7.35 ± 0.74	10.31 ± 1.2	9.20 ± 1.05	35.61 ± 3.63
Decoction (H_2O)	1.74 ± 0.13	1.84 ± 0.11	8.91 ± 0.94	22.24 ± 2.31
Soxhlet apparatus (EtOH)	9.91 ± 1.14	6.43 ± 0.60	8.75 ± 0.81	17.81 ± 1.82
Ultrasound-assisted extraction (EtOH)	2.74 ± 0.20	1.83 ± 0.22	4.41 ± 0.41	9.55 ± 0.93

Data are reported as mean ± standard deviation (n= 3). [a] Expressed as (g dried extract/ g plant materials) × 100

Extraction yields in the range 1.74-9.91% were obtained with fresh fruits. The extraction of fresh fruits by ethanol with Soxhlet apparatus gave the highest yield (9.91%), while the decoction gave lower yield with percentage of 1.74%. Taking into account some exceptions, generally the extraction of fruits after drying allows obtaining greater yields than the extraction of fresh matrix. Extraction yields (%) in the range 4.41-9.20% and 9.55-35.61% were obtained with the leaves. The following trend was observed: hydroalcoholic maceration > decoction > ethanol Soxhlet apparatus > ethanol maceration > ethanol ultrasound-assisted extraction, for both fresh and dried leaves. However, the best extractive results in terms of yield were obtained with dried materials.

Previously, Yousfbeyk et al. (2014) have described that the use of alcohol (methanol) for the extraction of dried fruits give high extractive yield, three-fold higher than that reported by using water, and two-fold higher compared with ethyl acetate.

Preliminary evaluation of total phytochemicals content namely, phenols, flavonoids, and iridoids was conducted on all fruits and leaves extracts. Data are summarised in Table 9.20 and 9.21.

Table 9.20. Total phytochemicals content of *C. sanguinea* fruits extracts

C. sanguinea	Total Phenols Content (mg CA/g)	Total Flavonoids Content (mg QE/g)	Total Iridoids Content (mg AU/g)
Fruits			
SF1	46.13 ± 0.6	30.20 ± 0.2	118.12 ± 0.5
SF2	37.27 ± 0.3	25.33 ± 0.3	82.67 ± 0.4
SF3	53.46 ± 0.4	41.33 ± 0.3	122.69 ± 0.7
SF4	34.22 ± 0.5	29.33 ± 0.3	104.67 ± 0.4
SF5	41.33 ± 0.5	19.33 ± 0.2	83.30 ± 0.3
SD1	37.07 ± 0.2	26.21 ± 0.2	86.13 ± 0.5
SD2	36.26 ± 0.4	20.62 ± 0.1	69.33 ± 0.4
SD3	34.27 ± 0.1	22.54 ± 0.1	86.66 ± 0.5
SD4	35.20 ± 0.3	27.23 ± 0.3	72.04 ± 0.5
SD5	38.27 ± 0.3	27.31 ± 0.2	95.12 ± 0.6

SF: fresh fruits, SD: dried fruits; 1. Ethanolic maceration; 2. Hydroalcoholic (60%) maceration; 3. Decoction; 4. Ethanol Soxhlet extraction; 5. Ethanol ultrasound extraction. Data are reported to mean ± Standard Deviation (SD) (n = 3). CA: Chlorogenic acid; QE: Quercetin; AU: Aucubin.

Fresh fruits showed a high content in phytochemical compounds compared with dried fruits. The decoction (SF3) has allowed to have the best content in polyphenols, flavonoids and iridoids with values of 53.46 mg CA equivalents/g extract, 41.33 mg QE equivalents/g extract and 122.69 AU equivalents/g extract, respectively.

Among extracts obtained from dried fruits the best results were attributed to ethanol ultrasound assisted-extraction (SD5) with the values of 38.27 mg CA equivalents/g extract, 27.31 mg QE equivalents/g extract and 95.12 mg AU equivalents/g extract respectively for polyphenols, flavonoids and iridoids.

Considering the polar nature of polyphenols, they are widely soluble in alcoholic and water solvents, thus our results are in agreement with Yousfbeyk et al. (2014) that evaluated the total phenols content of *C. sanguinea* dried fruits extracted by three solvents at different polarity such as ethyl acetate, methanol, and water. The trend observed was methanol > water > ethyl acetate with values of 88.56 mg gallic acid equivalents (GAE)/g dry extract, 74.91 mg GAE/g dry extract, and 20.13 mg GAE/g dry extract, respectively. In disagreement with these data are results obtained by Stanković et al. (2012). In this case, *C. sanguinea* fresh fruits were extracted by using methanol, water, ethyl acetate, acetone, and petroleum ether. Ethyl acetate provided extracts rich in polyphenols (45.34 mg GAE/g extract) and flavonoids (32.38 mg rutin equivalents (RUE)/g extract) compared with the other solvents. A less content in bioactive compounds (polyphenols

and flavonoids) was observed with aqueous (27.45 mg GAE/g extract) and methanol (14.40 mg RUE/g extract) extracts.

Table 9.21. Total phytochemicals content of *C. sanguinea* leaves extracts

C. sanguinea	Total Phenols Content (mg CA/g)	Total Flavonoids Content (mg QE/g)	Total Iridoids Content (mg AU/g)
PF1	355.80 ± 1.91	144.23 ± 1.03	123.33 ± 0.56
PF2	208.72 ± 1.22	80.93 ± 0.65	102.67 ± 1.10
PF3	296.27 ± 1.41	113.33 ± 0.75	85.33 ± 0.80
PF4	309.33 ± 2.25	141.33 ± 1.21	160.67 ± 1.05
PF5	316.43 ± 2.02	174.51 ± 1.62	132.05 ± 0.64
PD1	261.33 ± 2.43	167.07 ± 1.53	182.71 ± 1.54
PD2	307.20 ± 1.91	159.33 ± 1.12	116.68 ± 1.14
PD3	321.23 ± 1.65	120.76 ± 1.25	89.33 ± 0.71
PD4	297.35 ± 2.20	133.73 ± 1.23	166.34 ± 1.25
PD5	264.32 ± 2.12	171.07 ± 1.41	136.67 ± 1.01

PF: fresh leaves; PD: dried leaves. 1. Ethanolic maceration; 2. Hydroalcoholic (60%) maceration; 3. Decoction; 4. Ethanol Soxhlet extraction; 5. Ethanol ultrasound extraction. Data are reported to mean ± Standard Deviation (SD) (n = 3). CA: Chlorogenic acid; QE: Quercetin; AU: Aucubin.

In our study, leaves showed a greater content in phenols (208.72~355.80 mg CA/g extract) compared with fruits (34.22~53.46 mg CA equivalents/g extract) (Table 9.19). The same trend was observed for flavonoids with a content four-fold higher in the leaves extracts than the fruits extracts with values ranging from 80.93 to 174.51 and from 19.33 to 30.20 mg QE equivalents/g extract for leaves and fruits, respectively.

Values in the range of 69.33-118.12 and 85.33-182.71 mg AU equivalents/g extract were found for the total iridoids content of fruits and leaves, respectively.

The highest TPC was obtained by ethanolic maceration of fresh leaves (PF1; 355.80 mg CAE/g extract) and decoction of dried leaves (PD3; 321.23 mg CA equivalents/g extract). By contrast, the use of ultrasounds-assisted extraction has produced extracts rich in flavonoids for both fresh (PF5; 174.51 mg QE equivalents/g extract) and dried leaves (PD5; 171.07 mg QE equivalents/g extract). Instead, the extracts obtained by ethanol Soxhlet extractor of fresh leaves (PF4) and ethanolic maceration of dried leaves (PD1) presented higher iridoids content with values of 160.67 mg AU equivalents/g extract and 182.71 mg AU equivalents/g extract, respectively. To the best of our knowledge, this is the first report that reports the total iridoids content of *C. sanguinea* extracts.

The study conducted by Stanković et al. (2012) revealed a difference in phenols and flavonoids content of dried leaves depending on the nature of the solvent. Methanol was the election solvent for the phenolic compounds in the leaves (205.74 mg GAE/g extract), respect to other solvents (39.73~98.21 mg GAE/g extract). Moreover, ethyl acetate was the solvent providing extracts rich in flavonoid with value of 118.46 mg RUE/g extract. The concentration of total phenolic compounds and flavonoids depend on the polarity of solvents used and of type of plant material used for the extractions (fruits and leaves).

Generally, higher concentrations of total phenolic compounds and flavonoids were recorded in the leave extracts. Different authors have studied the difference between

different plant parts (fruits, leaves, flower, etc.) and phytochemical compounds content of many plants, confirmed higher concentration of phenols in leaves extracts compared to extracts from other plant parts (Barreira et al., 2008; Zhang et al., 2007). Phenolic contents of extracts is related on the plant part used, the extraction method and solvents employed, and not only the concentration of phenolic contents but also properties of these compounds contribute to the biological activities of different extracts.

9.2.2. LC-ESI-QTOF-MS analyses

Different phenolic acids, iridoids, and flavonoids are identified as main constituents of both fruits and leaves extracts analysed by LC-ESI-QTOF-MS (Tables 9.22 and 9.23). The chemical identification was based on bibliographic data, with compounds already described in the genus *Cornus*, but some compounds (not shown in the table) have not been identified. Quinic acid, chlorogenic acid, syringic acid, ferulic acid, gallic acid, ellagic acid, catechin, isoquercitrin, quercetin, rutin, hyperoside, and cornuside, were confirmed with authentic standards. Other compounds were identified based on UV spectra, and molecular weight (*m/z* ion $[M+H]^+$ or $[M+Na]^+$). Detection of chemical constituents of *C. sanguinea* extracts were influenced both solvents and procedures used. Chromatograms are reported in Appendix (Figures A9-A13).

Flavonoids

Eleven flavonoids are identified in all *C. sanguinea* extracts (Figure 9.17). However, there are differences in dependence of the extraction procedures. Hyperoside and isoquercitrin were found in all fruits and leaves extracts. Among extracts from *C. sanguinea* fruits, only the hydroalcoholic maceration of the dried matrix allowed the extraction of ten/eleven flavonoids. Quercitrin and rutin were revealed in all fruits extracts, while the presence of their aglycone (quercetin) was found in all extracts except for decoction, ethanol ultrasound-assisted extraction, and ethanol Soxhlet apparatus of fresh fruits. Kaempferol derivatives (glucoside and galactoside) together with quercetin derivatives (glucuronide and xyloside) were identified in extracts obtained by hydroalcoholic maceration and decoction of dried fruits. In addition, quercetin derivatives were found in decoction of fresh fruits (quercetin 3-*O*-glucuronide) and hydroalcoholic maceration of fresh fruits (quercetin 3-*O*-xyloside).

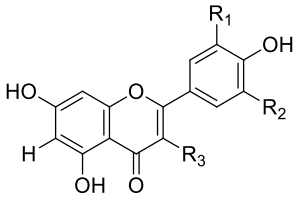
Myricetin 3-*O*-glucopyranoside was detected in extracts obtained by ethanol Soxhlet extractor of fresh fruits and hydroalcoholic maceration of dried fruits. Catechin was observed only in the decoction extract of fresh fruits.

A greater number of flavonoids has been detected in leaves extracts compared with fruits. Kaempferol derivatives (galactoside and glucoside) were found in all leaves extracts except fresh and dried decoction extracts while, kaempferol 3-*O*-glucuronide was not revealed in both hydroalcoholic and ethanol Soxhlet extracts of leaves. Rutin was present in fresh extract except to decoction, while it was found in dried extracts except to ethanol Soxhlet and decoction extracts. Isorhamnetin glucuronide and quercetin were

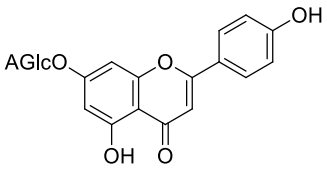
identified only in ethanol maceration and ultrasound extracts of fresh leaves. Myricitrin and quercetin 3-*O*-xyloside were detected in ethanol maceration, ultrasound extracts of fresh leaves and decoction of dried leaves; in addition myricitrin was found also in fresh leaves decoction and hydroalcoholic maceration of dried leaves.

Myricetin 3-*O*-α-L-arabinofuranoside was revealed in ethanol maceration and ultrasound extracts of fresh leaves and fresh and dried leaves extracts obtained by hydroalcoholic maceration. In ethanolic and hydroalcoholic maceration of fresh and dried leaves quercetin 3-*O*-glucuronide and galloyl norbergenin were identified. Instead, apigenin glucuronide was present only in the ethanol ultrasound extract of fresh leaves and Soxhlet extract of dried leaves.

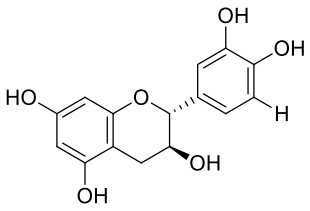
Myricetin galloylarabinopyranoside and quercetin acetylglycoside were specifically identified in fresh leaves obtained by ethanol and hydroalcoholic maceration. Few reports were present in literature for chemical composition of *C. sanguinea* extracts.



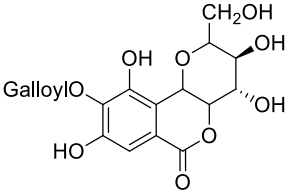
	R_1	R_2	R_3
Hyperoside	OH	H	*O*-Gal
Isoquercitrin	OH	H	*O*-Glc
Isorhamnetin 3-*O*-glucuronide	OCH_3	H	*O*-GlcA
Kaempferol 3-*O*-galactoside	H	H	*O*-Gal
Kaempferol 3-*O*-glucoside	H	H	*O*-Glc
Kaempferol 3-O-glucuronide	H	H	*O*-Glc A
Kaempferol 3-*O*-rutinoside	H	H	*O*-Glc(6←1)Rha
Myricetin 3-*O*-α–L-arabinofuranoside	OH	OH	*O*-Ara (f)
Myricetin galloylarabinoside	OH	OH	*O*-Gallolylarabinoside
Myricetin 3-*O*-glucopyranoside	OH	OH	*O*-MeGlc
Myricitrin	H	H	*O*-Rha
Quercetin	OH	H	OH
Quercetin acetylglicoside	OH	H	*O*-Acetylglicoside
Quercetin 3-*O*-glucuronide	OH	H	*O*-Glc A
Quercitrin	OH	H	*O*-Rha
Quercetin 3-*O*-xyloside	H	OH	*O*-Xyl
Rutin	OH	H	*O*-Glc(6←1)Rha



Apigenin 7-O-glucuronide



Catechin



Galloyl-norbergenin

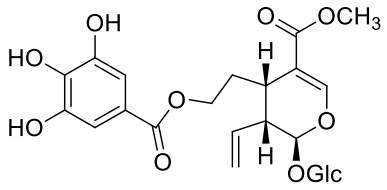
Figure 9.17. Flavonoids identified in *C. sanguinea* extracts.

In a recent study, Popović et al. (2017) indicated the presence of five quercetin glycoside (quercitrin, isoquercetin, hyperoside, rutin, and quercetin 3-*O*-glucuronide) in fresh hydroalcoholic fruits extract. The analysis of literature revealed that most of the

flavonoids identified in the present study were previous described in some *Cornus* species (Badalica-Petrescu et al., 2014; Pawlowska et al., 2010; Sochor et al., 2014; Dinda et al., 2016). Herein, to the best of our knowledge, for the first time, apigenin glucuronide, galloyl-norbergenin, isorhamnetin glucuronide, kaempferol 3-*O*-rutinoside and myricetin derivatives were described in the *Cornus* genus.

Iridoids

To the best of our knowledge, this is the first report on the iridoids identification in *C. sanguinea*. Cornuside is identified in *C. sanguinea* fruits and leaves (Figure 9.18). This compound is common in some *Cornus* species. In particular, it was found in the fruits of *C. mas* (Szumny et al., 2015) and *C. officinalis* (Hatano et al., 1990).



Cornuside

Figure 9.18. Iridoid identified in *C. sanguinea* extracts.

Cornuside characterised all fruits extracts except decoction of fresh and dried fruits, while in the leaves extracts was detected in extracts obtained by ethanol ultrasound-assisted extraction, ethanol and hydroalcoholic maceration of fresh leaves, and also in dried leaves hydroalcoholic maceration and Soxhlet (EtOH) extracts.

Table 9.22. Identification of chemical compounds in *C. sanguinea* fruits using LC-ESI-QTOF-MS technique.

Compounds	Rt (min)	Molecular Formula	MH^+/ MNa^+	Error (ppm)	Score (%)	MS fragment (*m/z*)	UV λ (nm)	Fresh fruits					Dried fruits					Reference
								SF1	SF2	SF3	SF4	SF5	SD1	SD2	SD3	SD4	SD5	
Phenolic acids																		
Abscisic acid	6.9	$C_{15}H_{20}O_4$	265.1439	2.3	98		260		√	√		√		√				Finkelstein, 2013
Ferulic acid°	1.7	$C_{10}H_{10}O_4$	195.0652	0.9	100		325		√					√	√			Krivoruchko, 2014
Gallic acid°	3.2	$C_7H_6O_5$	171.0287	1.1	100		217, 271			√		√			√	√	√	Deng et al., 2013
Shikimic acid	8.1	$C_7H_{10}O_5Na$	197.0425	1.3	99		215		√	√				√	√			Drkenka et al., 2014
Flavonoids																		
Catechin°	9.4	$C_{15}H_{14}O_6$	291.0868	1.2	100		280			√								Milenkovic-Andjelkovic et al., 2015
Hyperoside° (*)	12.8	$C_{21}H_{20}O_{12}$	465.1031	1.7	100	303.0499	217, 278, 350	√	√	√	√	√	√	√	√	√	√	Popović et al., 2017
Isoquercitrin° (*)	12.8	$C_{21}H_{20}O_{12}$	465.1031	1.7	100	303.0499	215, 253, 353	√	√	√	√	√	√	√	√	√	√	Popović et al., 2017
Kaempferol 3-*O*-galactoside (**)	13.2	$C_{21}H_{20}O_{11}$	449.1077	1.5	100	287.2287	210, 263, 344							√	√			Pawloska et al., 2010
Kaempferol 3-*O*-glucoside (**)	13.2	$C_{21}H_{20}O_{11}$	449.1077	1.5	100	287.2287	210, 265, 346							√	√			Pawloska et al., 2010
Myricetin 3-*O*-glucopyranoside	13.8	$C_{21}H_{20}O_{13}$	481.0982	1.5	99	318.0389	217, 262, 356					√		√				Guimarães et al., 2013
Quercetin°	16.8	$C_{15}H_{10}O_7$	303.0504	1.2	100		213, 255, 353	√	√				√	√	√	√	√	Sochor et al., 2014
Quercetin 3-*O*-glucuronide	13.1	$C_{21}H_{18}O_{13}$	479.0847	1.8	100	303.0499	216, 244, 354			√				√	√			Popović et al., 2017
Quercetin 3-*O*-xyloside	13.5	$C_{20}H_{18}O_{11}$	435.7749	0.6	100	303.0499	212, 254, 356		√					√	√			Pawloska et al., 2010
Quercitrin	13.6	$C_{21}H_{20}O_{11}$	449.1079	1.5	98	303.0499	213, 254, 356	√	√	√	√	√	√	√	√	√	√	Popović et al., 2017
Rutin°	12.4	$C_{27}H_{30}O_{16}$	611.1612	1.3	88	303.0499	213, 253, 352	√	√	√	√	√	√	√	√	√	√	Popović et al., 2017
Iridoids																		
Cornuside°	12.0	$C_{24}H_{30}O_{14}$	543.1713	0.5	100		218, 273	√	√		√	√	√	√		√	√	Szumny et al., 2015
Phenylethanoid glucoside																		
Cornoside	1.5	$C_{14}H_{20}O_8$	317.1236	0.9	100		215, 314							√				Nenadis et al., 2005

SF: fresh fruits, SD: dried fruits; 1. Ethanolic maceration; 2. Hydroalcoholic (60%) maceration; 3. Decoction; 4.Ethanol Soxhlet extraction; 5. Ethanol ultrasound extraction. ° identified with standard compounds; **in bold** not previously identified in the plant (or plant part); (*)(**) 2 peaks.

Table 9.23. Identification of chemical compounds in *C. sanguinea* leaves using LC-ESI-QTOF-MS technique

Compounds	Rt (min)	Molecular formule	MH^+	Error (ppm)	Score (%)	MS fragment (*m/z*)	UV λ (nm)	Fresh leaves					Dried leaves					Reference
								PF1	PF2	PF3	PF4	PF5	PD1	PD2	PD3	PD4	PD5	
Phenolic acids																		
Chlorogenic acid°	9.8	$C_{16}H_{18}O_9$	355.1029	1.6	100		242, 325	√	√	√	√	√	√	√	√	√	√	Deng et al., 2013
Ellagic acid°(*)	12.8	$C_{14}H_6O_8$	303.0140	1.2	100		255, 365	√	√	√		√	√	√	√			Deng et al., 2013
Gallic acid°	3.2	$C_7H_6O_5$	171.0287	1.1	100		217, 271	√	√	√			√	√	√			Deng et al., 2013
2-galloyl-4-caffeoyl-threonic acid(*)	12.1	$C_{20}H_{18}O_{12}$	451.0876	1.3	99		255, 294, 330	√				√		√				Lee et al., 2000
Neochlorogenic acid	6.7	$C_{16}H_{18}O_9$	355.1029	1.1	100		245, 325	√										Popović et al., 2017
Phenylacetic acid	2.1	$C_8H_8O_2$	137.0602	2.1	97		205, 259	√		√				√	√			Cook, 2019
Quinic acid°	0.9	$C_7H_{12}O_6$	193.0706	0.9	100		-	√										Drkenka et al., 2014
Syringic acid°	10.6	$C_9H_{10}O_5$	199.0606	2.3	89		218, 273	√						√				Guendouze-Bouchefa et al., 2015
Flavonoids																		
Apigenin glucuronide	13.4	$C_{21}H_{18}O_{11}$	447.0927	1.1	100		217, 266, 366					√				√		Sidorova., 2017
Galloyl-norbergenin	9.3	$C_{20}H_{18}O_{13}$	467.0825	1.5	97	314.0689	220, 285		√					√				Tangmouo et al., 2009
Hyperoside°(*)	12.8	$C_{21}H_{20}O_{12}$	465.1031	1.7	100	303.0499	217, 278, 350	√	√	√	√	√	√	√	√	√	√	Popović et al., 2017
Isoquercitrin°(*)	12.8	$C_{21}H_{20}O_{12}$	465.1031	1.7	100	303.0499	215, 253, 353	√	√	√	√	√	√	√	√	√	√	Popović et al., 2017
Isorhamnetin glucuronide	14.0	$C_{22}H_{20}O_{13}$	493.0982	2.2	97	316.2670	215, 256, 354	√				√						Li et al., 2014
Kaempferol 3-*O*-galactoside(**)	13.2	$C_{21}H_{20}O_{11}$	449.1077	1.5	100	287.2287	210, 263, 344	√	√		√	√	√	√		√	√	Pawloska et al., 2010
Kaempferol 3-*O*-glucoside(**)	13.2	$C_{21}H_{20}O_{11}$	449.1077	1.5	100	287.2287	210, 265, 346	√	√		√	√	√	√		√	√	Pawloska et al., 2010
Kaempferol 3-*O*-glucuronide	13.7	$C_{21}H_{18}O_{12}$	463.0876	1.1	100	287.2287	210, 264, 345	√		√		√	√		√		√	Badalica-Petrescu et al., 2014
Kaempferol 3-O-rutinoside	12.9	$C_{27}H_{30}O_{15}$	595.1663	2.2	81	287.2287	210, 265, 342					√	√					Li et al., 2014
Myricetin 3-*O*-*α*-L-arabinofuranoside(*)	12.6	$C_{20}H_{18}O_{12}$	451.0876	0.8	100	318.0436	219, 253, 365	√	√			√		√				Yan et al., 2002
Myricetin galloylarabinopyranoside	13.8	$C_{27}H_{22}O_{16}$	603.0986	1.5	98	318.0436	212, 265, 366	√	√									Korul'kina et al., 2004

Myricitrin	10.9	$C_{20}H_{18}O_{13}$	467.0825	0.8	100	318.0436	212, 257, 360	√		√		√		√	√			Hobbs et al., 2015
Quercetin°	16.8	$C_{15}H_{10}O_7$	303.0504	1.2	100		213, 255, 353	√				√						Sochor et al., 2014
Quercetin acetylglycoside	13.9	$C_{23}H_{22}O_{13}$	507.1138	1.1	100	303.0499	213, 252, 354	√	√									Bhattacharya et al., 2013
Quercetin 3-*O*-glucuronide	13.1	$C_{21}H_{18}O_{13}$	478.0747	1.8	100	303.0499	216, 244, 354	√	√				√	√				Popović et al., 2017
Quercetin 3-*O*-xyloside	13.5	$C_{20}H_{18}O_{11}$	435.7749	0.6	100	303.0499	212, 254, 356	√				√			√			Pawloska et al., 2010
Rutin°	12.4	$C_{27}H_{30}O_{16}$	611.1612	1.3	88	303.0499	213, 253, 352	√	√		√	√	√	√			√	Popović et al., 2017
Coumarin																		
Scopolin	9.1	$C_{16}H_{18}O_9$	355.1029	1.6	96		300, 345	√				√	√			√		Guzhva, 2008
Iridoids																		
Cornuside°	12.0	$C_{24}H_{30}O_{14}$	543.1713	0.5	100		218, 273	√	√			√		√		√		Szumny et al., 2015
Tannin																		
Gemin D	8.8	$C_{27}H_{22}O_{18}$	635.0884	1.1	100		221, 265	√										Hatano et al., 1989
Phenylethanoid glucoside																		
Cornoside	1.5	$C_{14}H_{20}O_8$	317.1236	0.9	100		215, 314					√	√			√		Nenadis et al., 2005

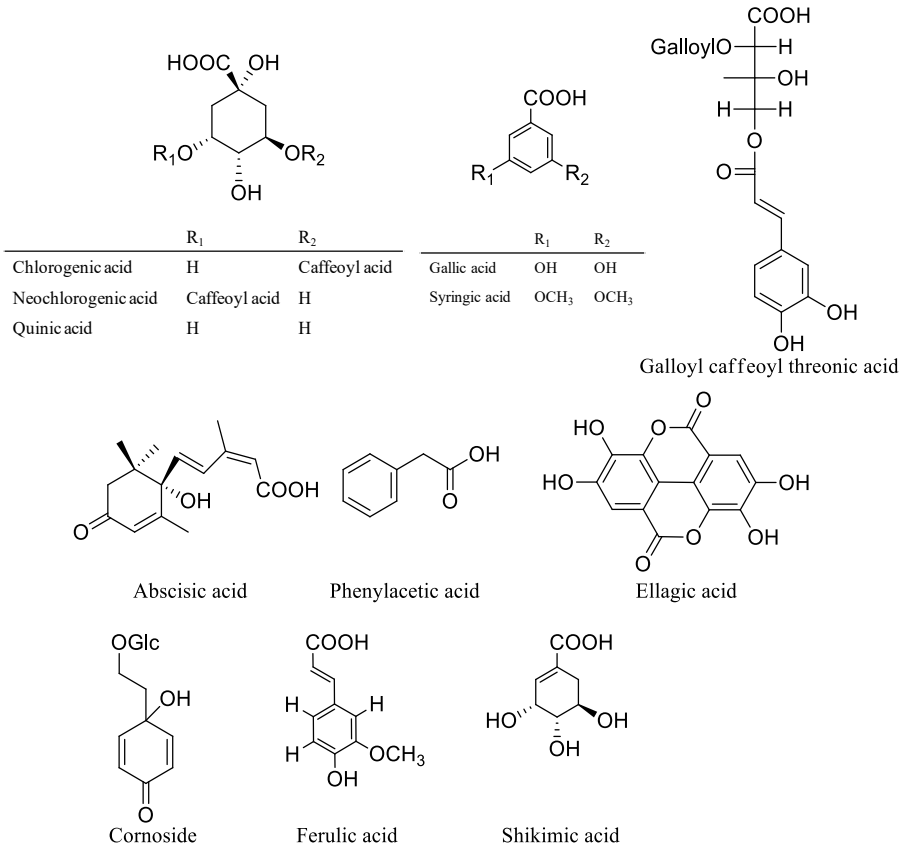
PF: fresh leaves; PD: dried leaves. 1. Ethanolic maceration; 2. Hydroalcoholic (60%) maceration; 3. Decoction; 4. Ethanol Soxhlet extraction; 5. Ethanol ultrasound extraction. ° identified with standard compounds; **in bold** not previously identified in the plant (or plant part); (*)(**) interchangeable 2 peaks

Other identified compounds

C. sanguinea fruits and leaves were characterised by the presence of different phenolic acids (Figure 9.19). Eight phenolic acids were detected in leaves extracts, four in fruits extracts. Phenolic acids were not detected in the extracts obtained by Soxhlet (EtOH) of fresh fruits and both (fresh and dried) ethanolic maceration. Moreover, both hydroalcoholic maceration, and both decoction extracts were characterised by presence of abscisic acid, ferulic acid and shikimic acid.

Abscisic acid was detected also in the ultrasound-assisted extraction (EtOH) of fresh fruits. Gallic acid was found in both fruits extracts obtained by ethanol ultrasound and decoction and also by Soxhlet (EtOH) of dried fruits.

All leaves extracts showed the presence of chlorogenic acid and ellagic acid. Ellagic acid is not identified only in extracts obtained by ethanol Soxhlet apparatus.



	R_1	R_2
Chlorogenic acid	H	Caffeoyl acid
Neochlorogenic acid	Caffeoyl acid	H
Quinic acid	H	H

	R_1	R_2
Gallic acid	OH	OH
Syringic acid	OCH_3	OCH_3

Figure 9.19. Phenolic acids and phenylethanoid glucoside identified in *C. sanguinea* extracts.

Gallic acid, phenylacetic acid, and 2-galloyl-4-caffeoyl treonic acid were identified in all extracts from fresh and/or dried materials obtained by conventional methods, such as ethanol and hydroalcoholic maceration and decoction.

2-Galloyl-4-caffeoyl treonic acid was found in the ethanol ultrasound extract of fresh leaves. Syringic acid was detected in the ethanol maceration of fresh leaves and

hydroalcoholic maceration of dried leaves. Quinic and neochlorogenic acid were specifically identified in fresh leaves obtained by ethanol maceration.

Except for abscisic, phenylacetic, and syringic acids, phenolic acids detected in this study were previously described in the *Cornus* genus (Deng et al., 2013; Drkenka et al., 2014; Krivoruchko, 2014; Lee et al., 2000; Popović et al., 2017; Szumny et al., 2015). Abscisic acid is one of the "classical" plant hormones, implicated in growth and development of plant (Finkelstein, 2013), as phenylacetic acid (Cook, 2019), while syringic acid is a phenolic acid that characterised other genus present in plant kingdom as *Arbutus* genus (Guendouze-Bouchefa et al., 2015).

The tannin gemin D was previously described in the extract obtained by ethanol maceration of *C. officinalis* fresh leaves (Hatano et al., 1989; Okuda et al., 1984).

Ethanolic extracts obtained by ultrasound of fresh leaves, Soxhlet of dried leaves and maceration of fresh and dried leaves reported the presence of scopolin, a secondary metabolite synthesized in plants as defence mechanism against various environmental stresses (Siwinska et al., 2014). Cornoside is identified in *C. sanguinea* fruits and leaves, but it is previous detected in other *Cornus* species, as *C. femina* (Nenadis et al., 2005). This phenylethanoid glucoside was found in leaves extracts; in particular, it is present in fresh leaves ethanol ultrasound extract, dried leaves extracts obtained by ethanol maceration and Soxhlet (EtOH) apparatus, and in dried fruits extract obtained by hydroalcoholic maceration.

9.2.3. Evaluation of antioxidant activity

The antioxidant effects of *C. sanguinea* extracts was estimated using ABTS and DPPH assays, which evaluate the capacity of antioxidants to act such as free radical scavengers.

The ABTS and DPPH antioxidant abilities of the extracts were essentially based on their capacity to quench free radicals by donating an electron (ABTS assay) or donating a hydrogen atom (DPPH assay). For these tests, the antioxidant ability is correlated to the degree of the decolourisation of the free radical (Prior et al., 2005). The colour from purple changes to yellow, upon the reduction of the radical DPPH• to the stable molecule.

In the ABTS test, the presence of sample with antioxidant ability was ascribed with the degree of cation radical decolourisation.

Moreover, we evaluated the ability of antioxidants to neutralise radical generate by *β*-carotene destruction initiated by degradation of linoleic acid through *β*-carotene bleaching test, and FRAP assay that assess the ability of antioxidants to perform as reducing agents. All four assays are reproducible, simple and inexpensive, employed together to estimate the antioxidant ability *in vitro*. Results (IC_{50} values) are summarised in the Table 9.24. Leaves extracts exhibited a strong antioxidant potential in all *in vitro* tests. Comparing fresh and dried plant materials, in general dried fruits were more active than fresh fruits in all tests (except DPPH assay). By contrast, fresh leaves resulted more active of dried leaves in all assays. Our results showed that all extracts have a concentration-dependent scavenging activity.

Table 9.24. *In vitro* antioxidant activity of *C. sanguinea* extracts

C. sanguinea	ABTS test	DPPH test	FRAP test	β-Carotene bleaching test	
	(IC_{50} μg/mL)	(IC_{50} μg/mL)	(μM Fe(II)/g)	(IC_{50} μg/mL)	
				30 min	60 min
Fruits					
SF1	15.05 ± 1.35****	365.61 ± 4.53****	22.14 ± 1.44****	40.27% [a]	30.77% [a]
SF2	13.90 ± 1.58****	19.56 ± 1.35	22.63 ± 1.39****	42.67% [a]	39.27%[a]
SF3	10.08 ± 0.61****	920.23 ± 12.58****	13.33 ± 1.23****	31.85% [a]	38.64% [a]
SF4	15.82 ± 1.25****	527.02 ± 7.03****	12.97 ± 1.12****	44.80% [a]	40.69% [a]
SF5	23.12 ± 1.15****	252.06 ± 6.76****	22.87± 1.19****	94.46 ± 1.20****	42.91% [a]
SD1	6.96 ± 0.50	31.39 ± 0.98****	25.99 ± 2.15****	15.63 ± 0.20****	31.02 ± 0.56****
SD2	6.23 ± 0.12	39.01 ± 1.02****	31.19 ± 2.65****	7.02 ± 0.10****	29.24 ± 0.60****
SD3	7.74 ± 0.19	798.23 ± 7.40****	23.22 ± 2.31****	80.85 ± 2.50****	72.22 ± 1.60****
SD4	15.82 ± 1.12	710.96 ± 8.23****	14.44 ± 1.29****	31.73% [a]	24.70% [a]
SD5	12.75 ± 0.88	37.93% [b]	NA	36.27% [a]	33.81% [a]
Leaves					
PF1	0.39 ± 0.03	6.89 ± 0.61	92.81 ± 2.59****	10.47 ± 0.98****	9.25 ± 0.86****
PF2	1.20 ± 0.02	13.87 ± 1.13	98.93 ± 3.01****	44.19%[a]	11.41 ± 0.98****
PF3	0.45 ± 0.01	4.89 ± 0.44	97.48 ± 3.09****	12.44 ± 0.53****	44.06 ± 2.33****
PF4	0.79 ± 0.06	13.87 ± 0.95	101.78 ± 4.45****	6.07 ± 0.32****	6.62 ± 0.11****
PF5	1.10 ± 0.07	9.88 ± 1.16	94.06 ± 2.81****	11.97 ± 0.82****	11.31 ± 0.57****
PD1	0.82 ± 0.01	12.87 ± 0.40	101.41 ± 4.13****	12.16 ± 0.77****	11.22 ± 0.45****
PD2	1.23 ± 0.02	9.88 ± 0.56	101.19 ± 4.89****	10.75 ± 1.03****	9.15 ± 0. 19****
PD3	1.02 ± 0.01	21.84 ± 1.31***	100.69 ± 4.71****	14.32 ± 1.08****	39.77%[a]
PD4	1.24 ± 0.03	10.87 ± 0.87	96.06 ± 3.93****	5.78 ± 0.65****	7.56 ± 0.76****
PD5	0.79 ± 0.08	15.86 ± 1.03	96.79 ± 4.06****	13.19 ± 0.99****	12.72 ± 1.05****
Positive control					
Ascorbic acid	1.71 ± 0.22	5.01 ± 0.84			
BHT			63.20 ± 4.32		
Propyl gallate				1.01 ± 0.01	1.02 ± 0.01

SF: Fresh fruits; SD: Dried fruits; PF: fresh leaves; PD: dried leaves. 1. Ethanolic maceration; 2. Hydroalcoholic (60%) maceration; 3. Decoction; 4. Ethanol Soxhlet extraction; 5. Ethanol Ultrasound extraction. [a] percentage of inhibition at a concentration of 100 μg/mL; [b] percentage of inhibition at a concentration of 1000 μg/mL; NA: not active. Data are expressed as means ± S.D. (n= 3). Ascorbic acid, BHT and Propyl gallate were used as positive control in antioxidant tests. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001,**p < 0.01, *p< 0.1 compared with the positive controls.

ABTS and DPPH tests

In the dataset of fresh fruit samples, the best results were obtained in ABTS test, with IC_{50} value of 10.08 μg/mL for the decoction extract (SF3), while in the DPPH test with the hydroalcoholic extract (SF2) with IC_{50} value of 19.56 μg/mL.

The best anti-radicals activity was found by extracts obtained with hydroalcoholic maceration (SD2) in ABTS test (IC_{50} value of 6.23 μg/mL) and by ethanol maceration (SD1) in DPPH test (IC_{50} value of 31.39 μg/mL). IC_{50} values in the range 0.39-1.20 and 0.79-1.24 μg/mL were found for fresh and dried leaves extracts, respectively in ABTS test. Interestingly, all leaves extracts are more active than the positive control ascorbic acid (IC_{50} value of 1.70 μg/mL).

In DPPH test, the extracts obtained by decoction of fresh leaves (PF3) and hydroalcoholic maceration of dried leaves (PD2) resulted the more active with IC_{50} values of 4.89 and 9.88 μg/mL, respectively.

Few studies that investigated the antioxidant properties of *C. sanguinea* fruits and leaves are present in literature. Antioxidant properties were evaluated through DPPH and FRAP tests. Yousfbeyk et al. (2014) assessed the influence of the extraction solvent on the antioxidant activity, using different solvent (ethyl acetate, methanol and water) for the extraction of dried fruits and evaluated *in vitro* antioxidant activity, describing a decidedly less interesting antioxidant activity than our samples. The methanol extract of dried fruits showed the high radical scavenging activity against DPPH with IC_{50} of 90.43 mg/mL, followed by aqueous extract (IC_{50} value of 269.75 mg/mL) and ethyl acetate extract (IC_{50} value of 762.3 mg/mL).

A significant correlation was found between TPC and antioxidant activity evaluated through DPPH test, as well as with the anthocyanin content. In the study by Stanković et al. (2012) the highest antioxidant activity (IC_{50} value of 247.83 μg/mL) is measured in the acetone extract of fresh fruits, followed by methanol extract (IC_{50} value of 358.59 μg/mL), water extract (IC_{50} value of 384.45 μg/mL),ethyl acetate extract (IC_{50} value of 537.83 μg/mL) and petroleum ether extract (IC_{50} value of 1202.85 μg/mL).

Same authors reported that leaves extracts appear to have higher antioxidant ability if compared to fruits extracts. In fact, the high ability to neutralise DPPH radicals was attributed to methanol and water extracts of dried leaves that neutralised DPPH free radicals in small concentration with IC_{50} values of 19.84 and 22.37 μg/mL, respectively; while the extract less active was obtained by ethyl acetate extraction (IC_{50} value of 738.20 μg/mL).

Previously, Serteser et al. (2009) reported the high antioxidant activity, measured by DPPH radical scavenging effects of hydroalcoholic fruits and leaves extracts of *C. sanguinea* compared with other plants grown in Turkey. The anti-radicals activity of fruits extract (IC_{50} value of 1.16 mg/mL) was high than leaves extract (IC_{50} value of 1.44 mg/mL). In conclusion, the use of conventional methods, as well in the *C. mas*, allows

obtaining the extracts (fruits and leaves) with high ability against radical ABTS and DPPH.

FRAP assay

The capacity of samples to reduce ferric ions is evaluated by FRAP assay. The yellow Fe^{3+}-TPTZ complex is reduced to blue Fe^{2+}-TPTZ complex in the presence of an antioxidant. In our study, all leaves extracts presented Fe^{3+}-reducing power with values in the range 92.81-101.78 μM Fe (II)/g. These results feature the greater capacity of leaves to reduce iron, better than BHT (63.20 μM Fe (II)/g), used as positive control.

Fresh and dried fruits showed values ranging from 12.97 to 31.19 μM Fe (II)/g. Only the extract obtained by ethanol ultrasound-assisted extraction of dried fruits (SD5) resulted not active in the iron reduction.

Previously, Yousfbeyk et al. (2014) assessed the influence of the extraction solvent on the antioxidant activity, using different solvents (ethyl acetate, methanol and water) for the extraction of dried fruits and evaluated *in vitro* antioxidant activity. Methanol extract showed the highest antioxidant capacity in FRAP method with 1419.167 mmol Fe (II)/g dry extract, followed by water extract (432.5 mmol Fe (II)/g dry extract) and ethyl acetate extract (157.51 mmol Fe (II)/g dry extract).

Serteser et al. (2009) have tested plant extracts from Turkey in a metal chelating assay. Chelating activity of fruits extracts was higher than leaves extracts. Percentages of 54.24 and 52.34% for the fruits of *C. mas* and *C. sanguinea*, respectively, were obtained.

The same trend was observed with leaves extracts, with percentage of 44.64 and 45.72% for *C. mas* and *C. sanguinea*, respectively.

β-carotene bleaching test

The anti-lipids peroxidation activity of *C. sanguinea* samples were determined by the linoleic acid/*β*-carotene system. Generally, leaves are more active than fruits. The extracts obtained by ethanol Soxhlet apparatus of both fresh and dried leaves (PF4 and PD4, respectively) were the most active with IC_{50} values of 6.07 and 6.62 (PF4) and 5.78 and 7.56 μg/mL (PD4), after 30 and 60 min of incubation, respectively. Except for PF2 and PD3, the other IC_{50} values are in the range 10.47-14.32 μg/mL after 30 min of incubation and 9.15-44.06 μg/mL after 60 min of incubation. Among fresh fruits samples, SF5 showed an IC_{50} value of 94.46 μg/mL after 30 min of incubation. All the other extracts exhibited an antioxidant activity in the range from 24.70 to 44.80% of inhibition at the maximum concentration tested (100 μg/mL). Among dried fruits samples, hydroalcoholic maceration (SD2) showed the best activity with IC_{50} values of 7.02 and 29.24 μg/mL, after 30 and 60 min of incubation, respectively.

This is the first report that shown the antioxidant activity evaluated through *β*-carotene bleaching test of *C. sanguinea* extracts. Except for the dried fruits, ethanol

Soxhlet apparatus and ethanol ultrasound-assisted extraction allow obtaining the extracts with remarkable anti-lipids peroxidation activity.

GAS and RACI determination were calculated to select the extract with the best antioxidant activity (Figures 9.20 and 9.21).

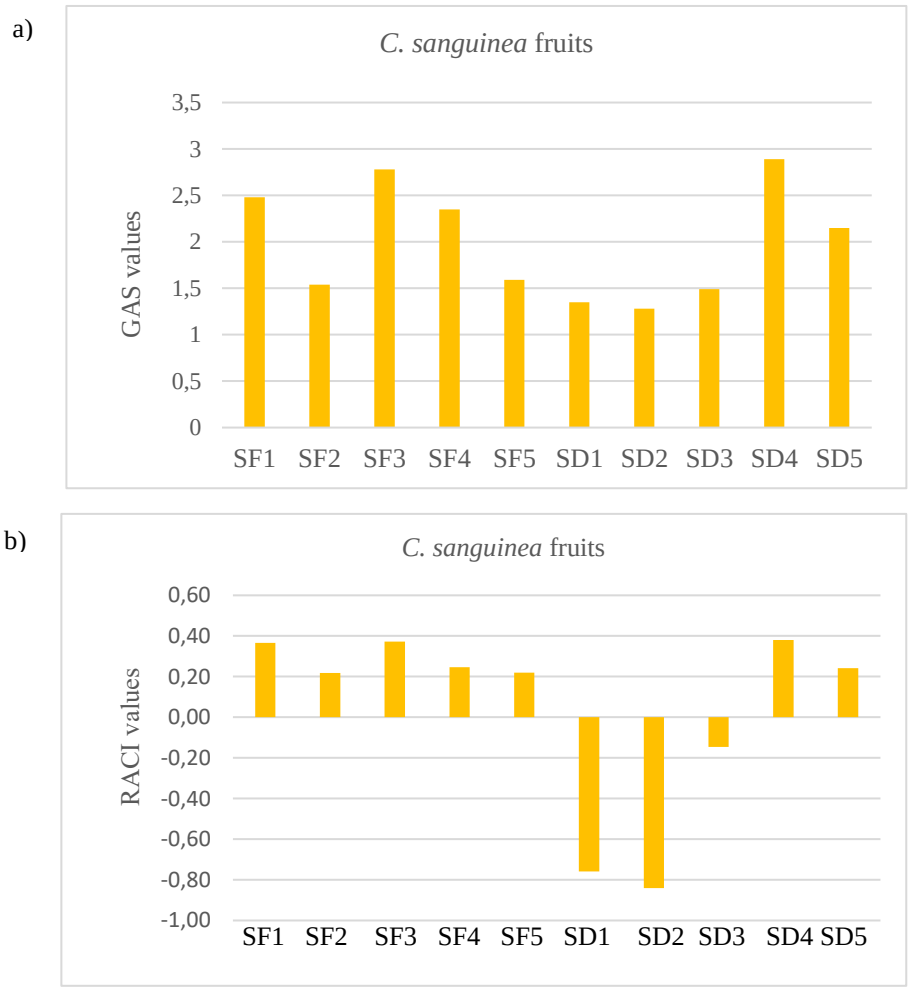
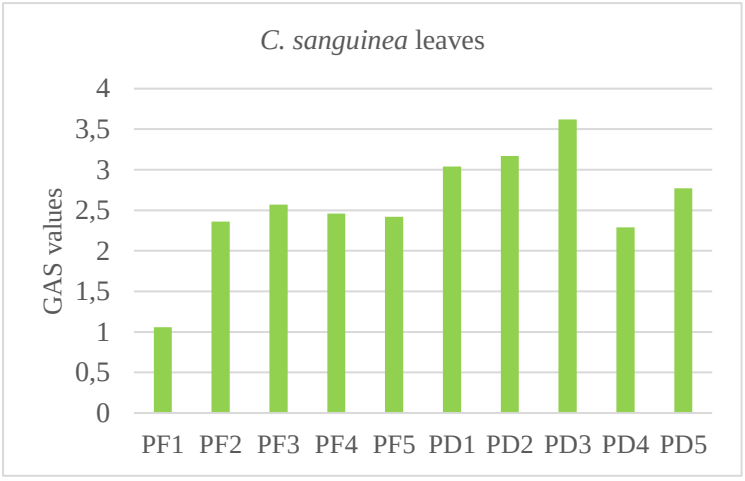


Figure 9.20. Evaluation of total antioxidant activity of fruits through RACI (a) and GAS (b). SF: Fresh fruits; SD: Dried leaves. 1. Ethanolic maceration; 2.Hydroalcoholic (60%) maceration; 3.Decoction; 4.Ethanol Soxhlet extraction; 5. Ethanol ultrasound extraction.

Among fruits extracts, SD2 (GAS= 1.28; RACI = −0.84) and SD1 (GAS= 1.35; RACI = −0.76) samples reported the highest antioxidant potential.

Among leaves extracts the highest activity was attributed to PF1 (GAS and RACI values of 1.06 and −0.89, respectively).

a)



b)

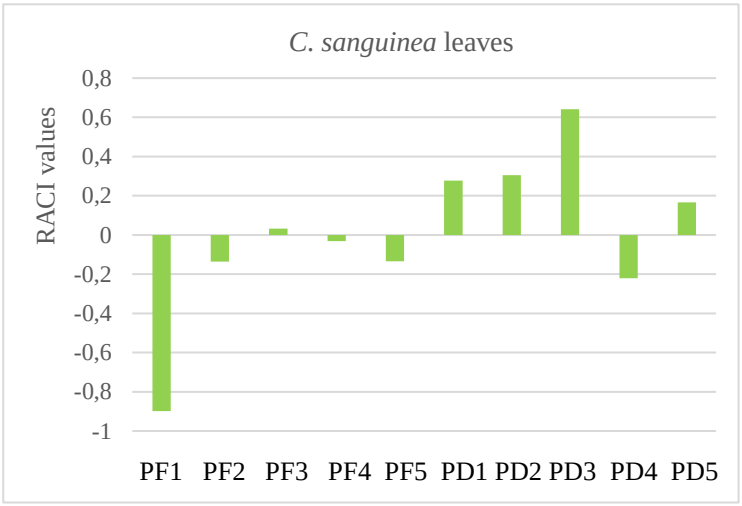


Figure 9.21. Evaluation of total antioxidant activity of leaves through RACI (a) and GAS (b). PF: Fresh leaves; PD: Dried leaves. 1. Ethanolic maceration; 2. Hydroalcoholic (60%) maceration; 3. Decoction; 4. Ethanol Soxhlet extraction; 5. Ethanol ultrasound extraction.

In conclusion, leaves extracts were more active than fruits extracts. Chemically comparing PF1, the most active extract, and PD3, the less active extract, PF1 resulted characterized by the presence of myricetin 3-*O*-arabinofuranoside, quercetin 3-*O*-glucuronide, myricetin galloylarabinopyranoside, and cornuside. These molecules were not identified in PD3. Moreover, PF1 resulted enriched in gallic acid, myricitrin, quercetin 3-*O*-xyloside, and kaempferol 3-*O*-glucuronide.

9.2.4. Hypoglycaemic activity

C. sanguinea extracts were tested for their inhibition against two enzymes involved in carbohydrates digestion, α-amylase and α-glucosidase. Data are summarised in the Table 9.25. Against α-amylase the most active extracts were SF3 that exhibited an IC_{50} value of 147.77 μg/mL and PF2 that showed an IC_{50} value of 174.05 μg/mL. However, both samples are less active than the positive control (IC_{50} value of 50.01 μg/mL).

Table 9.25. Carbohydrates-hydrolysing enzymes inhibitory activity of *C. sanguinea* fruits and leaves extracts.

C. sanguinea	IC_{50}, μg/mL		Selectivity Index (SI)	
	α-Amylase	α-Glucosidase	α-Amylase[a]	α-Glucosidase[b]
Fruits				
SF1	450.82 ± 4.56****	178.42 ± 1.89****	0.40	2.53
SF2	263.38 ± 2.63****	197.69 ± 2.54****	0.75	1.33
SF3	147.77 ± 1.57****	287.91 ± 3.25****	1.95	0.51
SF4	705.70 ± 7.36****	56.46 ± 0.36****	0.08	12.50
SF5	343.09 ± 3.56****	179.30 ± 2.58****	0.52	1.91
SD1	306.30 ± 6.35****	230.10 ± 2.45****	0.75	1.33
SD2	267.76 ± 4.69****	68.35 ± 1.26****	0.25	3.92
SD3	180.18 ± 2.35****	157.77 ± 0.96****	0.88	1.14
SD4	890.50 ± 9.65****	251.12 ± 1.54****	0.28	3.55
SD5	358.85 ± 2.54****	277.40 ± 2.69****	0.77	1.29
Leaves				
PF1	568.19 ± 5.63****	9.79 ± 0.05****	0.02	58.03
PF2	174.05 ± 1.89****	341.34 ± 2.67****	1.96	0.51
PF3	449.07 ± 4.53****	182.80 ± 1.92****	0.41	2.46
PF4	259.88 ± 2.47****	27.44 ± 2.36**	0.11	9.47
PF5	904.52 ± 6.34****	554.17 ± 4.69****	0.61	1.63
PD1	682.05 ± 3.84****	63.43 ± 1.25****	0.09	10.75
PD2	446.44 ± 3.56****	137.26 ± 3.65****	0.31	3.25
PD3	277.40 ± 2.45****	142.51 ± 2.59****	0.51	1.95
PD4	241.49 ± 1.25****	177.55 ± 2.46****	0.73	1.36
PD5	444.69 ± 4.69****	227.47 ± 1.78****	0.51	1.95
Positive control				
Acarbose	50.0 ± 1.4	35.5 ± 1.1	0.41	1.41

SF: Fresh fruits; SD: Dried fruits; PF: fresh leaves; PD: dried leaves. 1. Ethanolic maceration; 2. Hydroalcoholic (60%) maceration; 3. Decoction; 4. Ethanol Soxhlet extraction; 5. Ethanol ultrasound extraction. Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001,**p < 0.01, *p< 0.1 compared with the positive controls. [a]SI for α-amylase is defined as IC_{50} (α-glucosidase)/IC_{50} (α-amylase). [b]SI for α-glucosidase is defined as IC_{50} (α-amylase)/IC_{50} (α-glucosidase).

As found for *C. mas*, results that are more interesting have been obtained in the inhibition test of α-glucosidase. The most promising inhibitors of this enzyme were fresh leaves extracted by ethanol maceration (PF1) (IC_{50} values of 9.79 μg/mL) with a SI for α-glucosidase of 58.03, and ethanol Soxhlet apparatus (PF4) (IC_{50} value of 27.44 μg/mL) with a SI for α-glucosidase of 9.47. These values are better than to the positive control acarbose (IC_{50} value of 35.50 μg/mL). Of interest is also the α-glucosidase inhibitory activity of PD1 with an IC_{50} value of 63.43 μg/mL and a selectivity of 10.75.

The most active extract among *C. sanguinea* fruits extracts were SF4 and SD2 with IC_{50} values of 56.46 and 68.35 μg/mL, respectively, and a SI value of 12.50 and 3.92, respectively. To the best of our knowledge, no previous works analysed *C. sanguinea* fruits and leaves as carbohydrates-hydrolysing enzymes inhibitory agents.

In conclusion, leaves (PF1, PF4) and fruits (SF4, SD2) extracts showed an interesting α-glucosidase inhibition and a moderate α-amylase inhibition.

9.2.5. Inhibitory effects on nitric oxide (NO) production

Various anti-inflammatory drugs have showed to have an antioxidant mechanism as base of their activity (Delaporte et al., 2002). Thus locking free radicals as antioxidants can decrease inflammation status. Since the ancient time, use of plant, their part or extracts as antioxidant and anti-inflammatory compounds is known. In this work, the positive effects of *C. sanguinea* extracts on the inhibition of NO production was evaluated in HFF1 cells (fibroblasts).

Previous treatment of fibroblasts with IL-2β induces NO production. Its quantification was detected indirectly through the measure of nitrite, a stable metabolite of NO, using the chromogenic Griess reaction. Data were summarised in Table 9.26. All extracts of *C. sanguinea* are able to reduce NO production. Fruits were more active than leaves. IC_{50} values in the range of 27.85-31.12 μg/mL and 29.93-32.25 μg/mL were found for fresh and dried fruits, respectively. The most active samples were SF5 and SD1.

Table 9.26. Inhibition of NO production by *C. sanguinea* fruits and leaves extracts

C. sanguinea	IC_{50}, μg/mL
fruits	
SF1	31.12 ± 2.22
SF2	28.31 ± 2.87
SF3	30.08 ± 1.88
SF4	30.12 ± 2.32
SF5	27.85 ± 1.36
SD1	29.93 ± 2.11
SD2	31.04 ± 2.24
SD3	32.16 ± 2.34
SD4	32.25 ± 1.11
SD5	30.26 ± 2.24
Leaves	
PF1	37.97 ± 1.14
PF2	38.91 ± 1.93
PF3	34.33 ± 2.55
PF4	37.34 ± 2.12
PF5	41.55 ± 1.83
PD1	38.33 ± 1.73
PD2	44.32 ± 2.08
PD3	39.26 ± 1.12
PD4	36.51 ± 2.96
PD5	34.82 ± 0.82

SF: Fresh fruits; SD: Dried fruits; PF: fresh leaves; PD: dried leaves. 1. Ethanolic maceration; 2. Hydroalcoholic (60%) maceration; 3. Decoction; 4. Ethanol Soxhlet extraction; 5. Ethanol-ultrasound-assisted extraction. Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001 compared with the negative control (0 μg/mL).

IC_{50} values ranged from 34.33 to 44.32 μg/mL were reported for leaves, being PF3 and PD5 the most active ones.

C. sanguinea samples did not show any cytotoxicity up to the concentration of 250 μg/mL. Leaves extracts showed a more slight toxicity respect to fruits extracts (Figures 9.22 and 9.23).

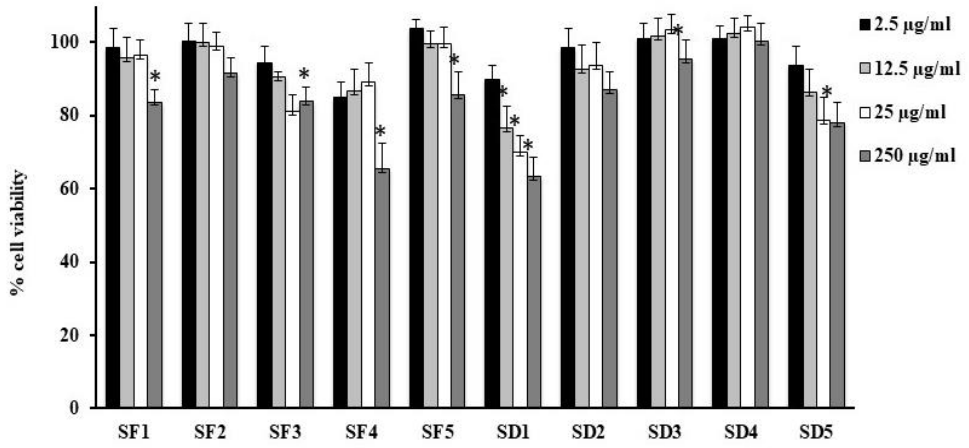


Figure 9.22. Cell viability in HFF1 cells untreated and treated for 24h with *C. sanguinea* fruits extracts at different concentrations (12.5-250 μg/mL). Values are the mean ± S.D. of four experiments in triplicate. Control cells were incubated only with medium and considered as 100% of cell viability. *Significant *vs* untreated control cells and *vs* other concentrations of the same extract $p< 0.001$.

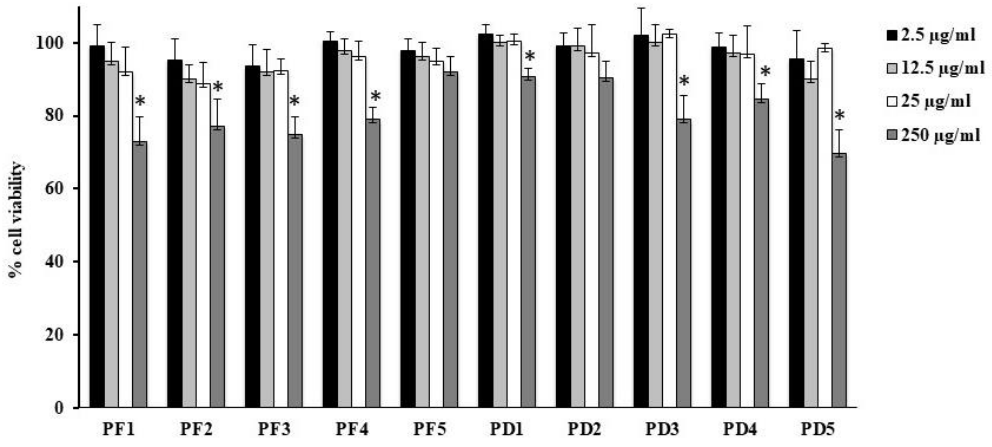


Figure 9.23. Cell viability in HFF1 cells untreated and treated for 24h with *C. sanguinea* leaves extracts at different concentrations (12.5-250 μg/mL). Values are the mean ± S.D. of four experiments in triplicate. Control cells were incubated only with medium and considered as 100% of cell viability. *Significant *vs* untreated control cells and *vs* other concentrations of the same extract $p< 0.001$.

9.2.6. Correlation between biological activity and chemical profile

Pearson's correlation coefficients were evaluated to show the antioxidant and anti-inflammatory activities displayed by the extracts were related to TPC, TFC and TIC.

Generally, the total phenolic and flavonoids contents are associated to biological activities reported in the plant extracts. A notable correlation was found also with TIC.

In fresh fruits, a positive correlation (r= 0.65) was found between TPC and DPPH test, but strongly correlation was registered between TFC and TIC with DPPH test with values of r= 0.85 and 0.81, respectively. In addition, TPC showed a strong correlation with α-glucosidase inhibitory activity (r= 0.84) Interesting correlation was reported between inhibition production of NO of fresh fruits and TFC and TIC, in particular very strongly correlation (r= 0.91) was found with TIC, followed by TFC (r= 0.67), while TPC present a less correlation (r= 0.37). Moreover, in dried fruits good correlation was found only with TFC and ABTS test (r= 0.74), but also less correlation with inhibition of enzyme α-amylase and α-glucosidase with values of r= 0.59 and 0.63, respectively.

Considering fresh leaves extracts, statistical analysis reveals a good correlation between β-carotene bleaching test after 30 min of incubation and phenolic content (r= 0.79) and flavonoid content (r= 0.69). In addition antioxidant activity (DPPH test) and iridoids content reported a less correlation with value (r= 0.55).

Moreover, a good correlation was found between the radical scavenging activity against ABTS of dried leaves with TPC (r= 0.74), while the inhibition of lipid peroxidation after 60 min of incubation present a strong correlation with TFC (r= 0.93) and less correlation with TIC (r= 0.66).

Of interest is also the correlation between TFC and α-amylase inhibitory activity of both fresh and dried leaves *C. sanguinea* extracts with values of r= 0.73 and 0.80, respectively. Moreover, a positive correlation was observed between phenolic content and inhibition production of NO of dried leaves (r= 0.55).

Phenolic compounds as flavonoids are likely to contribute biological activities, as confirmed from correlation obtained in this study. A strong correlation with phenolic and flavonoids contents was found, but the contribution of biological activities was correlated also with iridoids content, in particular for the inhibition of NO production of fresh fruits.

9.2.7. Selective separation of *C. sanguinea* flavonoids and iridoids-rich fractions

The most interesting extracts such as PF1 (ethanol maceration of fresh leaves) and SD2 (hydroalcoholic maceration of dried fruits) were subjected to bio-fractionation in order to obtain flavonoids and iridoids rich-fractions by using the XAD-16 resin.

Previous experiments with different resins such as XAD-4, XAD-16, and HLB described in the paragraphs on *C. mas* have allow us to choice XAD-16 for best affinity in separation to flavonoids and iridoids. Yield obtained with XAD-16 were summarised in Table 9.27.

Table 9.27. Yield (g) after separation on XAD-16 resin

XAD-16	Matrix (g)	Fraction I (g)	Fraction II (g)	Fraction III (g)	Recovery (g)
SD2	2.17	1.3	0.73	0.14	2.17
PF1	4.0	1.7	1.80	0.48	3.98

Fraction I: H_2O; Fraction II: 80% EtOH ; Fraction III: 100% EtOH.

After separation, fractions were analysed to LC-MS. Chromatograms are reported in Appendix (Figures A18 and A19). Identified compounds were summarised in Table 9.28.

Flavonoids and iridoids were detected in both fractions obtained by 80% ethanol (SD2 II) and 100% ethanol (SD2 III). 1-*O*-galloyl-D-Sedoheptulose, cornoside, and myricetin 3-*O*-glucopyranoside were identified in the aqueous fraction (SD2 I).

Specifically, trigalloylglucose isomers, quercetin 3-*O*-xyloside, myricetin 3-*O*-glucopyranoside, hyperoside, isoquercitrin, quercetin 3-*O*-glucuronide and quercetin were identified in SD2 (II) and SD2 (III). Interesting is the presence of the iridoid cornuside only in SD2 (II).

Table 9.28. Identification of chemical compounds in SD2 fractions (from hydroalcoholic maceration of *C. sanguinea* dried fruits) using LC-MS technique

Compounds	Rt (min)	Molecular Formula	MM	UV λ (nm)	SD2 (I)	SD2 (II)	SD2 (III)	Reference
Cornoside	1.9	$C_{14}H_{20}O_8$	316	215, 314	√			Nenadis et al., 2005
Gallic acid	3.7	$C_7H_6O_5$	170	216, 270	√			Deng et al., 2013
1-*O*-galloyl-*D*-sedoheptulose	4.7	$C_{14}H_{18}O_{11}$	362	216, 275	√			Lee et al., 1989
Trigalloylglucose(*)	8.6	$C_{27}H_{24}O_{18}$	636	217		√	√	Lee et al., 1989
Trigalloylglucose(*)	9.2	$C_{27}H_{24}O_{18}$	636	217		√	√	Lee et al., 1989
Quercetin 3-*O*-xyloside(*)	13	$C_{20}H_{18}O_{11}$	434	202, 250, 344		√	√	Pawloska et al., 2010
Cornuside	21	$C_{24}H_{30}O_{14}$	542	220, 277		√		Szumny et al., 2015
Myricetin 3-*O*-glucopyranoside(*)	21.2	$C_{21}H_{20}O_{13}$	480	208, 265, 356	√	√	√	Guimarães et al., 2013
Hyperoside(*)	23.4	$C_{21}H_{20}O_{12}$	464	205, 258, 358		√	√	Popović et al., 2017
Isoquercitrin(*)	24.5	$C_{21}H_{20}O_{12}$	464	212, 255, 367		√	√	Popović et al., 2017
Quercetin 3-*O*-glucuronide(*)	24.7	$C_{21}H_{18}O_{13}$	478	205, 258, 356		√	√	Popović et al., 2017
Quercetin	27.7	$C_{15}H_{10}O_7$	302	203, 257, 375		√	√	Sochor et al., 2014

SD2:hydroalcoholic maceration of dried fruits; I:fraction H_2O; II:fraction 80% EtOH; III:fraction 100% EtOH. (*) or isomer

Fractions obtained by leaves are generally richer in compounds (Table 9.29). However, phenolic acids, flavonoids, and iridoids are mainly present in the fraction obtained by 80% ethanol (PF1 II). Isoquercitrin, quercetin 3-*O*-xyloside, and quercetin, together with some unknown compounds were reported in PF1 (III).

Table 9.29. Identification of chemical compounds in PF1 fractions (from ethanol maceration of *C. sanguinea* fresh leaves) using LC-MS technique

Compounds	Rt (min)	Molecular Formula	MM	UV λ (nm)	PF1 (I)	PF1 (II)	PF1 (III)	Reference
Unknown	1.4		294	200	√			
Unknown	1.9		308	200	√			
Unknown	2.15			210		√	√	
Gallic acid	3.7	$C_7H_6O_5$	170	216, 270	√			Deng et al., 2013
1-*O*-galloyl-*D*-sedoheptulose	4.7	$C_{14}H_{18}O_{11}$	362	216, 275	√			Lee et al., 1989
Unknown	8.9		184	216, 275		√	√	
Digalloylglycoside(*)	9.9	$C_{20}H_{20}O_{14}$	484	216, 275		√		Lee et al., 1989
Quercetin 3-*O*-xyloside(*)	13	$C_{20}H_{18}O_{11}$	434	202, 250, 344		√	√	Pawloska et al., 2010
Digalloylglycoside(*)	13.7	$C_{20}H_{20}O_{14}$	484	216, 275		√		Lee et al., 1989
Cornuside	21	$C_{24}H_{30}O_{14}$	542	220, 277		√		Szumny et al., 2015
2-galloyl-4-caffeoyl-threonic acid(*)	21.5	$C_{20}H_{18}O_{12}$	450	217, 250, 329		√		Lee et al., 2000
2-galloyl-4-caffeoyl-threonic acid(*)	21.7	$C_{20}H_{18}O_{12}$	450	217, 250, 329		√		Lee et al., 2000
Hyperoside(*)	23.4	$C_{21}H_{20}O_{12}$	464	217, 278, 350		√		Popović et al., 2018
Hyperoside or Isoquercitrin(*)	23.7	$C_{21}H_{20}O_{12}$	464	219, 278, 350		√		Popović et al., 2018
Quercitrin(*) or Kaempferol 3-*O*-glucoside(*)	24.3	$C_{21}H_{20}O_{11}$	448	217, 278, 350		√		Pawloska et al., 2010; Popović et al., 2018
Isoquercitrin(*)	24.5	$C_{21}H_{20}O_{12}$	464	212, 255, 367		√	√	Popović et al., 2017
Quercetin 3-*O*-glucuronide(*)	24.7	$C_{21}H_{18}O_{13}$	478	205, 258, 356		√		Popović et al., 2017
Cornuskoside A	24.9	$C_{25}H_{32}O_{10}$	492	218, 280		√		Lee et al., 2008
Kaempferol 3-*O*-glucuronide(*)	25.2	$C_{21}H_{18}O_{12}$	462	200, 267, 300		√		Badalica-Petrescu et al., 2014
4-caffeoyl-2,3-digalloyl-L-threonic acid	25.6	$C_{27}H_{22}O_{16}$	602	218, 285, 330		√		Lee et al., 2000
Quercetin	27.7	$C_{15}H_{10}O_7$	302	203, 257, 375		√	√	Sochor et al., 2014

PF1:ethanol maceration of fresh leaves; I:fraction H_2O; II:fraction 80% EtOH; III:fraction 100% EtOH. (*) or isomer.

All fractions were tested for their biological properties (Table 9.30).

In ABTS test, the fractions (IC_{50} values in the range from 0.20 to 0.62 μg/mL) exhibited greater ABTS radicals scavenging activity than the positive control (IC_{50} of 1.71 μg/mL). PF1 (II) was the most promising fraction also in DPPH assay with the highest activity (IC_{50} value of 8.94μg/mL). Interesting data were reported also for SD2 (II). The greater activity of this fraction compared to SD2 (III) can be linked to the presence of cornuside, which was not identified in SD2 (III). In ABTS test, fractions presented comparable IC_{50} values (0.58 and 0.62 μg/mL, for SD2 (II) and SD2 (III) respectively), while significant difference was reported in DPPH test (Figure 9.24).

A two fold higher antiradical activity was registered with SD2 (II) (IC_{50} value of 55.04 μg/mL) compared to SD2 (III) (IC_{50} value of 120.68 μg/mL).

The antioxidant activity was assessed also by FRAP assay that evaluates the ability the reducing potential of bioactive compounds. The reducing ability was related with presence of compounds giving a hydrogen atom to free radical, breaking free radical chain (Duh et al., 1999). The ferric reducing ability of fractions was expressed as FRAP values

(*μ*M Fe(II)/g). SD2 (II) showed high FRAP value of 33.03 *μ*M Fe(II)/g, while SD2 (III) lower activity with value of 5.01 *μ*M Fe(II)/g.

Table 9.30. Antioxidant profile of *C. sanguinea* flavonoids and iridoids enriched-fractions

C. sanguinea	ABTS test	DPPH test	FRAP test[#]	*β*-Carotene bleaching test	
	(IC_{50} *μ*g/mL)	(IC_{50} *μ*g/mL)	(*μ*M Fe(II)/g)	(IC_{50} *μ*g/mL)	
				30 min	60 min
Fruits Fractions					
SD2 (II)	0.58 ± 0.06****	55.04 ± 1.64****	33.03 ± 0.68****	13.19 ± 0.40****	20.98 ± 1.98****
SD2 (III)	0.62 ± 0.04****	120.68 ± 9.45****	5.01 ± 0.75****	90.52 ± 5.69****	41.86%[a]
Leaves Fractions					
PF1 (II)	0.20 ± 0.03****	8.94 ± 0.29	102.17 ± 1.14****	8.40 ± 0.89*	12.72 ± 0.20****
PF1 (III)	0.50 ± 0.07****	16.93 ± 0.99***	60.41 ± 0.40	10.75 ± 0.70***	15.82 ± 0.50****
Positive control					
Ascorbic acid	1.71 ± 0.22	5.01 ± 0.84			
BHT			63.20 ± 4.32		
Propyl gallate				1.01 ± 0.01	1.02 ± 0.01

Data are expressed as means ± S.D. (n= 3). [a] at concentration of 100 *μ*g/mL. [#]at the concentration of 1 mg/mL. Ascorbic acid, BHT and propyl gallate are used as positive control in antioxidant tests. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001, **p < 0.01, *p< 0.1 compared with the positive controls.

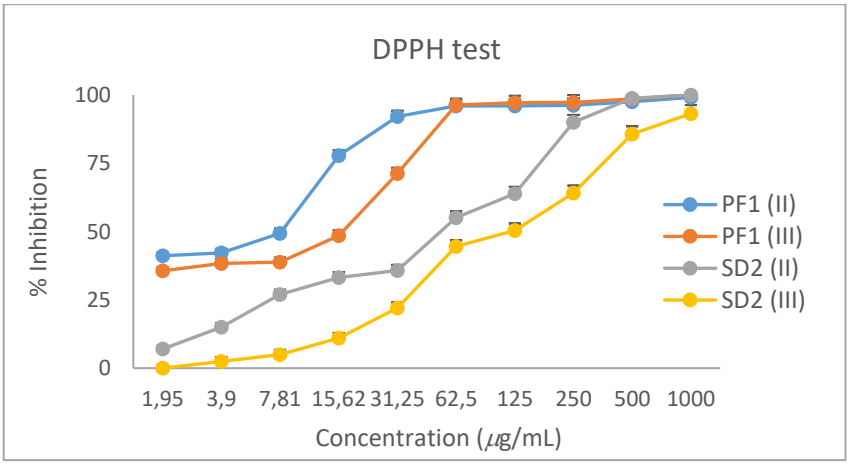


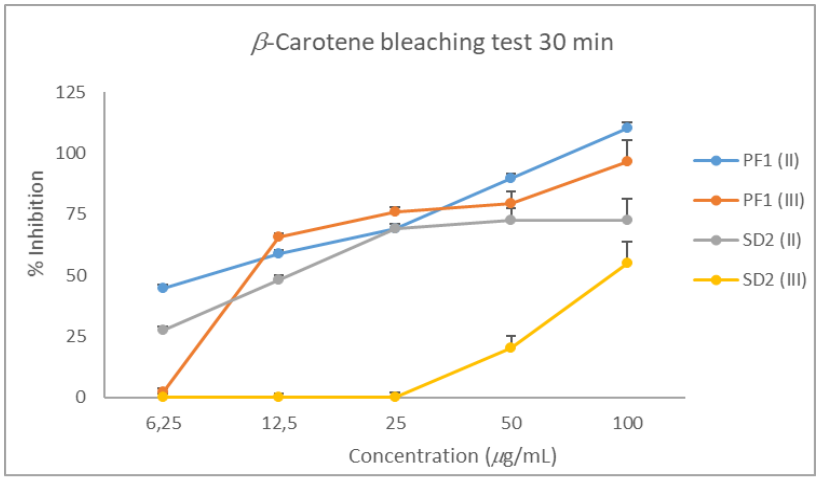
Figure 9.24. DPPH free radical-scavenging activity of fractions PF1 (II), PF1 (III), SD2 (II), and SD2 (III) of *C. sanguinea*. Data are mean ± SD (n = 3).

The high antioxidant activity of SD2 (II) was confirmed also in the *β*-carotene bleaching test with IC_{50} values of 13.19 and 20.98 *μ*g/mL after 30 and 60 min of incubation, respectively (Figure 9.25).

SD2 (III) reported activity after 30 min of incubation with IC_{50} value of 90.52 *μ*g/mL.

In the FRAP test, high ability to iron reduce was reported by PF1 (II) with FRAP value of 102.17 *μ*M Fe(II)/g. This value is better than found with the positive control BHT (63.2 *μ*M Fe(II)/g). PF1 (II) is 1.7 fold more active than PF1 (III) (60.41 *μ*M Fe(II)/g).

a)



b)

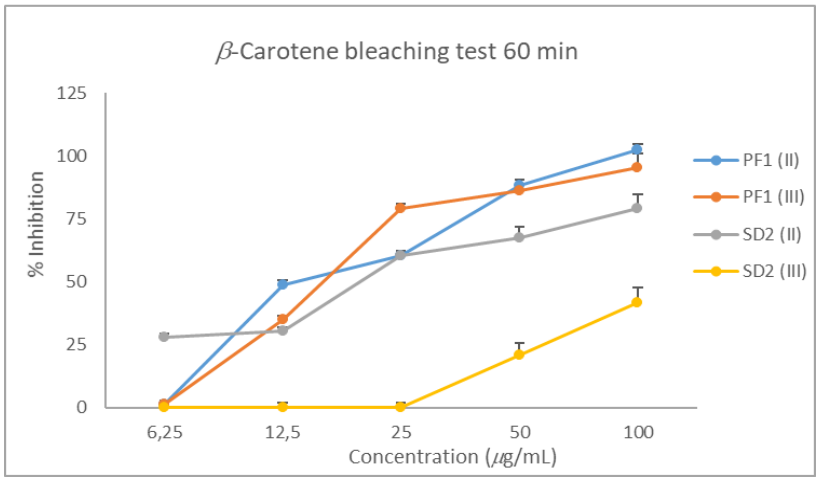


Figure 9.25. Lipid peroxidation inhibition using *β*-carotene-linoleic acid system a) after 30 and b) 60 min of incubation of fractions PF1 (II), PF1 (III), SD2 (II), and SD2 (III) of *C. sanguinea*. Data are mean ± SD (n = 3).

PF1 (II) exerted the highest activity also in the *β*-carotene bleaching test, with IC_{50} values of 8.40 and 12.72 *μ*g/mL at 30 and 60 min of incubation, respectively (Figure 9.26). A promising inhibition of lipid peroxidation was also found for PF1 (III) that showed IC_{50} values of 10.75 and 15.82 *μ*g/mL at 30 and 60 min of incubation, respectively.

An interesting antioxidant activity were reported for PF1 (II) and SD2 (II). In this latter a greater activity can be linked to the presence of cornuside.

The abilities of fruits and leaves fractions to inhibit *α*-amylase and *α*-glucosidase enzymes are presented in Table 9.31.

Table 9.31. Hypoglycaemic activity of fruits and leaves fractions

C. sanguinea	IC_{50}, μg/mL		Selectivity Index (SI)	
	α-Amylase	α-Glucosidase	α-Amylase	α-Glucosidase
Fruits Fractions				
SD2 (II)	165.29 ± 2.52****	NA	-	-
SD2 (III)	52.58 ± 0.87	NA	-	-
Leaves Fractions				
PF1 (II)	858.10 ± 1.08****	143.39 ± 1.84****	0.17	5.98
PF1 (III)	341.34 ± 2.60****	146.02 ± 1.71****	0.43	2.34
Positive control				
Acarbose	50.01 ± 1.43	35.50 ± 1.10	0.41	1.41

Data are expressed as means ± S.D. (n= 3). NA: not active. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001,**p < 0.01, *p< 0.1 compared with the positive controls. . [a]SI for α-amylase is defined as IC_{50} (α-glucosidase)/IC_{50} (α-amylase). [b]SI for α-glucosidase is defined as IC_{50} (α-amylase)/IC_{50} (α-glucosidase).

Both fruits fractions exhibited inhibition of α-amylase with IC_{50} values of 165.29 and 52.58 μg/mL for SD2 (II) and (III), respectively, but resulted inactive against α-glucosidase. Conversely, leaves fractions exhibited an interesting activity of inhibition against α-glucosidase with IC_{50} values of 143.39 and 146.02 μg/mL, for PF1 (II) and PF1 (III), respectively. A weak activity against α-amylase with IC_{50} values of 858.10 and 341.34 μg/mL for PF1 (II) and PF1 (III), respectively, was found.

Inflammatory disorders are characterised among other events, by the production of significant amounts of various pro-inflammatory mediators including TNF-α, PGE2, IL-1β, IL-6, and nitric oxide (NO).

When these mediators are over-produced, they cause excessive inflammatory responses. Thus, inhibition of pro-inflammatory mediator release may be beneficial in attenuating the inflammatory response. NO is a highly reactive oxidant that is produced through the action of *i*NOS and participates in diverse biological effects such as the regulation of vascular inflammation, neurotransmission, and apoptosis. Numerous studies have revealed that excessive NO production is important in the pathogenesis of inflammation and can lead to tissue damage. In fact, several NO induction inhibitors have been reported to exert anti-inflammatory effects.

In order to explore the mechanism underlying the anti-inflammatory effect of *C. sanguinea* fractions, our attention was focused on the NO inhibition and NF-κB signal pathway.

NO released from cells can be detected and quantified spectrophotometrically as its stable product nitrite by a simple colorimetric reaction (Griess reaction) (Dirsch et al., 1998). Table 9.32 summarizes obtained data (IC_{50} values, μg/mL).

All fractions exhibited activity in a concentration-dependent manner. The most interesting results were obtained by SD2 (III) with an IC_{50} value of 9.19 μg/mL, followed by PF1 (III) (IC_{50} value of 9.35 μg/mL).

Table 9.32. Inhibition of NO production by *C. sanguinea* fruits and leaves extracts.

C. sanguinea	IC_{50} μg/mL
Fruits fractions	
SD2 (II)	10.24 ± 1.26
SD2 (III)	9.19 ± 0.92
Leaves fractions	
PF1 (II)	9.81 ± 0.21
PF1 (III)	9.35 ± 0.63

Data are expressed as means ± S.D. (*n*= 3).

Noteworthy is that SD2 (III) that showed a weak antioxidant activity exhibited the highest inhibitory effects of NO production.

To assess any potential toxic effects, fractions and pure compounds were subjected to MTT test. Data were reported in Figures 9.26 and 9.27. SD2 (II) and SD2 (III) at lower concentrations (2.5-12.5-25 μg/mL) showed no significant cytotoxic effects. A decrease in cell viability was detected only at the concentration of 250 μg/mL with 60 and 36% reduction for SD2 (II) and SD2 (III), respectively.

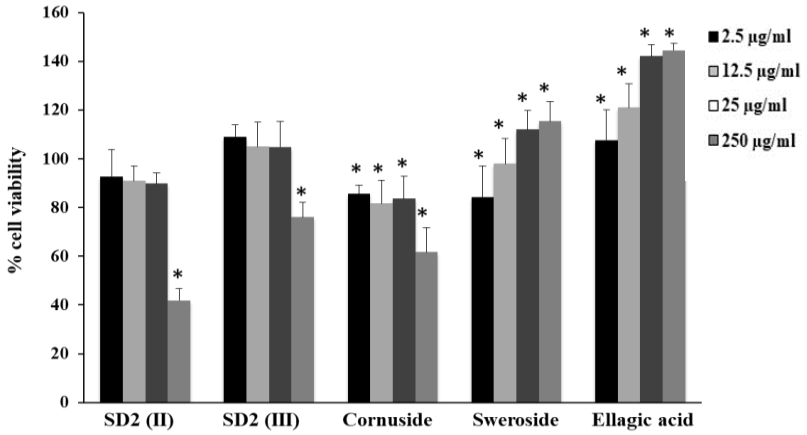


Figure 9.26. Cell viability in HFF1 cells untreated and treated for 24h with *C. sanguinea* fruits fractions and pure compounds at different concentrations (12.5-250 μg/mL). Values are the mean ± S.D. of four experiments in triplicate. Control cells were incubated only with medium and considered as 100% of cell viability. *Significant *vs* untreated control cells and *vs* other concentrations of the same extract $p< 0.001$.

PF1 (II) and PF1 (III) did not affect cell viability at the lower concentrations (2.5-12.5-25 μg/mL) but significantly reduced cell viability only at the concentration of 250 μg/mL with a decrease of approximately of 42 and 67%, respectively. Both fruits and leaves fractions reported cytotoxic effects only at high concentration tested (250 μg/mL).

Cornuside decreased cell viability at all concentrations tested compared to the untreated control. The decrease was approximately 20% at concentrations of 2.5-12.5-25

μg/mL and reached 40% at 250 μg/mL. Ellagic acid increased cell viability in a concentration-dependent manner, reaching the most significant effect at the highest dose. The exposure to sweroside at the concentration of 2.5 μg/mL reduced cell viability by about 15% while at the concentrations of 12.5-25-250 μg/mL, cell viability increased by about 15% compared to the untreated control cells. Ellagic acid, on the other hand, increased cell viability by 20% at the concentration of 12.5 μg/mL and by 40% at 25 and 250 μg/mL.

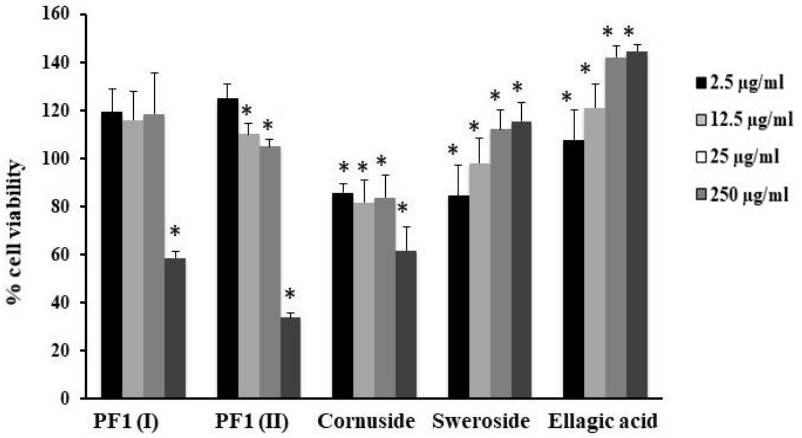


Figure 9.27. Cell viability in HFF1 cells untreated and treated for 24h with *C. sanguinea* leaves fractions and pure compounds at different concentrations (12.5-250 μg/mL). Values are the mean $\pm$ S.D. of four experiments in triplicate. Control cells were incubated only with medium and considered as 100% of cell viability. *Significant *vs* untreated control cells and *vs* other concentrations of the same extract $p< 0.001$.

Recent studies have reported that nuclear factor-kappa B (NF-κB) regulates the expression of inducible nitric oxide synthase (*i*NOS) and cyclooxygenase-2 (COX-2) (Xie et al., 1994). NF-κB is one of ubiquitous eukaryotic transcription factors that regulate gene expression of cytokines and enzymes involved in controlling inflammatory responses. The activation of NF-κB is critically required for the activation of pro-inflammatory mediators in LPS-stimulated macrophages.

Considering the antioxidant properties and the effects exerted by PF1 (II) and SD2 (II) fractions on NO production, we tested the ability of these two selected fractions to reduce the activation of NF-κB in LPS-stimulated RAW 264.7 cells.

In particular, after the treatment of LPS-stimulated RAW 264.7 cells, the translocation of NF-κB into the nucleus was monitored. As shown in figure 9.28, CTRL cells appears with no fluorescence into the nuclei reflecting the NF-κB inactivation. At the same time, CTRL LPS cells appears with cytoplasmic and nuclear fluorescence, confirming the translocation of NF-κB into the nuclei.

The results obtained in this study highlighted the ability of these fractions to reduce the LPS-induced translocation of NF-κB into the nucleus (Figure 9.28) that was more marked for SD2 (II) than PF1 (II) suggesting a different ability to reduce the NF-κB translocation.

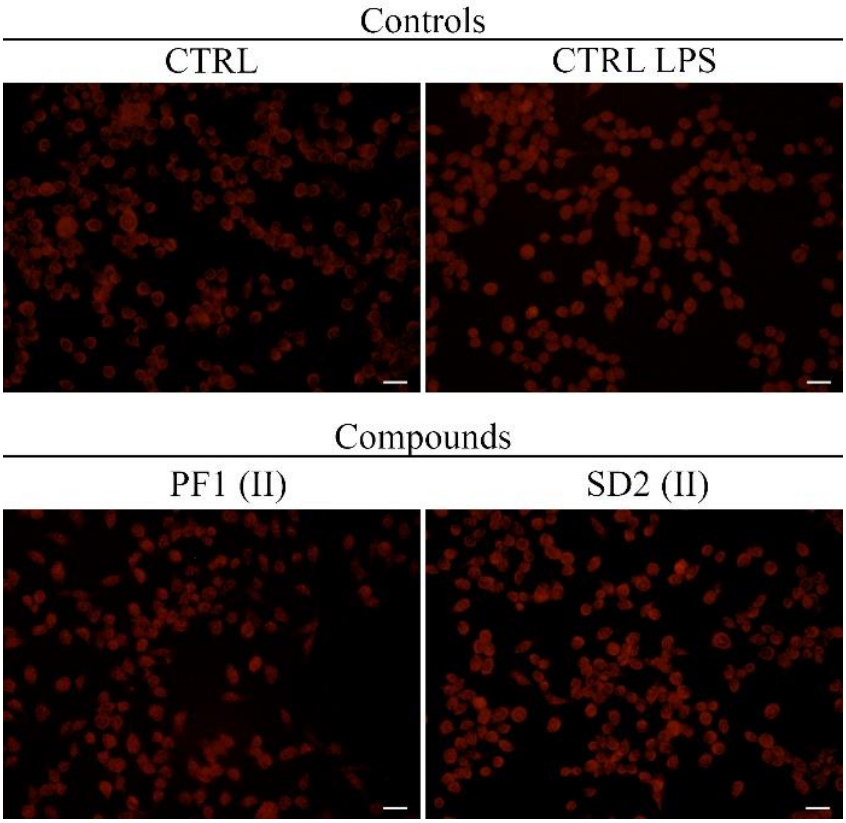


Figure 9.28. Immuno-fluorescent localization of NF-κB in RAW 264.7 cells treated for 1 h with DMSO (CTRL), 1 μg/mL LPS + DMSO (CTRL LPS), 1 μg/mL LPS + PF1 (II) at IC_{50} value (PF1 (II)), 1 μg/mL LPS + SD2 (II) at IC_{50} value (SD2 (II)). Scale bar: 25 μm.

To check the toxicity direct on blood cells, in particular on red blood cells (RBCs), PF1 (II) and SD2 (II) fractions were subjected to haemolysis assay on human blood. The fractions did not show a significant release of haemoglobin, resulting non-toxic towards the RBCs. PF1 (II) exhibited a percentage of haemolysis of 0.20 and 0.96% after 1 and 24 h, respectively. Comparable results were obtained with SD2 (II) fraction (percentage of haemolysis of 0.20 and 1.18% after 1 and 24 h, respectively).

Cornuside is the secoiridoid glucoside that specifically characterised this fraction. Previous studies have been demonstrated that cornuside inhibited LPS-induced NO production in cultured macrophages and suppressed the expression of cytokine-induced pro-inflammatory and adhesion molecules in human endothelial cells, and protected cultured rat cortical cells against damage induced by oxygen-glucose deprivation (Kang et al., 2007; Jiang et al., 2009). Choi et al. (2011) demonstrated that cornuside inhibited NF-κB activation by suppressing the phosphorylation and degradation of IkB-α, and the subsequent translocation of the p65 subunit of NF-κB from the cytosol to the nucleus in LPS-induced RAW 264.7 cells. The interesting properties of cornuside were confirmed in several other studied. Song et al. (2014) evaluated the protective effect of cornuside against carbon tetrachloride (CCl_4) in hepatic injury. The hepatotoxicity induced by CCl_4 is associated with pro-inflammatory mediator production; the treatment with cornuside

decreased TNF-α levels, suppressing its production from Kupffer cells and thus protecting the liver against hepatotoxicity. The effects of cornuside were evaluated on rat peritoneal mast cells, indicating none cytotoxicity after a treatment at 100 μM and inhibiting histamine release at 30 μM. In the human mast cells, cornuside locked the production and secretion of pro-inflammatory cytokines (Li et al., 2016). *In vivo* studies demonstrated that the anti-inflammatory properties of cornuside might protect the heart from myocardial ischemia and reperfusion (Jiang et al., 2011). The treatment with cornuside improved the myocardial function in rats with decrease of infarct size compared with control group (rats with myocardial ischemia and reperfusion), furthermore cornuside at 20 or 40 mg/kg concentration reduced TNF-α, IL-6, and troponin-t (Tn-T; myocardial damage biomarker) levels. Yang et al. (2016) have evaluated the effects of sweroside on pro-inflammatory responses induced by cholestatic liver in mice. Cholestatic liver is characterised by increased transaminases levels, alteration of bile acid flux with overload in the liver, and release of pro-inflammatory cytokines.

9.3. Conclusion

In this chapter, the chemical characterisation and the *in vitro* biological properties of *C. mas* and *C. sanguinea* fruits and leaves extracts were reported. The beneficial health effects of the fruits extracts of both *Cornus* species are correlated to their chemical compounds, in particular iridoids and flavonoids. However, leaves, until now considered vegetable by-products, may be a potential source of phytochemicals with hypoglycaemic, anti-inflammatory, and antioxidant properties, confirming the role of plant-derived compounds as a source of bioactive drugs. Interesting results were in fact obtained with the leaves extracts.

The first goal of this work is the identification of the extraction procedures that allow to well preserve and isolate the most bioactive compounds of *Cornus* species.

Hydroalcoholic maceration of *C. mas* dried fruits and dried leaves and hydroalcoholic maceration of *C. sanguinea* dried fruits and ethanol maceration of fresh leaves resulted the most promising procedure to obtain extracts characterised by the highest of bioactivity in terms of antioxidant effects, carbohydrate-hydrolysing enzyme inhibitory activity, and inhibition of NO production. Interestingly, leaves extracts resulted more active compared with fruits extracts. All samples were able to reduce NO production. Generally, extracts of both *Cornus* species showed high inhibition against α-glucosidase and moderate inhibition against α-amylase. This selectivity can will be considerate an election characteristic for products employed in the treatment of type 2 diabetes because high inhibition against α-amylase could cause a possible accumulation of undigested starch in the colon with stomach distension.

The second goal of this investigation is related to the enhanced bioactivity when selected *C. mas* and *C. sanguinea* extracts are enriched in flavonoids and iridoids content by the use of selective resins. The most promising results were obtained by *C. sanguinea* fractions, SD2 (II) and PF1 (II).

The observed activities of *Cornus* species provide some basic evidence for the potential biological effects of flavonoids and iridoids enriched-fractions and suggest that studies of *Cornus* extracts should be carried out in appropriate animal models and ultimately in human prevention trials of degenerative diseases such as type 2 diabetes.

Moreover, taking into account that *Cornus* leaves are a very rich source of bioactive compounds and that are considered as agrochemical waste, it is possible to prospect an innovative valorisation of these by-products as nutraceutical-pharmaceutical agents.

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General conclusions and future perspectives

In the field of drugs discovery and development, natural products have proven to be a rich source of therapeutic compounds. Over the last 30 years, there is a great number natural-derived new drug discovered, mainly because of intense studies conducted by pharmaceutical company and academia. In recent years, fruits and vegetables have been consistently reported in epidemiological surveys as naturally rich sources of antioxidants, helping to reduce the incidence of several degenerative diseases, besides reducing the mortality risks. Evidences point out that the antioxidant activity of fruits extracts is due to the additive and synergistic effects of phytochemicals present in the fruits, which might help to explain why no single synthetic antioxidant is able to replace the combination of natural phytochemicals found in fruits in achieving the desired health benefits.

Despite the many previous studies dealing with the composition of *A. unedo*, *V. corymbosum*, *C. mas*, and *C. sanguinea* species, the goal of the present research is to investigate the contribution of flavonoids and iridoids to the biological properties of the selected species, exploring the most promising extractive techniques and analysing some *in vitro* biological properties of fruits and leaves.

Leaves are considered by-products of berries cultivation. However, the scientific interest regarding their composition and beneficial properties grows, documenting that leaves may be considered an alternative source of bioactive compounds. In fact, analytical studies reveal that the leaves chemical composition is similar to that of the fruits or even richer and higher, indicating that they may be used as an alternative source of bioactive compounds for the development of functional foods, nutraceuticals, and/or food supplements. Few studies present in literature investigated these by-products of *A. unedo*, *V. corymbosum*, *C. mas*, and *C. sanguinea*.

The analysis of the chemical composition by LC/ESI/QTOF/MS has proved to be a powerful tool for screening extracts for the occurrence of phenolic acids, flavonoids and iridoids. Thus, 20 and 37 compounds were identified in *A. unedo* fruits and leaves, respectively. Of these, naringenin 7-*O*-glucoside, kaempferol 3-*O*-glucoside, isovitexin 7-*O*-glucoside, myricetin 3-*O*-rhamnopyranoside, norbergenin, and myricetin were herein identified for the first time in *A. unedo*. Flavonoids are identified in all extracts. However, there are difference in dependence of the extraction processes. Quercetin derivatives were detected in all extracts. Afzelin, kaempferol 3-*O*-glucoside, naringenin 7-*O*-glucoside, rutin, isovitexin 7-*O*-glucoside, myricetin 3-*O*-xyloside, and kaempferol were found only in the leaves extracts. In contrast to the literature, proanthocyanidins detected in the present study were identified in the leaves, but not in the fruits. In particular, epicatechin-4,6-catechin and epicatechin-4,8-epicatechin were found in all leaves extracts, while gallocatechin was identified in some extracts (FL3, FL4, DL1-3).

Six iridoids were identified in *A. unedo*. Three of these, gardenoside, geniposide and unedoside, are specifically produced by the leaves, while all the others have been found in fruit extracts.

Interesting results were obtained with *V. corymbosum*. Generally, leaves have greater chemical diversity than fruits and only some of the identified compounds are biosynthesized by both fruits and leaves. Five phenolic acids, seven flavonoids, and three iridoids characterised fruits extracts, while six phenolic acids, thirteen flavonoids and four iridoids were reported in leaves extracts. To the best of our knowledge, based on LC-ESI-Q-TOF-MS analyses, geniposide, dihydromonotropein, scandoside, and vaccinoside were detected for the first time in *V. corymbosum*.

Also in the *Cornus* genus leaves extracts resulted richer in bioactive compounds than fruits extracts, with 32 and 16 compounds identified in *C. mas* leaves and fruits, respectively. Of these, the presence of ellagic acid 4-*O*-rutinoside, ethyl caffeate, 3-(3,4,5-trimethoxyphenyl) propanoic acid, kaempferol 3-*O*-glucuronide, kaempferol 3-*O*-rutinoside, and quercetin 3-*O*-*β*-D-glucuronide-6”-methyl ester is described herein for the first time.

In contrast to the literature, the phenolic acids 5-*O*-caffeoylquinic acid, vanillic acid, salicylic acid and *p*-coumaric acid were not detected in this study. Flavonoids represented majority of compounds found in this genus, in particular kaempferol and quercetin derivatives are principal flavonoids detected in fruits and leaves extracts. Six iridoids loganin, loganic acid, sweroside, cornuside, secologanin and α-dihydrocorninic acid were found in leaves extracts, while four of these, loganin, loganic acid, sweroside, cornuside characterised fruits extracts. Interesting for the first time in the leaves of *C. mas* were detected major iridoids content respect to fruits extracts, in fact in literature only secologanin was found in the leaves.

The fruits of *C. sanguinea* showed seventeen of compounds. Twenty-nine constituents characterised the leaves extracts. Of these, in literature were found only quercetin glycoside (quercitrin, isoquercetin, hyperoside, rutin and quercetin 3-*O*-glucuronide) in fruits extracts. Consequently, for the first time, comprehensive qualitative analysis and iridoids identification of *C. sanguinea* in leaves and fruit extracts was reported. Quercetin and kaempferol derivatives resulted the main constituents of extracts, together with cornuside and various phenolic acids. In addition, presence of cornoside was detected in *C. sanguinea*. To the best of our knowledge, this is the first report on the iridoids identification in *C. sanguinea*.

Degenerative diseases occur due to the continuous deterioration of cells and tissues, which affects major organs. Both oxidative stress and inflammation are considered major players in the pathogenesis of degenerative diseases, such as type 2 diabetes mellitus (T2DM), and their complications. Growing evidence suggests a positive association between reduction in the incidence of T2DM and the consumption of diets rich in fruits and vegetables, natural source of antioxidant compounds able to protect cell constituents against oxidative damage and to reduce the risk of degenerative diseases related to oxidative stress. Likewise, inflammation is responsible for the pathogenesis of T2DM.

Thus, due to the multifactorial aetiology of T2DM, the search for new extracts/molecules that can act as multi-target agents represents an attractive prospect for the development of new drugs.

A. unedo leaves extracts showed high antioxidant and hypoglycaemic activity compared to fruit extracts. Interestingly, in ABTS and FRAP tests, leaves extracts were more active compared to positive control. Fruit extracts exhibited a high anti-inflammatory activity and moderate toxicity compared to leaves extracts. Furthermore, alcoholic and hydroalcoholic maceration extracts showed the highest ability in reducing mitochondrial dehydrogenases activity.

The extracts obtained by *V. corymbosum* fruits and leaves not presented significant differences in the chemical composition and biological activity excepted for hypoglycaemic potential. Indeed, hydroalcoholic and Soxhlet extracts of fresh leaves and dried leaves showed against α-amylase an inhibition 2.4 and 3 times higher than positive control. While, against α-glucosidase, decoction of dried leaves exhibited the highest inhibitory activity, 4.4 times higher than positive control. In addition, promising inhibition of NO production was found in all samples. In particular, highest activity was observed with decoction of fruits. Generally, the biological evaluation demonstrated the implication of phenolic compounds and iridoids. Considering that *V. corymbosum* fruits are edible, their consumption may be helpful for the treatment of chronic disorders.

The *in vitro* biological properties of *C. mas* and *C. sanguinea* fruits and leaves reveled the highest promising biological activity. Hydroalcoholic maceration of *C. mas* dried fruits and dried leaves and hydroalcoholic maceration of *C. sanguinea* dried fruits and ethanol maceration of fresh leaves resulted the most promising procedure to obtain extracts characterised by the highest of bioactivity in terms of antioxidant effects, carbohydrates-hydrolysing enzymes inhibitory activity, and inhibition of NO production.

Interestingly, leaves extracts resulted more active compared with fruits extracts. All samples were able to reduce NO production.

Extracts of both *Cornus* species showed high inhibition against *α*-glucosidase and moderate inhibition against *α*-amylase. This selectivity can will be considerate an election characteristic for products employed in the treatment of type 2 diabetes because high inhibition against *α*-amylase could cause a possible accumulation of undigested starch in the colon with stomach distension.

The contribution of flavonoids and iridoids to biological activity was evaluated on the most promising *C. mas* and *C. sanguinea* extracts by the use of selective resins.

The most promising results were obtained by *C. sanguinea* fractions, SD2 (II) and PF1 (II).

In conclusion, relevant are the multi-target properties of *C. sanguinea* and *C. mas* fractions that may allow them to be potential agents in prevention and treatment of the degenerative diseases related to oxidative stress such as type 2 diabetes.

The analytical method applied in the present research study could provide new and interesting chemical information on *A. unedo, V. corymbosum, C. mas,* and *C. sanguinea*

fruits and leaves composition, which may be useful for further research into understanding the effects of these plants on humans. Moreover, the information presented should help consumers and food technologists to become aware of the benefits of using these traditionally used plants in current diets as potential sources of healthy compounds.

Nevertheless, analysis reported in this study let understanding that a general extraction method does not seem to exist to obtain the most active extracts for all plants, but each plants have to be considered on a case by case basis.

Furthermore, future studies should focus on determination in vivo of real efficacy of different extracts and on the contribution of the identified compounds to the biological activity to prospect a potential use as nutraceuticals and/or functional foods.

Appendix

Legend of Figures A1-A19

A1. Chromatogram of the extract of *A. unedo* obtained by ethanol maceration of dried leaves (DL1).

A2. Chromatogram of the extract of *A. unedo* obtained by ethanol maceration of fresh fruits (FF1).

A3. Chromatogram of the extract of *A. unedo* obtained by ethanol Soxhlet apparatus extraction of dried fruits (DF4).

A4. Chromatogram of the extract of *V. corymbosum* obtained by ethanol maceration of fresh leaves (L1A).

A5. Chromatogram of the extract of *V. corymbosum* obtained by ethanol maceration of fresh fruits (F1A).

A6. Chromatogram of the extract of *C. mas* obtained by ethanol maceration of fresh leaves (TFA)

A7. Chromatogram of the extract of *C. mas* obtained by hydroalcoholic maceration of dried leaves (TDB).

A8. Chromatogram of the extract of *C. mas* obtained by hydroalcoholic maceration of dried fruits (MDB).

A9. Chromatogram of the extract of *C. sanguinea* obtained by ethanol maceration of fresh leaves (PF1).

A10. Chromatogram of the extract of *C. sanguinea* obtained by ethanol ultrasound assisted-maceration of fresh leaves (PF5).

A11. Chromatogram of the extract of *C. sanguinea* obtained by hydroalcoholic maceration of dried leaves (PD2).

A12. Chromatogram of the extract of *C. sanguinea* obtained by decoction of fresh fruits (SF3).

A13. Chromatogram of the extract of *C. sanguinea* obtained by hydroalcoholic maceration of dried fruits (SD2).

A14. Fractions obtained by separation through HLB SPE from the extract of *C. mas* obtained by hydroalcoholic maceration of dried leaves (TDB).

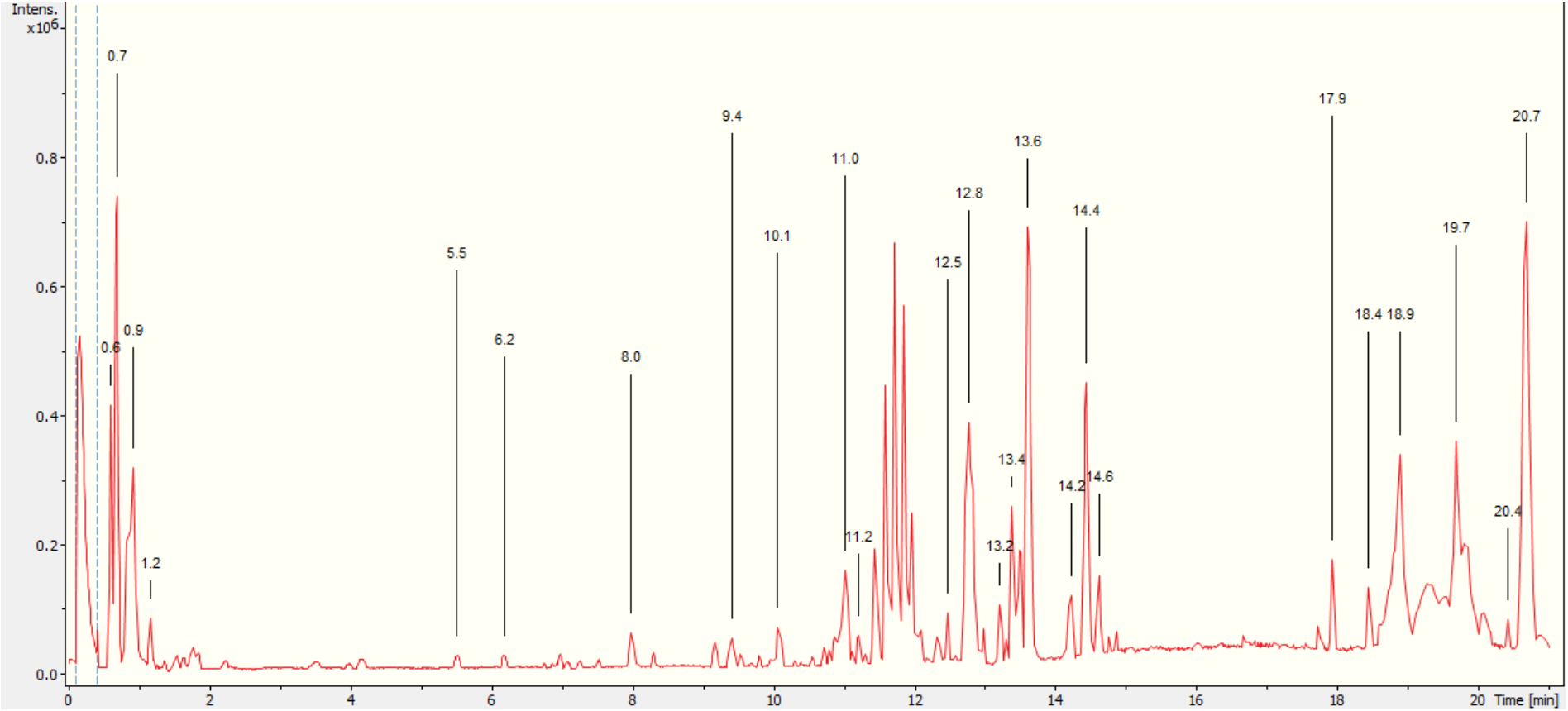
A15. Fractions obtained by separation through XAD-4 from the extract of *C. mas* obtained by hydroalcoholic maceration of dried leaves (TDB).

A16. Fractions obtained by separation through XAD-16 from the extract of *C. mas* obtained by hydroalcoholic maceration of dried leaves (TDB).

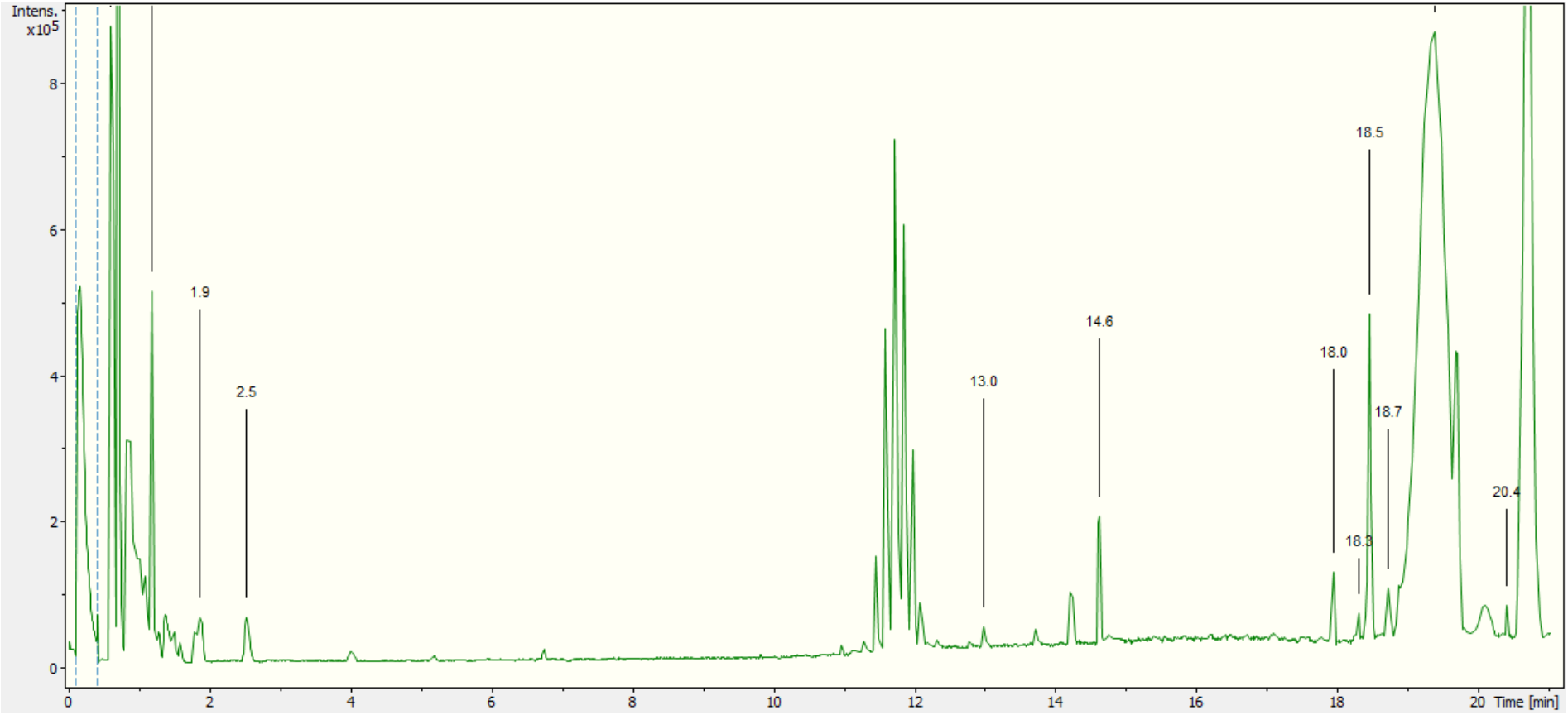
A17. Fractions obtained by separation through XAD-16 from the extract of *C. mas* obtained by hydroalcoholic maceration of dried fruits (MDB).

A18. Fractions obtained by separation through XAD-16 from the extract of *C. sanguinea* obtained by ethanol maceration of fresh leaves (PF1).

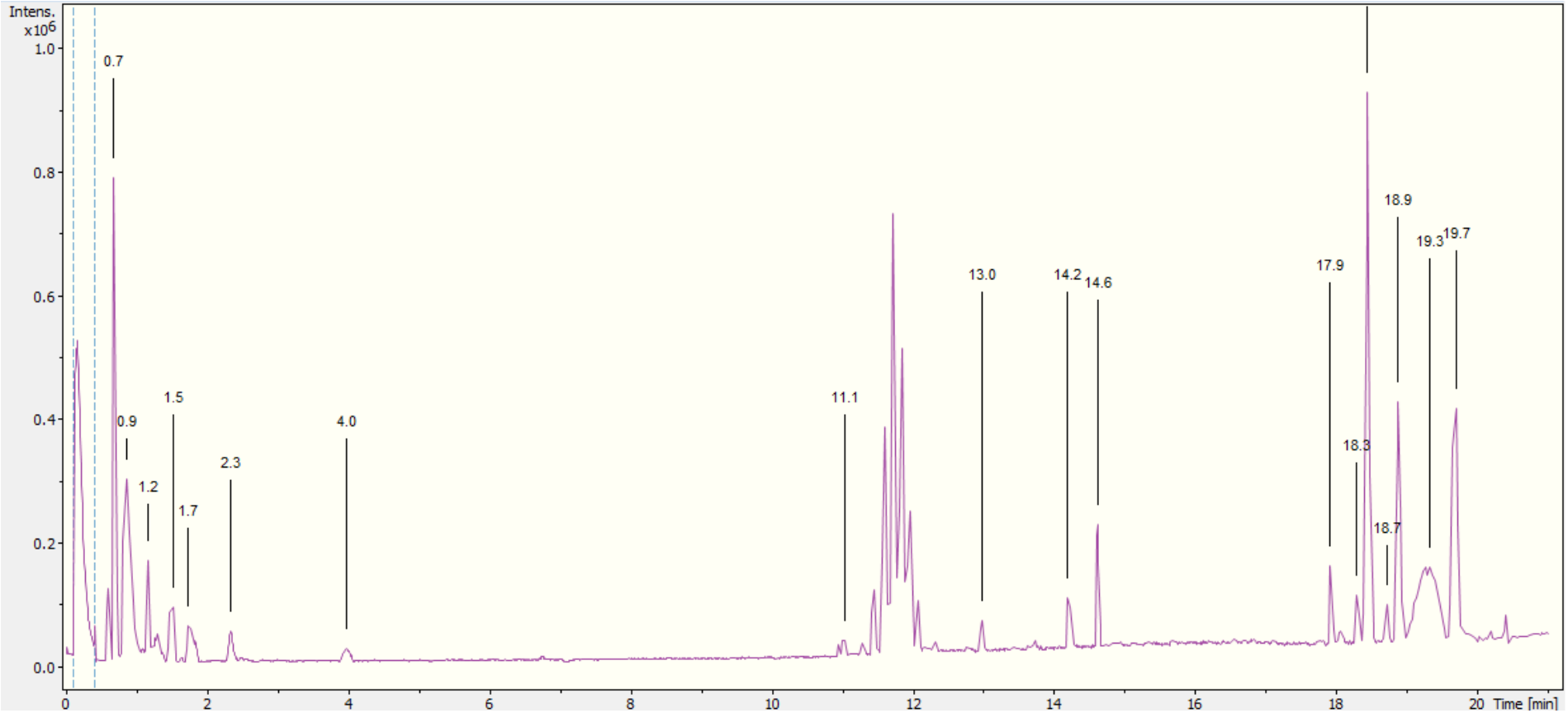
A19. Fractions obtained by separation through XAD-16 from the extract of *C. sanguinea* obtained by hydroalcoholic maceration of dried fruits (SD2).



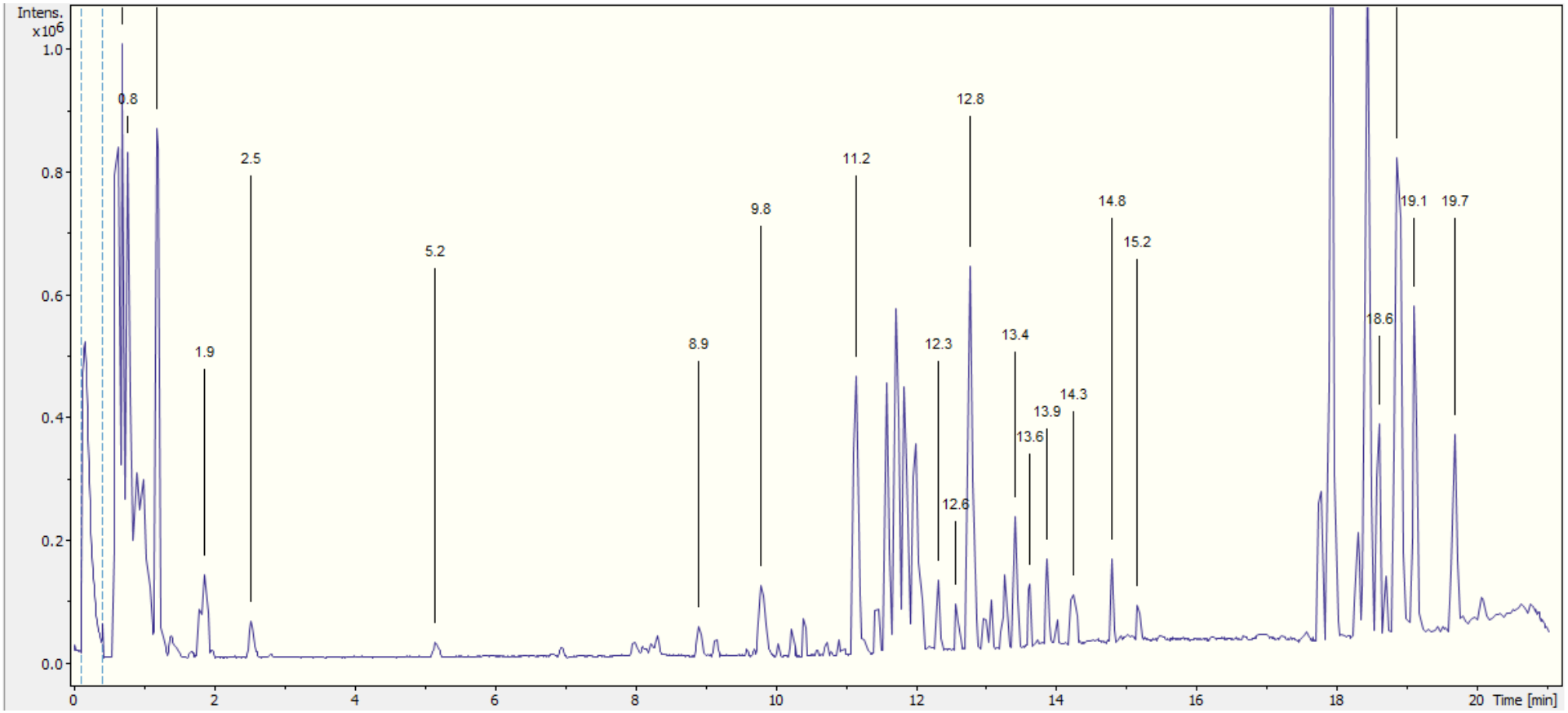
A1. Chromatogram of the extract of *A. unedo* obtained by ethanol maceration of dried leaves (DL1).



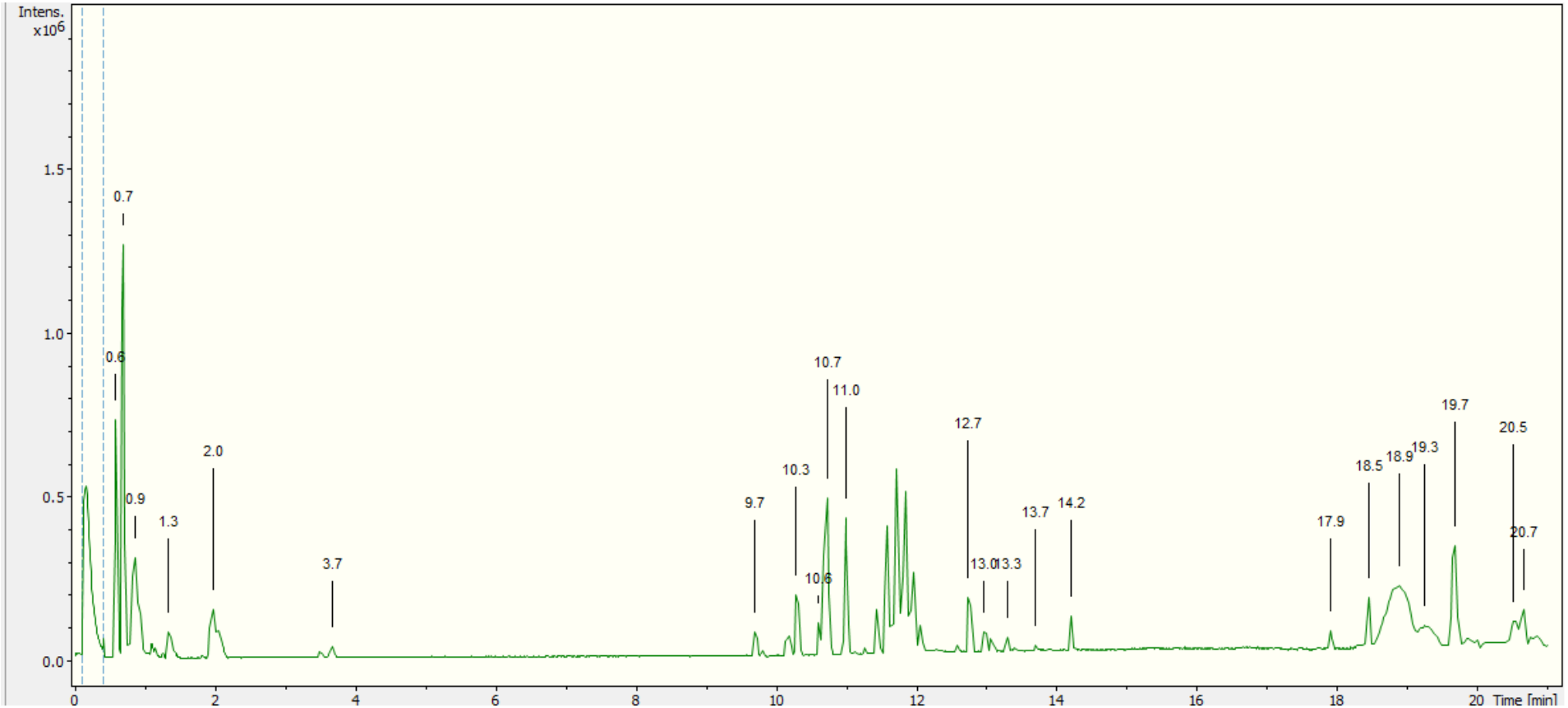
A2. Chromatogram of the extract of *A. unedo* obtained by ethanol maceration of fresh fruits (FF1).



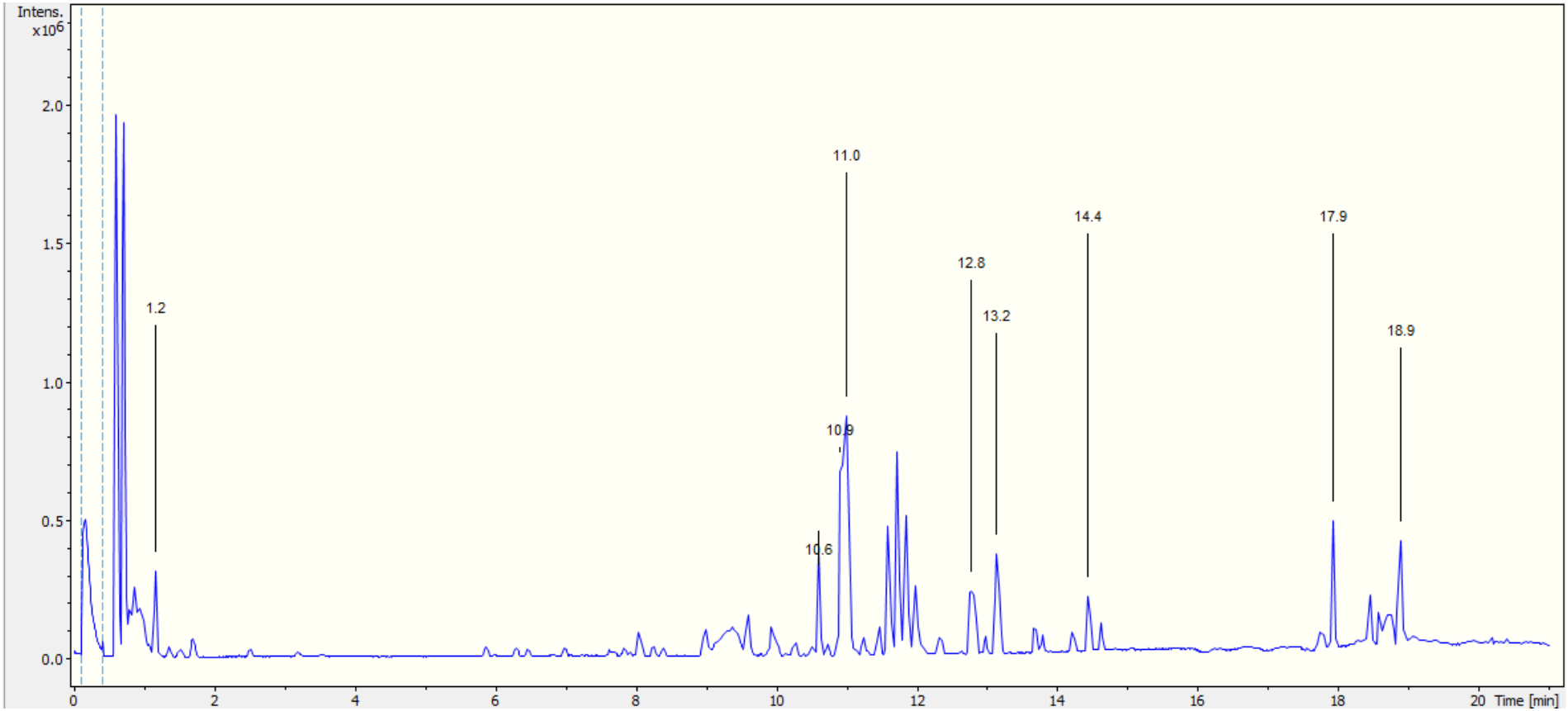
A3. Chromatogram of the extract of *A. unedo* obtained by ethanol Soxhlet apparatus extraction of dried fruits (DF4).



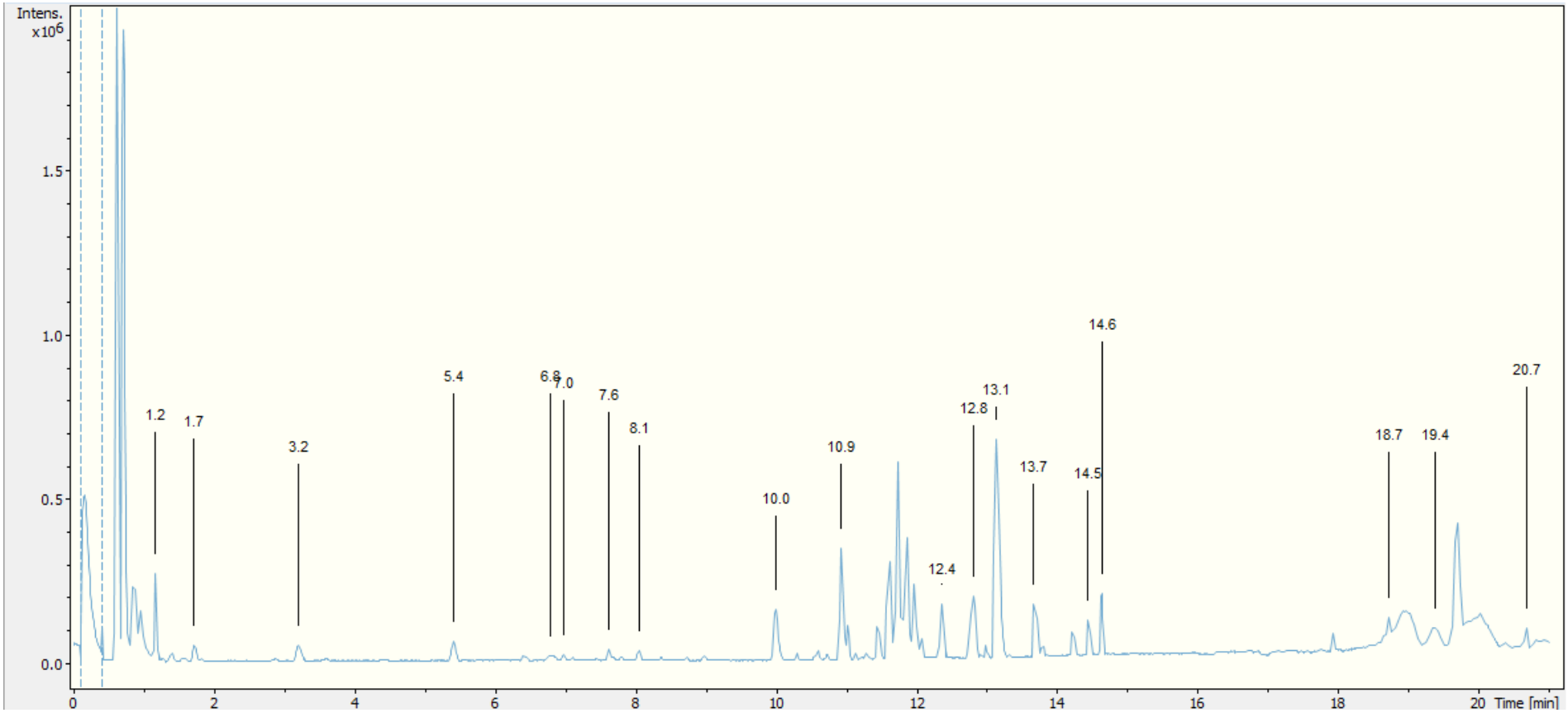
A4. Chromatogram of the extract of *V. corymbosum* obtained by ethanol maceration of fresh leaves (L1A).



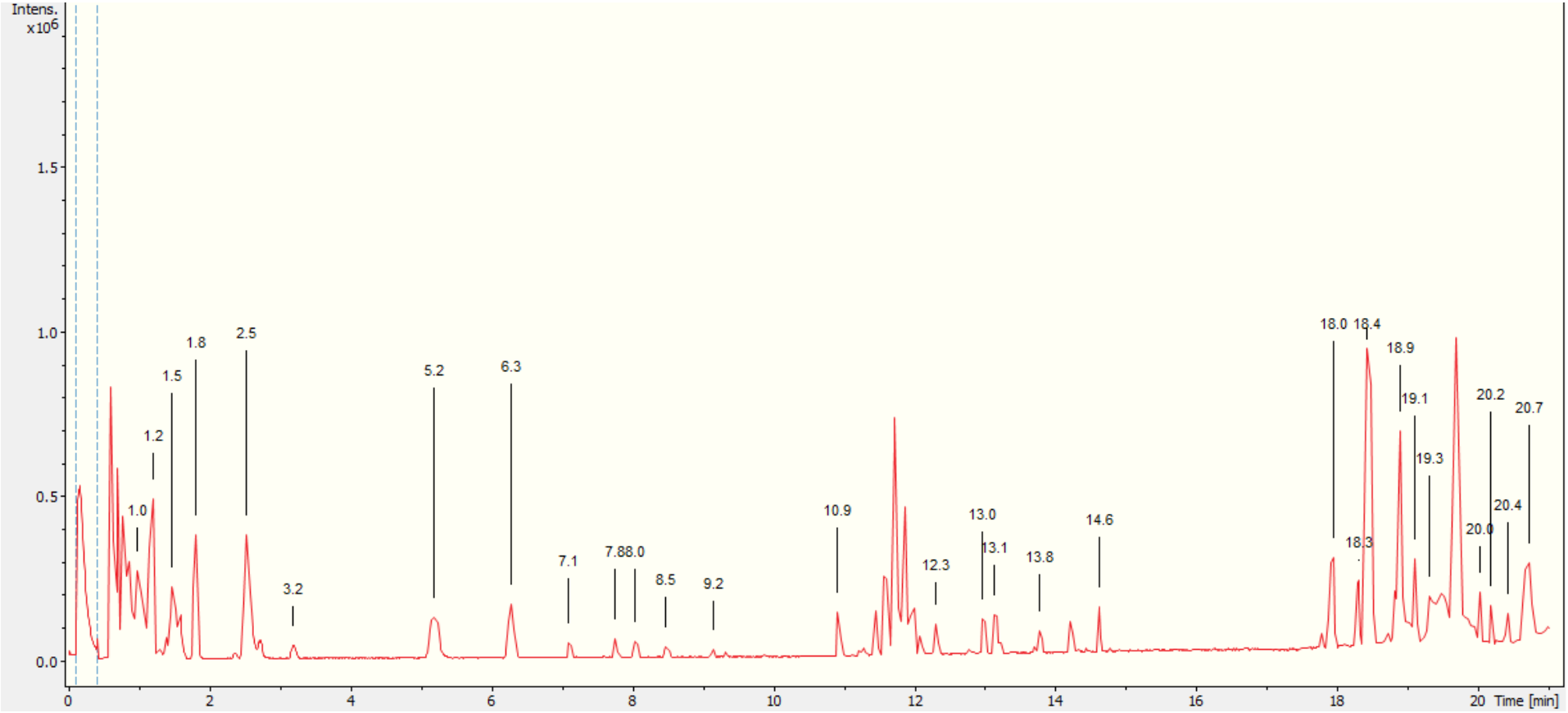
A5. Chromatogram of the extract of *V. corymbosum* obtained by ethanol maceration of fresh fruits (F1A).



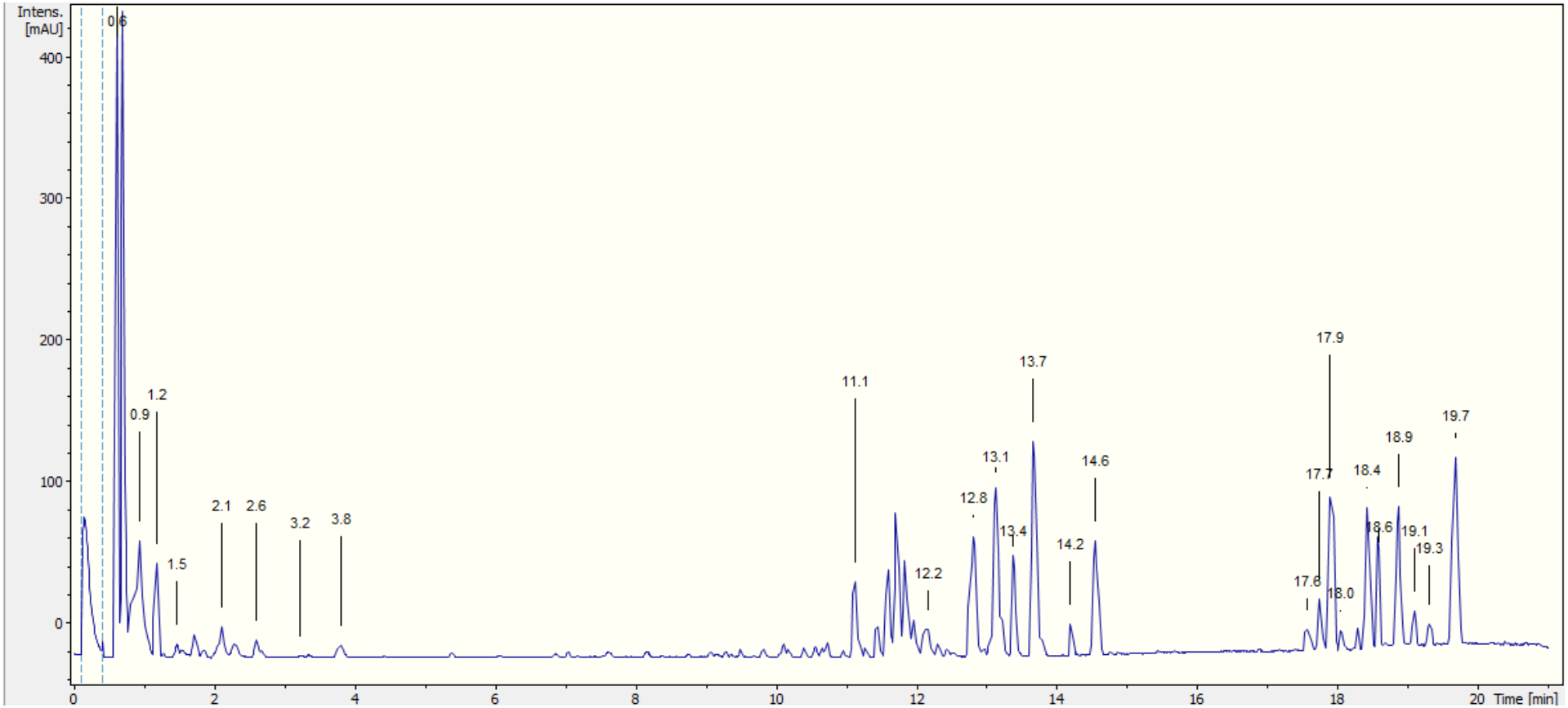
A6. Chromatogram of the extract of *C. mas* obtained by ethanol maceration of fresh leaves (TFA).



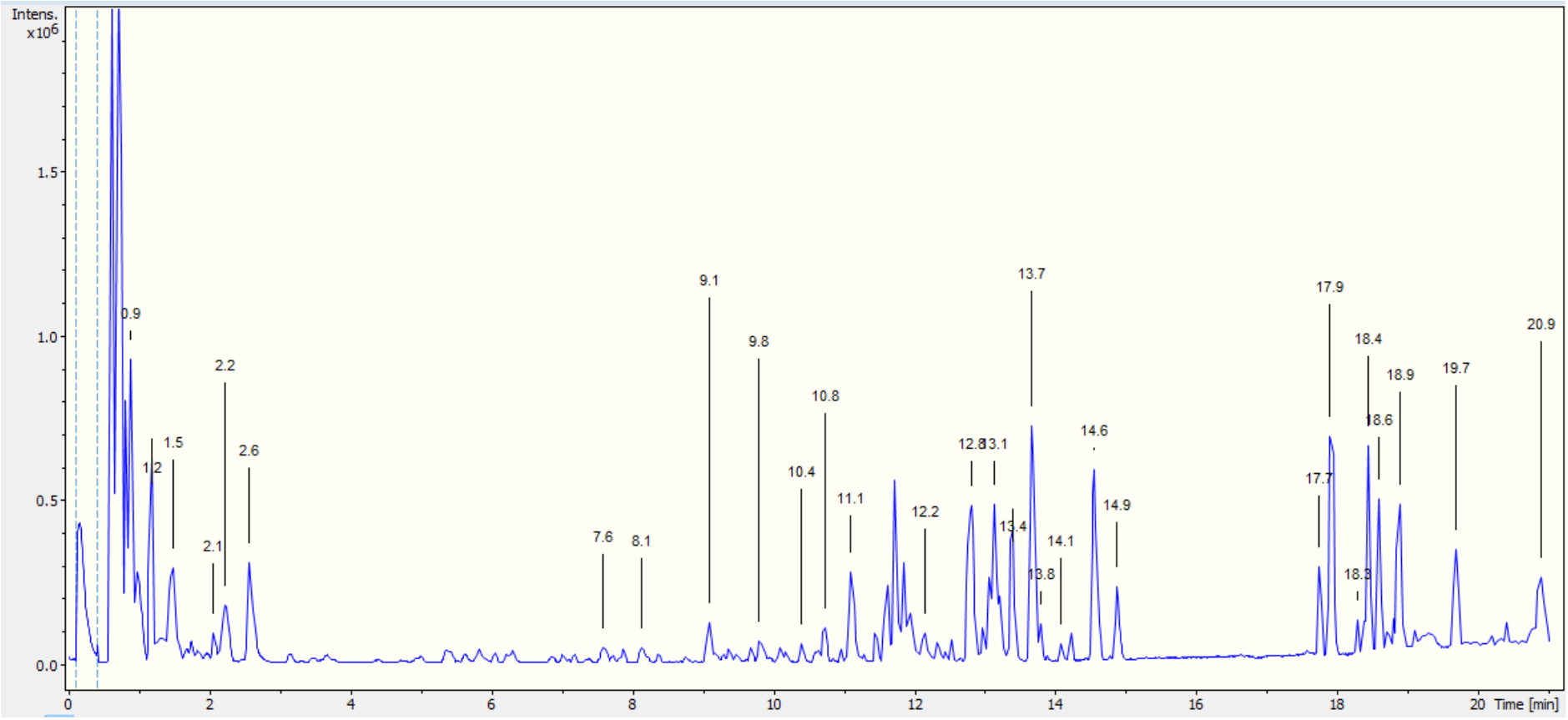
A7. Chromatogram of the extract of *C. mas* obtained by hydroalcoholic maceration of dried leaves (TDB).



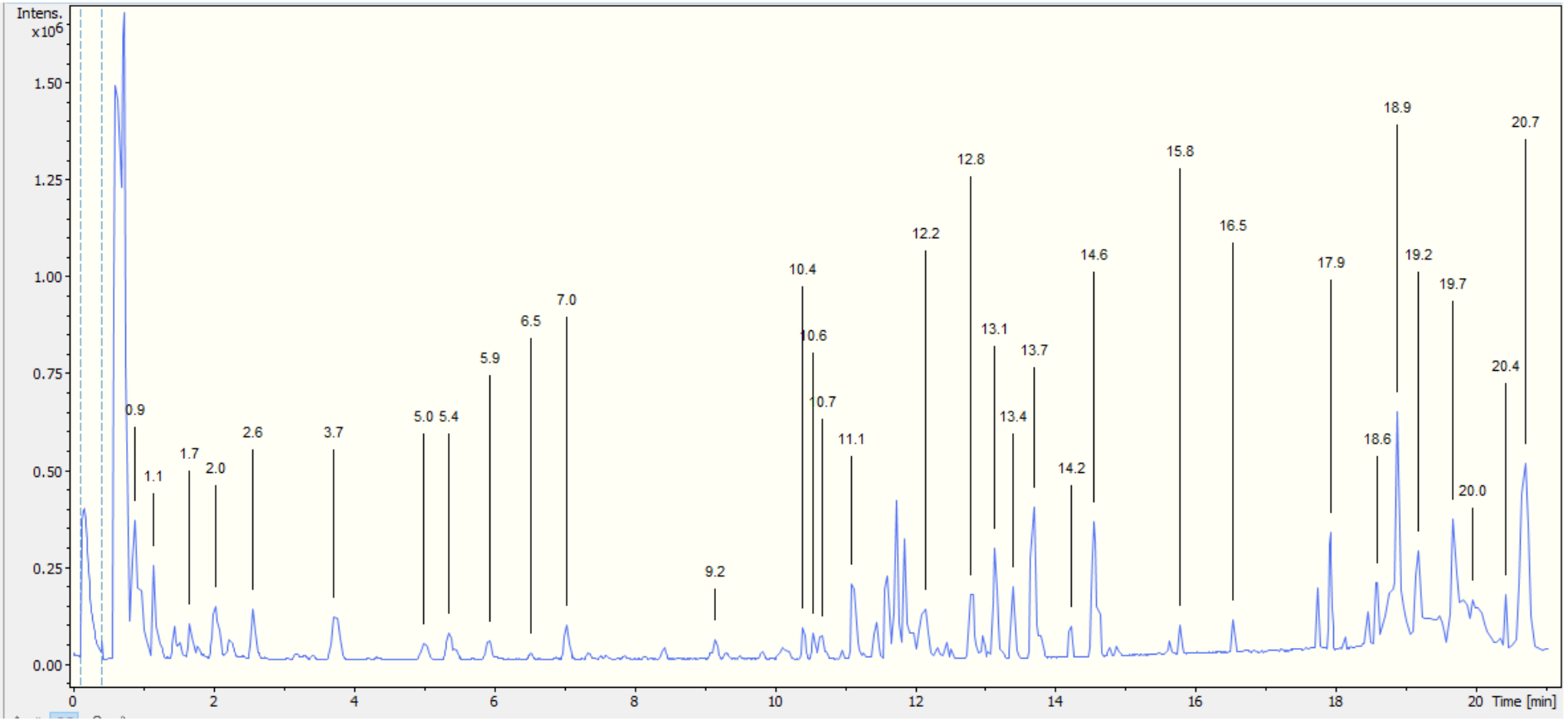
A8. Chromatogram of the extract of *C. mas* obtained by hydroalcoholic maceration of dried fruits (MDB).



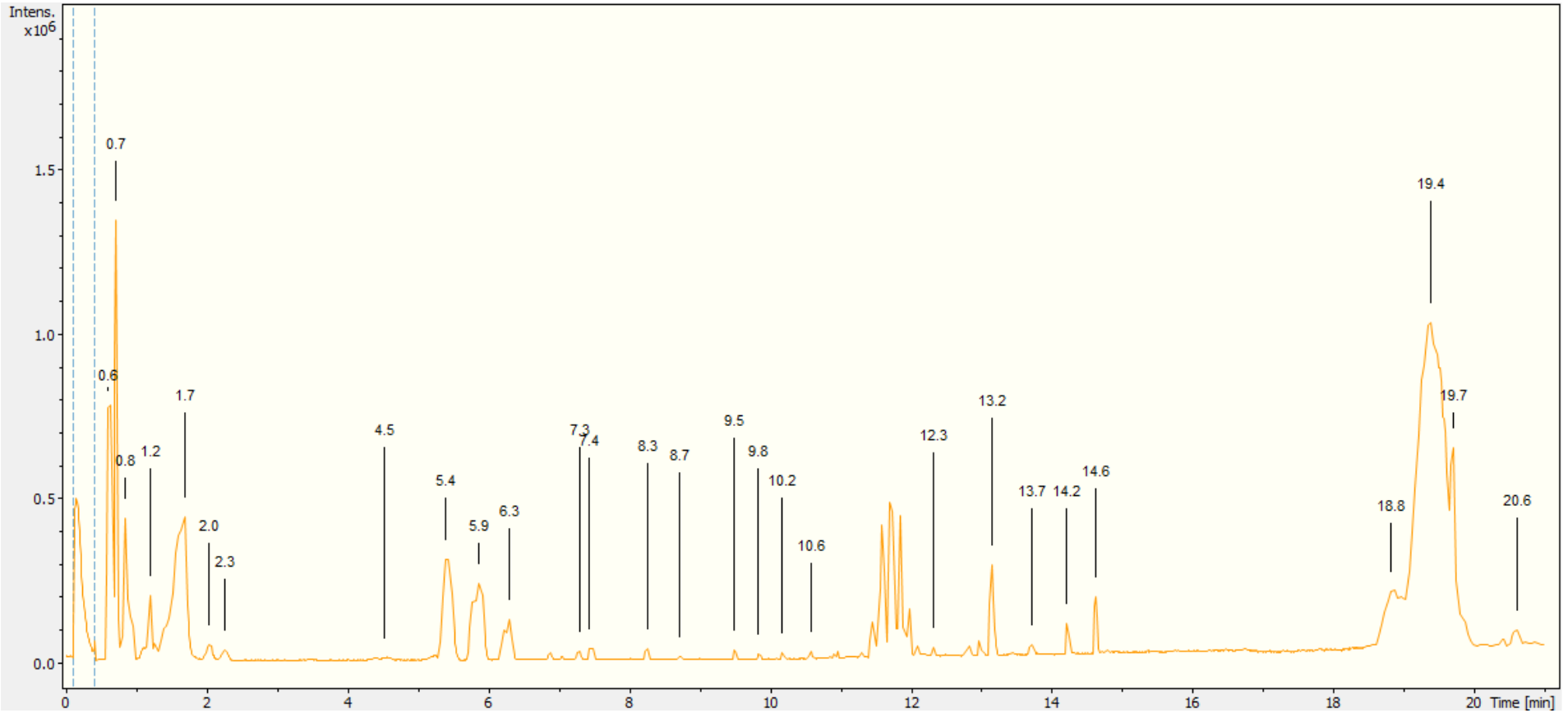
A9. Chromatogram of the extract of *C. sanguinea* obtained by ethanol maceration of fresh leaves (PF1).



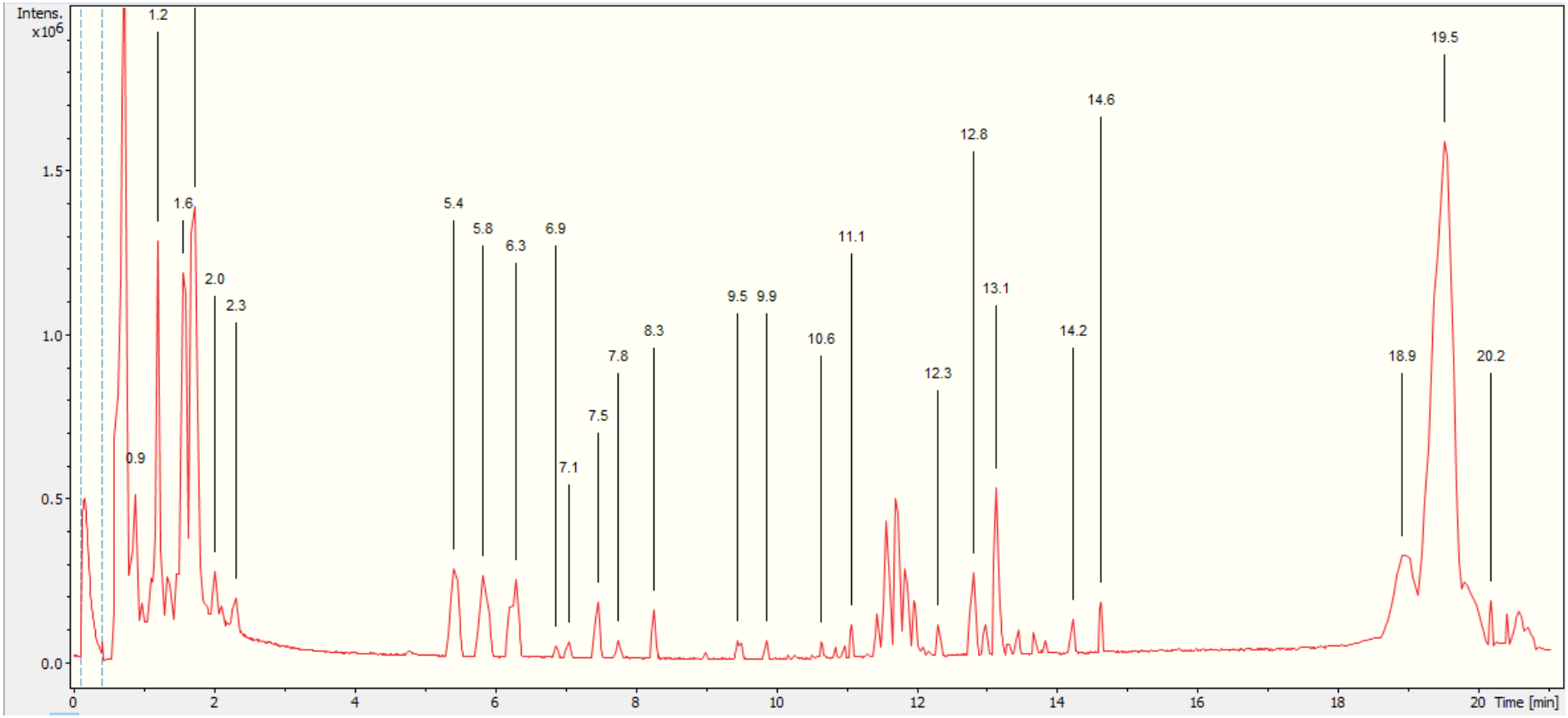
A10. Chromatogram of the extract of *C. sanguinea* obtained by ethanol ultrasound assisted-maceration of fresh leaves (PF5).



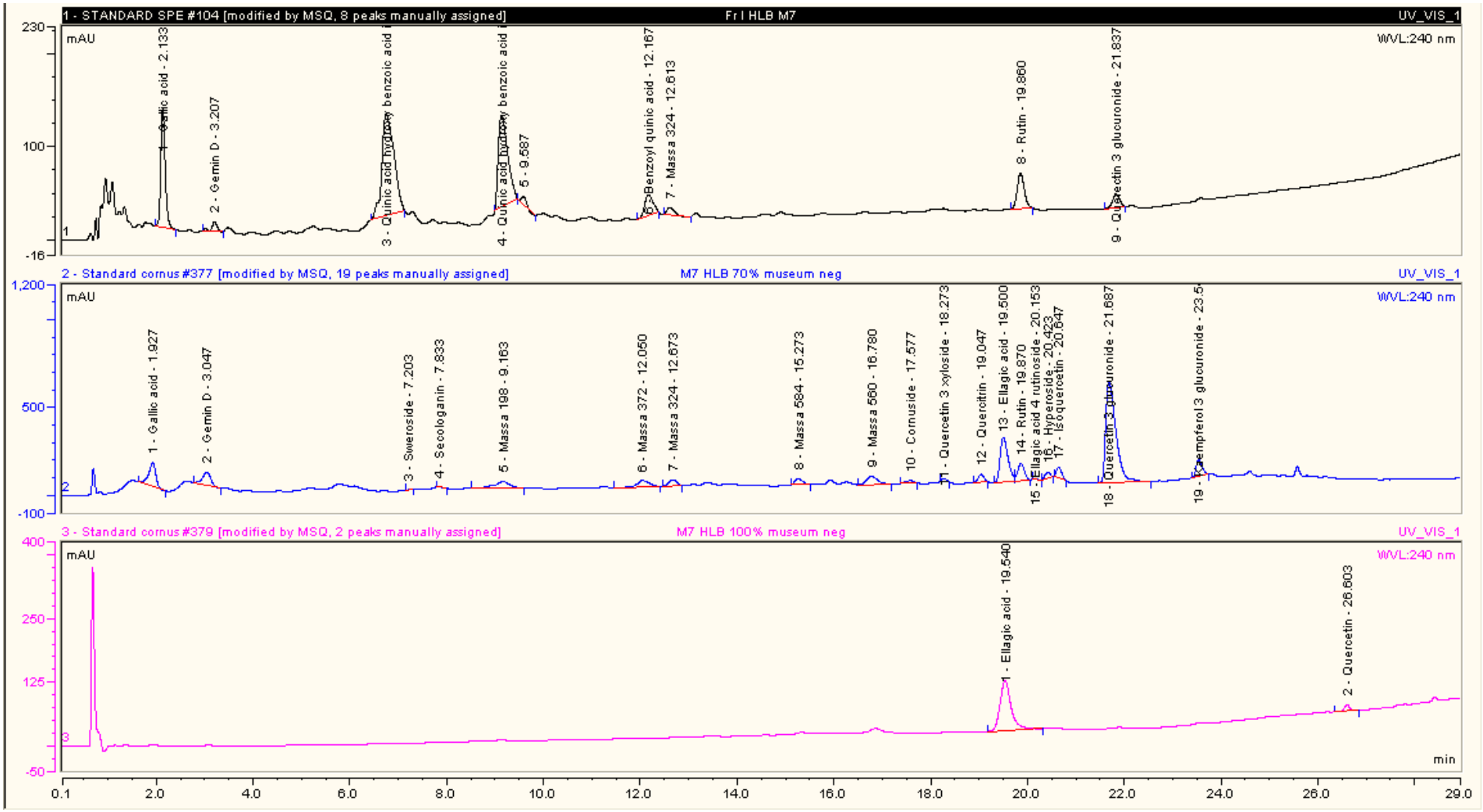
A11. Chromatogram of the extract of *C. sanguinea* obtained by hydroalcoholic maceration of dried leaves (PD2).



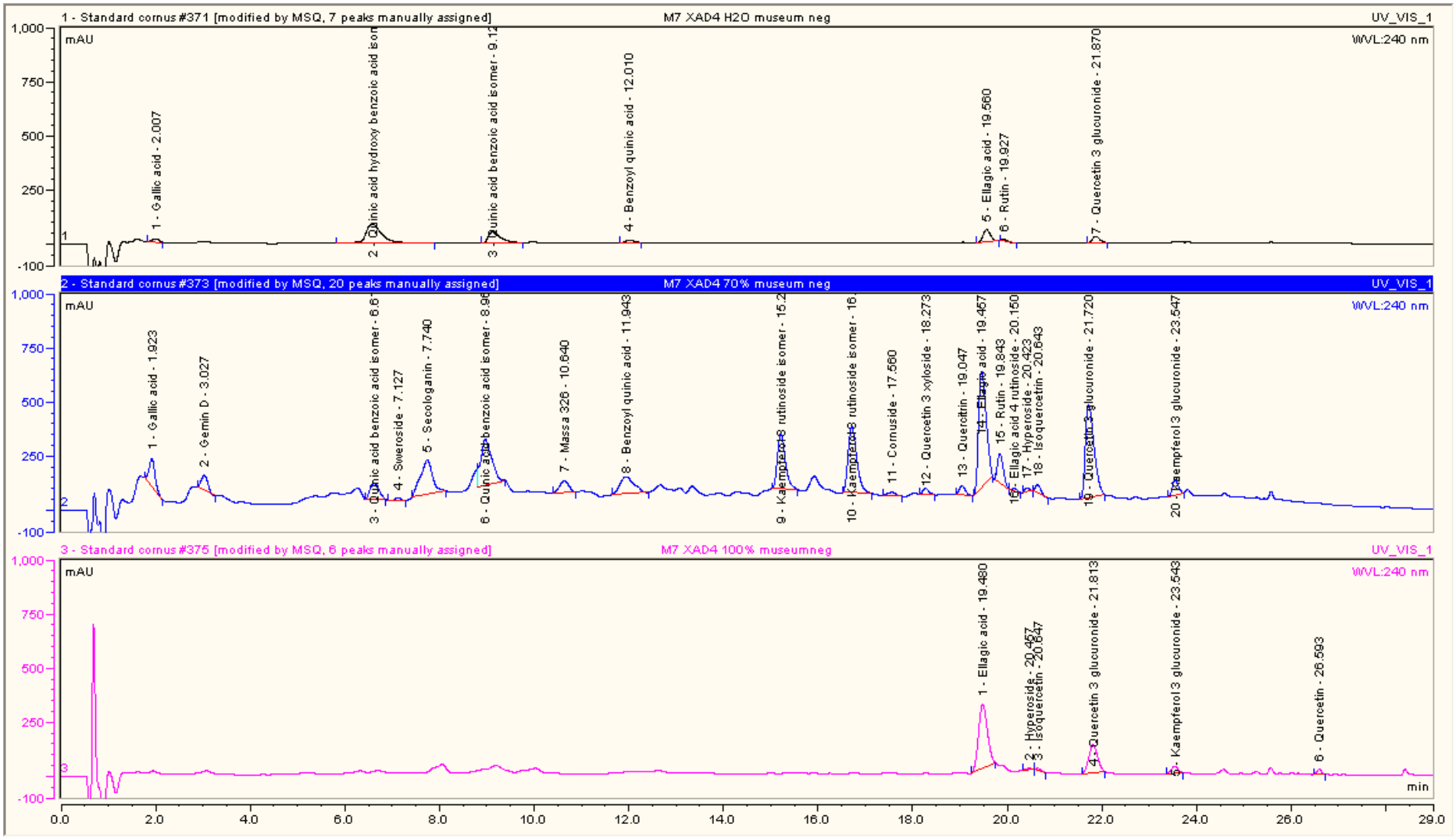
A12. Chromatogram of the extract of *C. sanguinea* obtained by decoction of fresh fruits (SF3).



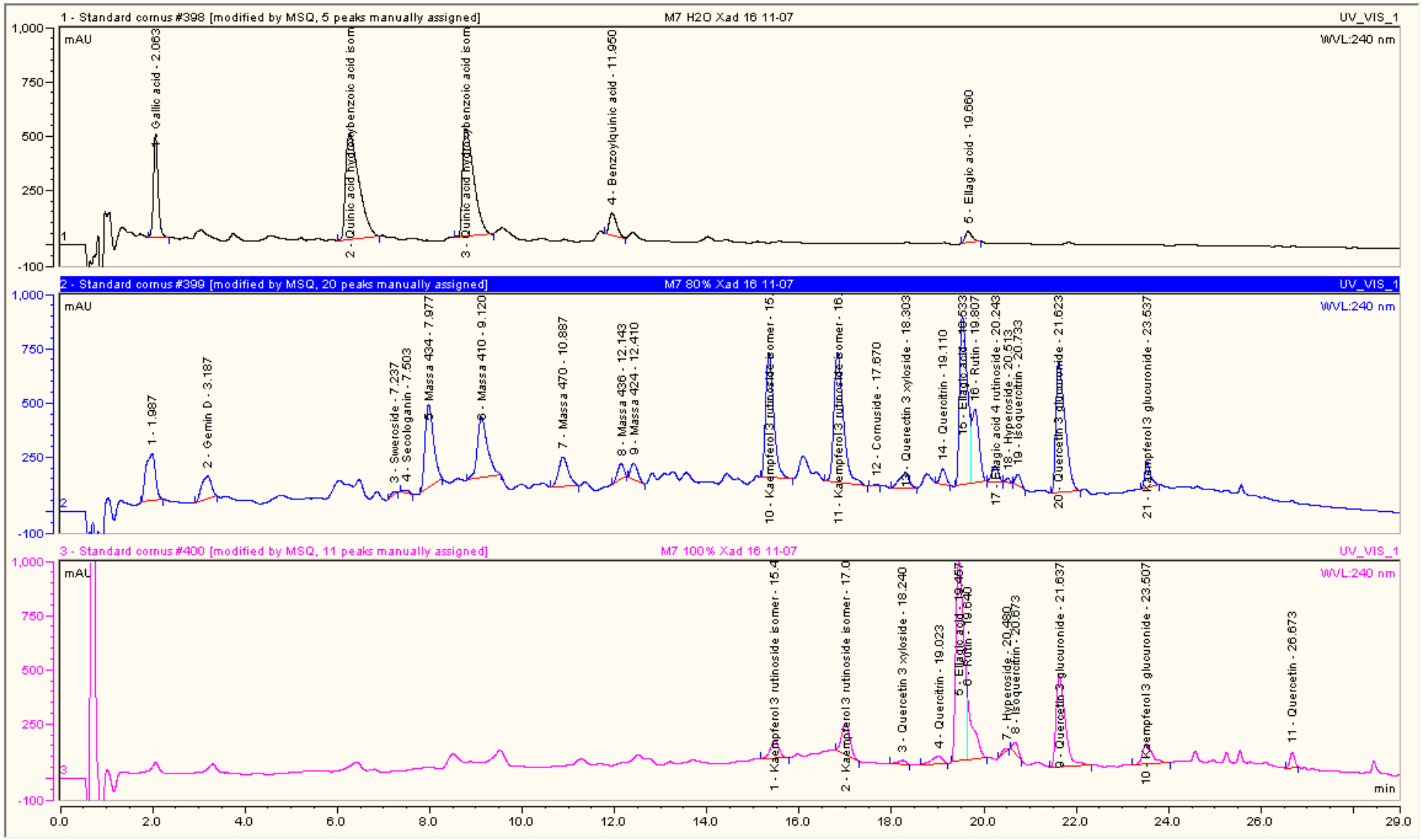
A13. Chromatogram of the extract of *C. sanguinea* obtained by hydroalcoholic maceration of dried fruits (SD2).



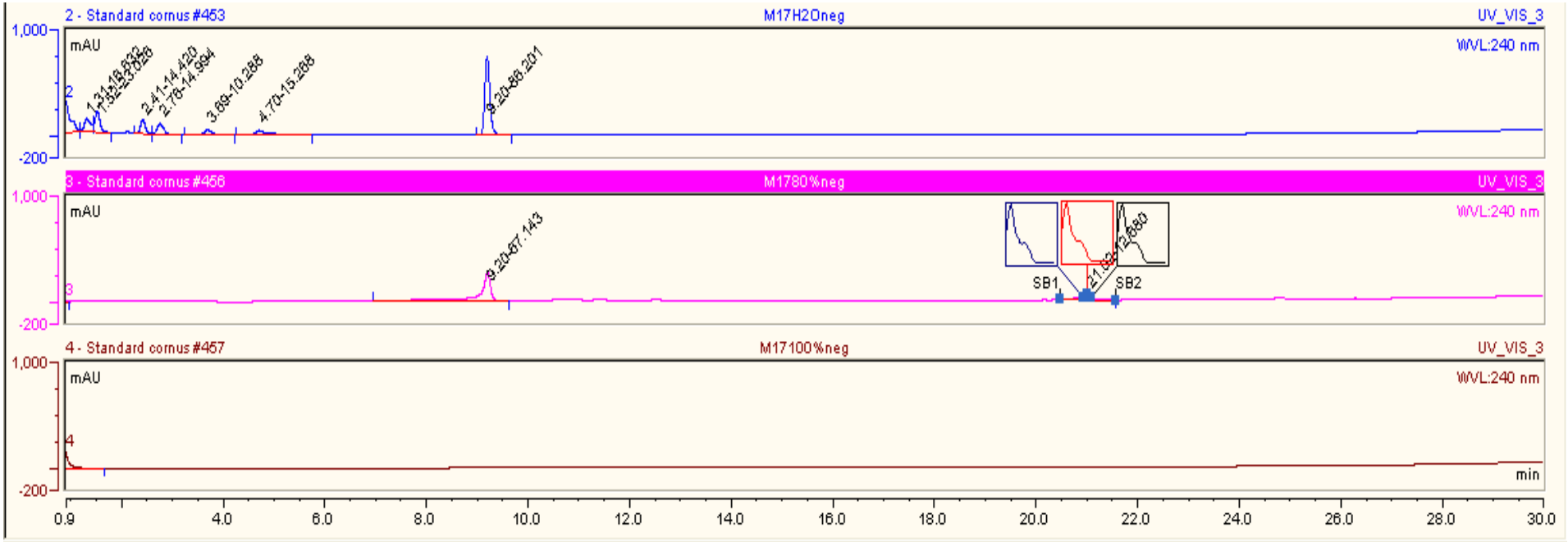
A14. Fractions obtained by separation through HLB SPE from the extract of *C. mas* obtained by hydroalcoholic maceration of dried leaves (TDB).



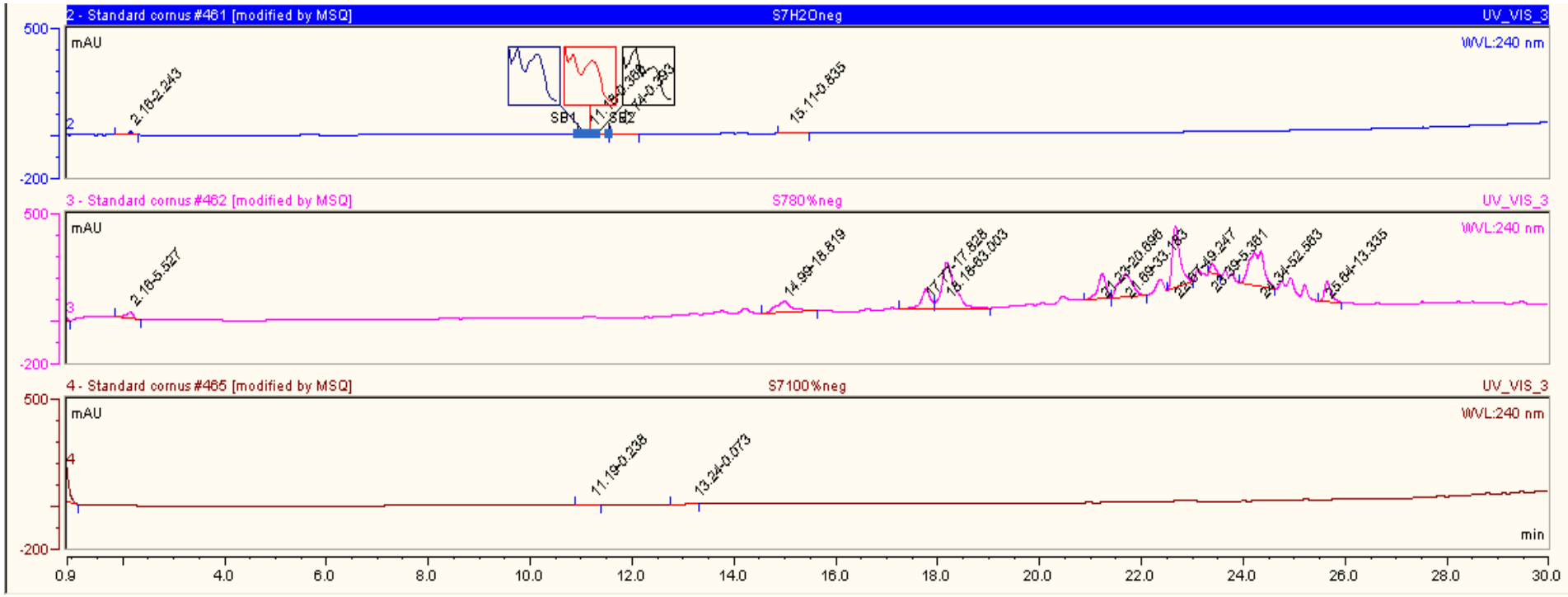
A15. Fractions obtained by separation through XAD-4 from the extract of *C. mas* obtained by hydroalcoholic maceration of dried leaves (TDB).



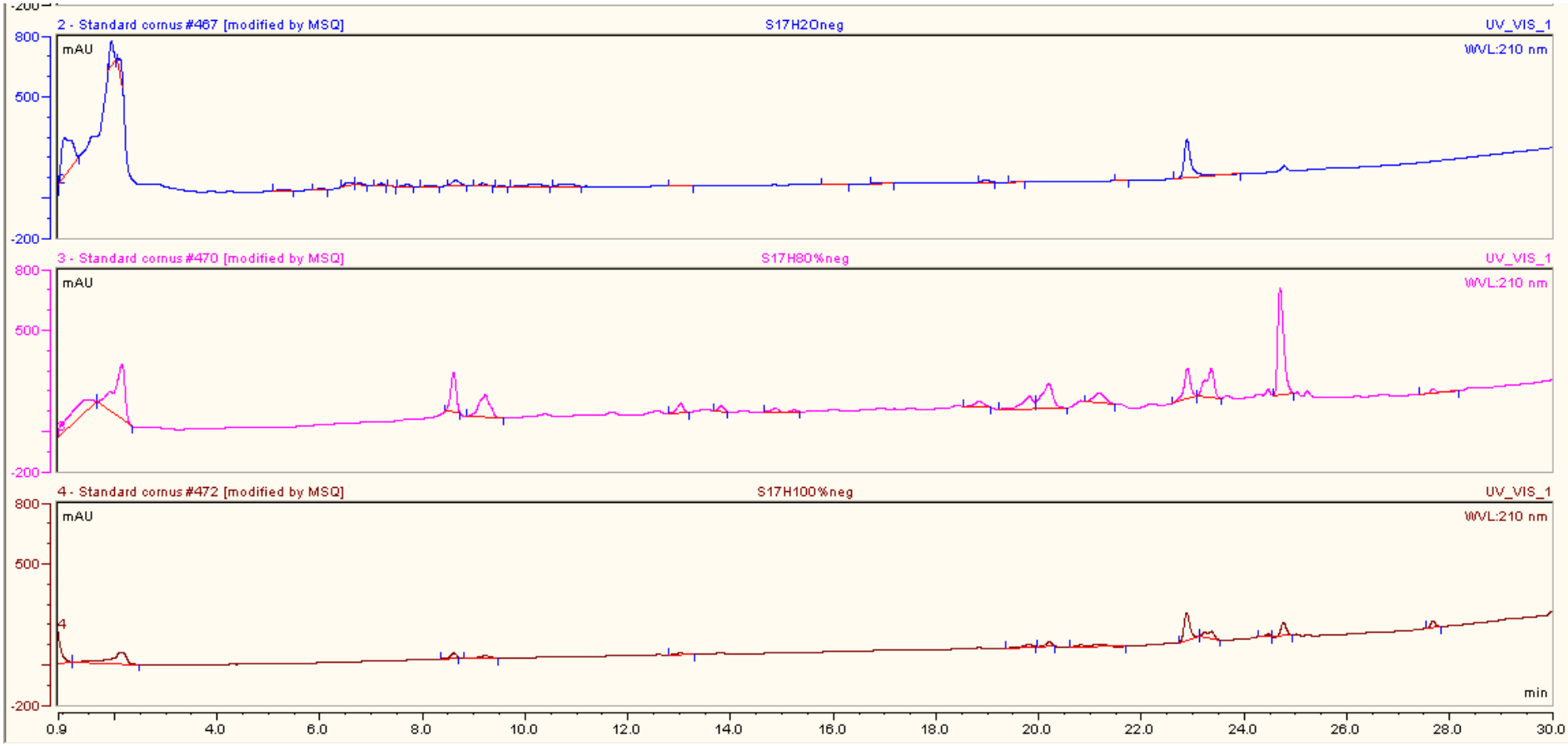
A16. Fractions obtained by separation through XAD-16 from the extract of *C. mas* obtained by hydroalcoholic maceration of dried leaves (TDB).



A17. Fractions obtained by separation through XAD-16 from the extract of *C. mas* obtained by hydroalcoholic maceration of dried fruits (MDB)



A18. Fractions obtained by separation through XAD-16 from the extract of *C. sanguinea* obtained by ethanol maceration of fresh leaves (PF1)



A19. Fractions obtained by separation through XAD-16 from the extract of *C. sanguinea* obtained by hydroalcoholic maceration of dried fruits (SD2)