DOVILĖ GRAUZDYTĖ

PHYTOCHEMICAL COMPOSITION AND *IN VITRO* BIOACTIVITY OF LOW INVESTIGATED *PHYLLANTHUS PHILLYREIFOLIUS* AND *APHLOIA THEIFORMIS* PLANT SPECIES INDIGENOUS TO REUNION ISLAND

DOCTORAL DISSERTATION

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2020

KAUNAS UNIVERSITY OF TECHNOLOGY

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KAUNO TECHNOLOGIJOS UNIVERSITETAS

DOVILĖ GRAUZDYTĖ

MAŽAI TIRTŲ *PHYLLANTHUS PHILLYREIFOLIUS* IR *APHLOIA THEIFORMIS* RŪŠIŲ FITOCHEMINĖ SUDĖTIS IR *IN VITRO* BIOAKTYVUMAS

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TABLE OF CONTENTS

ABBREVIATIONS 7
1. INTRODUCTION 9
2. LITERATURE REVIEW 12
2.1. Application of plants products in food, cosmetics and medicine 12
2.2. Characterization of selected medicinal plants indigenous to Reunion Island 13
2.3. Plants as natural sources of bioactive compounds and antioxidants 15
2.4. Biosynthetic pathway of phenolic compounds 19
2.5. Extraction of phytochemical compounds 21
2.6. Oxidative stress and antioxidant defenses 24
2.7. Evaluation of antioxidant activity 26
2.8. Gene expression 29
2.9. Cytotoxicity 30
3. MATERIALS AND METHODS 32
3.1. Materials 32
3.1.1. Research objects 32
3.1.2. Chemicals and reagents 32
3.2. Preparation of extracts and fractions 33
3.2.1. Soxhlet extraction 33
3.2.2. Maceration by stirring 33
3.2.3. Supercritical fluid extraction (SFE-CO_2) 33
3.2.4. Pressurized liquid extraction (PLE) 36
3.2.5. Liquid-liquid extraction 36
3.2.6. Fractionation by cooling 36
3.3. Antioxidant activity evaluation *in vitro* 37
3.3.1. Analysis of the total phenolic content (TPC) by Folin-Ciocalteu assay 37
3.3.2. $DPPH^{\bullet}$ scavenging assay 38
3.3.3. $ABTS^{\bullet+}$ scavenging assay 38
3.3.4. Ferric reducing antioxidant power (FRAP) assay 38
3.3.5. Oxygen radical absorbance capacity (ORAC) assay 39
3.4. Determination of tocopherols by high performance liquid chromatography (HPLC) 39
3.5. Identification and quantification of phenolic compounds by UPLC-QTOF-MS analysis 39
3.6. Assays in cells 41
3.6.1. MTT cell viability assay 41
3.6.2. Trypan blue exclusion assay 41
3.6.3. Cellular antioxidant activity (CAA) 42
3.6.4. RNA isolation, reverse transcription and quantitative real time PCR (QPCR) 42
3.7. Statistical analysis 42
4. RESULTS AND DISCUSSION 43

4.1. Valorisation of *P. phillyreifolius* by different extraction methods and evaluation of antioxidant properties and phytochemical composition................ 43
4.1.1. The effects of different extraction methods on the yield of extracts........ 43
4.1.2. Tocopherol content and antioxidant properties of lipophilic fractions..... 44
4.1.3. Total phenolic content and antioxidative properties of higher polarity fractions.. 46
4.1.4. Antioxidant properties of solid *P. phillyreifolius* samples measured by *QUENCHER* method.. 48
4.1.5 Characterization of phytochemicals by chromatography mass spectrometry 50
4.2. The effects of phytochemical antioxidants rich *P. phillyreifolius* extracts against hydrogen peroxide induced oxidative stress 57
4.2.1. Cytotoxic effect of *P. phillyreifolius* extracts in HEK293 cells............... 57
4.2.2. Effect of *P. phillyreifolius* extracts on intracellular ROS level in HEK293 cells.. 59
4.2.3. Expression of antioxidant enzymes in HEK293 cells 61
4.3. Comprehensive evaluation of antioxidant potential and mangiferin content of *A. Theiformis* extracts and fractions isolated by applying different methods.. 62
4.3.1. Extract yields... 62
4.3.2. Effect of extraction methods on the antioxidant activity of extracts........ 64
4.3.3. Fractionation of crude hydroethanolic extract and characterization of the obtained fractions 66
4.3.4. Characterization of phytochemicals by chromatography-mass spectrometry 67
4.3.5. Correlation between different values.. 74
4.4. Perspective of the investigated plant as valuable ingredients for nutraceuticals, pharmaceuticals, and cosmetic formulations.................................... 75
CONCLUSIONS 77
REFERENCES 78
LIST OF PUBLICATIONS ON THE THEME OF THIS DISERTATION 95
INFORMATION ABOUT THE AUTHOR 97
ACKNOWLEDGEMENTS.. 97

ABBREVIATIONS

AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABTS	2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)
AC	Acetone
AUC	Net area under the curve
CAT	Catalase
C4H	Cinnamate 4-hydroxylase
DAHP	3-deoxy-D-arabino-heptulosonate-7-phosphate
DCF	2',7'-dichlorofluorescein
DCFH	2',7'-dichlorofluorescin
DCFH-DA	Dichloro-dihydro-fluorescein diacetate
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified eagle medium
DPPH	2,2-diphenyl-1-picrylhydrazyl
DWE	Dry weight extracts material
DWF	Dry weight fraction
DWP	Dry weight plant material
DWR	Dry weight residue
E	Extract
EA	Ellagic acid
ECf	Ethyl acetate
EH	Ethanol:water (70:30)
E4P	Erythrose-4-phosphated
F	Fraction
FCR	Folin–Ciocalteu reagent
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
GPx	Glutathione peroxidase
GRx	Glutathione reductase
GSH	Oxidize reduced glutathione
GSSG	Oxidized glutathione
HAT	Hydrogen atom transfer
HE	Hexane
HEK	Human embryonic kidney
HPLC	High performance liquid chromatography
IC50	The concentration of 50% inhibition
iNOS	Nitric oxide synthase
LOX	Lipoxygenase
MAO	Monoamine oxidase
MBS	Maceration by stirring
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
N-B	N-butanol
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
ORAC	Oxygen radical absorbance capacity

OD	Optical density
OS	Oxidative stress
PAL	Phenylalanine ammonia lyase
PBS	Phosphate-buffered saline
PDA	Photodiode array detector
PEP	Phosphoenolpyruvate
PLE	Pressurised liquid extraction
QENCHER	Quick, easy, new, cheap and reproducible
QPCR	Quantitative real time polymerase chain reaction
QTOF	Quadrupole time-of-flight
R	Plant residue after extraction
RNA	Ribonucleic acid
RSC	Radical scavenging capacity
RT-QPCR	Real time polymerase chain reaction
ROS	Reactive oxygen species
RT	Retention time
SE	Soxhlet extraction
SET	Single electron transfer
SAE	Stirring assisted extraction
SC-CO_2	Supercritical carbon dioxide
SFE	Supercritical fluid extraction
SFE-CO_2	Supercritical extraction carbon dioxide
SOD1	Superoxide dismutase (Cu-Zn)
SOD2	Superoxide dismutase (Mn)
Sto1g	Green fraction (storage 1)
Sto1p	Precipitated fraction (storage 1)
Sto2p	Precipitated fraction (storage 2)
T	Transparent
TE	Trolox equivalent
TPC	Total phenolic content
TPTZ	2,4,6-tripyridyl-s-triazine
W	Water
XO	Xanthine oxidase
3-DHS	3-dehydroshikimic acid
4CL	4-coumaroyl CoA ligase

1. INTRODUCTION

Throughout the ages, plants have formed the basis of various traditional medicine systems and folk medicine. Until now, plants are still highly valued as a rich source of therapeutic agents. Most clinical drugs that are currently in use (e.g., aspirin, codeine, morphine, atropine) were derived from plants and were discovered because of their use in the traditional medicine (Gilani and Atta-ur-Rahman, 2005). It is well known that the medicinal value of plants depends on the presence of biologically active ingredients (Pan *et al*., 2013) which can be utilized in the pharmaceutical industry as natural drug agents and/or for the purposes of synthesis, while in the food and cosmetic industry, many of them are regarded as health promoting/disease preventing compounds and as antioxidant and antimicrobial additives. It is therefore not surprising that, in the last few decades, the identification of new phytochemicals and the development of bioactive ingredients from various plants has become an important area in the health and medicinal food related research (Dai and Mumper, 2010). The trend of potential applications for natural compounds is constantly growing, and it may be expected that newly discovered compounds could possess even stronger bioactivities than the already known substances.

Nevertheless, a large number of botanicals still remain poorly investigated, and, therefore, they lack scientific valorization for wider application of their phytochemical constituents. From this point of view, the tropical flora, which is more seriously threatened with extinction than many other species in different parts of the world, is of particular interest. Considering all these aspects, systematic scientific evaluation of less studied plant species is an important objective which may lead to finding new sources of valuable natural substances (Jonville *et al*., 2008). Reunion Island, a French volcanic island in the Indian Ocean, is listed among the world's top biodiversity areas. *Phyllanthus phillyreifolius* and *Aphloia theiformis* belong to the plant species which are indigenous to this island. Although both species are locally used in the folk medicine, scientific information on them is fairly limited.

In order to obtain valuable ingredients, they must be efficiently and safely extracted from their plant sources (Rosa, Moreno-Escamilla, Rodrigo-García and Alvarez-Parrilla, 2019). Various extraction methods are used for this purpose including conventional (Soxhlet, maceration by stirring, distillation) and innovative (supercritical fluid, pressurized liquid, ultrasound, microwave assisted) extractions. The assessment of the phytochemical composition and activity of the obtained products is a very important task in finding further application for these products. The evaluation of antioxidant activity is usually a financially cheap procedure which provides valuable information about plant samples.

The aim and objectives of the research

The aim of this work was to comprehensively evaluate the phytochemical composition and antioxidant potential of *Phyllanthus phillyreifolius* and *Aphloia*

theiformis leaves extracts isolated by conventional and innovative extractions methods with solvents of various polarity.

The following objectives were outlined in order to achieve this aim:

1. To evaluate the effectiveness of various extraction schemes when using different polarity solvents and applying the traditional and high pressure extraction methods.
2. To investigate the antioxidant properties of the obtained extracts, fractions and extraction residues by using the selected *in vitro* antioxidant activity assays.
3. To determine the phytochemical composition of the obtained extracts and fractions by using chromatographic and mass spectrometric methods.
4. To evaluate the cytotoxicity and the protective effect of selected *P. phillyreifolius* extracts on human embryonic kidney (HEK293) cells against H_2O_2 induced oxidative stress.

Scientific novelty

1. Application of comprehensive extraction procedures to obtain valuable extracts of *P. phillyreifolius* and *A. theiformis* has not been reported previously.
2. To the best of our knowledge, the evaluation of cytotoxicity, antioxidant properties and phytochemical characterization of *P. phillyreifolius* extracts has been performed for the first time.
3. The fractionation of crude hydroethanolic extract of *A. theiformis* by precipitation at a low temperature has not been performed previously.
4. Six isomers of tormentic and hydroxytormentic acids have been observed for the first time in *A. theiformis* extracts.
5. The quantity evaluation of mangiferin, tormentic and hydroxytormentic acids in *A. theiformis* extracts and fractions has not been reported previously.

Practical significance

The study revealed that both investigated plant species are good sources of antioxidant active compounds which have high potential for their practical application in the pharmaceutical, nutraceutical, food and cosmetics industries. It is known that major compounds – mangiferin and geraniin – identified in *A. theiformis* and *P. phillyreifolius*, respectively – are valued for their various bioactivities. The present study may foster further studies on the isolation of these compounds and further investigation of their practical application. It is important to underline that the fractionation of the crude hydroethanolic extract of *A. theiformis* yielded some quite pure mangiferin rich fractions where the content of mangiferin was up to 5 times higher than in the crude extract.

Key points of the thesis

Application of various extraction techniques and concepts is feasible with the objective to obtain natural products of poorly investigated medicinal plants which are indigenous to Reunion Island, namely *Phyllanthus phillyreifolius* and *Aphloia theiformis*, as they may be further used as valuable functional ingredients in

pharmaceutical, nutraceutical and cosmetical industries due to the richness in bioactive phytochemicals and antioxidant activity.

Publication of the research results

The results of this research have been presented in 3 publications in the journals indexed by *Clarivate Analytics Web of Science* and reported at 6 international scientific conferences.

Structure and content of the dissertation

The thesis is written in English. It consists of the list of abbreviations; introduction; literature review; materials and methods; results and discussion; conclusions; references (in total, 187 references were used), and a list of publications on the dissertation topic. The dissertation contains 98 pages, 14 tables and 33 figures.

Statements presented for the defense

1. The application of the conventional and high pressure extraction methods allows the extraction of extracts of *P. phillyreifolius* and *A. theiformis* with strong antioxidant properties and valuable phytochemicals.
2. The preconditioning of HEK293 cells with *P. phillyreifolius* extracts has a positive effect on protecting cells against oxidative stress.
3. By the application of different fractionation methods, mangiferin rich fractions of the crude *A. theiformis* extract can be obtained.

2. LITERATURE REVIEW

2.1. Application of plant products in food, cosmetics and medicine

The rising awareness among the consumers towards the health benefits and efficacy of naturally derived products has increased the use of herbal extracts or phytochemicals in foods, pharmaceuticals, cosmetics and nutraceuticals. The market of herbal extracts has been constantly growing and is expected to continue to grow.

Dietary supplements are nowadays a sizable branch of the food industry, and their consumption has increased in the recent years. Antioxidant dietary supplements are sold as isolated substances or as mixtures of the natural or synthetic origin in a variety of forms including tablets, pills, capsules, powders, drinks and supplement bars. Antioxidant formulations use various ingredients, including antioxidant vitamins (tocopherols, ascorbic acid), bioactive compounds of the plant origin (polyphenols and carotenoids), plant and algae extracts, fruits and vegetables concentrates, enzymes, minerals (selenium, zinc, manganese), polysaccharides, etc. (Almeida, Barreira, Oliveira and Ferreira, 2011). Some of plants derived dietary supplements are resveratrol (from the extract of *Reynoutria japonica*), extracts of green coffee and goji fruit, turmeric rhizome powder (Sieniawska *et al*., 2017).

Functional ingredients, such as purified bioactive compounds or concentrated extracts from natural sources, can be successfully incorporated into foods thus providing novel functional product categories and new commercial opportunities. One of the successful examples could be rosemary extracts (in free and microencapsulated forms) incorporation into cottage cheese in order to enhance phenolic compounds and the antioxidant activity of cheese (Sousa *et al*., 2016). Incorporation of plant extracts into meat-based products in order to control the microbiological activity as well as to increase nutritional properties has been extensively studied in previous research (Hayrapetyan, Hazeleger and Beumer, 2012; Odedina, Vongkamjan and Voravuthikunchai, 2016; Das, Rajkumar, Verma and Swarup, 2012).

Cosmetic products that are derived from a natural source (such as plants) are preferable to consumers because of their numerous advantages for the human skin. Carotenoids such as lutein and zeaxanthin help to protect human skin and eyes from photo damage (Shegokar and Mitri, 2012). Cosmetic and cosmeceutical formulations with extracts from coffee by-products implicate positive results for skin care (Bessada, Alves and Oliveira, 2018). The extract from *P. guajava* by-product increases the photoprotection efficacy in the cosmetic formulations and can be used to develop innovative products (Milani *et al*., 2018).

Plant-based medicine and traditional healing has formed the basis for new drug discovery and development. Some natural products may have direct medicinal application as drug entities, whereas many others can serve as chemical models or templates for the design, synthesis, and semi synthesis of novel substances for the treatment of various diseases (Veeresham, 2014). According to Newman and Cragg (2016), in the area of cancer over the timeframe from around the 1940s to the end of 2014, about 49% of the approved molecules were either natural products, or were directly derived therefrom. Some examples of well-known drugs derived from plants

could be aspirin (from the willow, *Salix spp.*), chemotherapy drugs – taxol (from *Taxus brevifolia*), antimalarial drugs – artemisinin (from *Artemisia annua*) (Mahdi, 2010; Wall, McPhail, Taylor, Wani and Coggon, 2005; Tu, 2011).

2.2. Characterization of selected medicinal plants indigenous to Reunion Island

Reunion Island is a French volcanic island which emerged from the Indian Ocean about three million years ago (Fig. 2.1). This tropical island together with Mauritius and Rodrigues islands forms the Mascarene Archipelago in the Indian Ocean east of Madagascar. A large variety of microclimates with radical changes in the sunlight, precipitation and temperature allows the development and evolution of a wide diversity of plant species. Reunion Island is listed among the world's top biodiversity hotspots with an endemic rate of approximately 40 (Dorla *et al*., 2018).

Fig. 2.1. Reunion Island and its indigenous plant species

*P. phillyreifolius var. phillyreifolius (*common names Bois de négresse, Bois de cafrine, Bois de ravine, Bois de chien), a species that is indigenous to Reunion Island, belongs to the genus *Phyllanthus* (Euphorbiaceae). The genus *Phyllanthus* consists of 2084 species of shrubs, trees and herbs spread over the American, African, Australian, and Asian continents (Mahomoodally *et al*., 2018; Sarin, Verma, Martín and Mohanty, 2014; Chai *et al*., 2016). *P. phillyreifolius* is an extremely branched shrub with a height from 2 m to 6 m. Plants belonging to the genus *Phyllanthus* are widespread in most tropical and subtropical countries and have been traditionally used for treating various ailments, such as gonorrhea, dysentery, diabetes, tumors, hypertension, obesity, asthma, malaria, liver and kidney diseases (Kumaran and Karunakaran, 2006; Paithankar, Raut, Charde and Vyas, 2011; Patel, Tripathi, Sharma, Chauhan and Dixit, 2011). In Reunion Island, the leaves and stems of *P. phillyreifolius* are used as a diuretic and against diarrhea (Poullain, Girard-Valenciennes and Smadja, 2004). The most extensively studied species among *Phyllanthus* herbs are *P. amarus*, *P. debilis*, *P. urinaria*, *P. virgatus*, and *P. emblica*. The previous studies demonstrated a wide range of pharmacological

activities of these species, including antidiabetic (Shabeer, Srivastava and Singh, 2009), hepatoprotective (Pramyothin, Ngamtin, Poungshompoo and Chaichantipyuth, 2007), antioxidant, anti-inflammatory (Chouhan and Singh, 2011) and antiviral effects (Eldeen, Seow, Abdullah and Sulaiman, 2011). These activities are associated with the presence of phenolic compounds in these plants. More than 510 compounds have been isolated from *Phyllanthus* plants, of which, lignans and tannins due to their various activities are considered to be the biologically active compounds of this genus. Corilagin, geraniin, phyllanthin and gallic acid, which are the most prevalent compounds of this genus, are characterized by their multiple biological activities (Table 2.1).

Table 2.1. Biological activities of the most prevalent phenolic compounds which are present in *Phyllanthus* genus

Class	Phytoconstituent	Biological activities	Reference
Lignan	Phyllanthin	Hepatoprotective Antioxidant	(Krithika *et al*., 2009)
		Anticancer	(Parvathaneni, Battu, Gray and Gummalla, 2014)
Ellagitannin	Geraniin	Antibacterial	(Ribeiro *et al*., 2019)
		Antiviral	(Yang, Zhang, Fan, Qin and Liu, 2012)
		Antioxidant Anticancer	(Liu *et al*., 2012)
		Antihypertensive	(Phang, Palanisamy and Kadir, 2019)
	Corilagin	Antibacterial	(Funatogawa *et al*., 2004)
		Antioxidant Antidiabetic	(Nandini and Naik, 2019)
		Anti-inflammation	(Guo *et al*., 2010)
Phenolic acid	Gallic acid	Antidiabetic	(Garud and Kulkarni, 2018)
		Anti-inflammation	(Fan *et al*., 2019)

Aphloia theiformis, previously included in the *Flacourtiaceae* family, currently is the only one species of the *Aphloiaceae* family (Danthu *et al*., 2010). *A. theiformis* is indigenous to Madagascar, Comoros Island, the Mascarenes Islands, the Seychelles Archipelago and tropical Africa (Antoine, Bosser and Ferguson, 1993; Gurib-Fakim and Brendler, 2004). In Madagascar, *A. theiformis* is represented by trees measuring around ten meters in the tropical forests and shrubs in high-altitude forests. *A. theiformis* has been traditionally used for treating dysentery, febrifuge, paludism, cataract, diabetes as an anti-inflammatory, antipyretic, wound healing agent (Poullain, Girard-Valenciennes and Smadja, 2004; Mootoosamy and Mahomoodally, 2014; Danthu *et al*., 2010).

A. theiformis has been previously studied for its biological properties. Studies by Picot and Mahomoodally (2017) supported the traditional uses of this plant to treat diabetes mellitus and indicated that the crude methanolic extract of *A. theiformis* as well as its ethyl acetate and n-butanol fractions showed potent inhibitory activities against enzymes relevant to diabetes (α-amylase and α-glucosidase). It was also reported by Carene *et al*., 2017 that *A. theiformis* exhibits inhibitory activity against some key enzymes linked to obesity, kidney stone formation, Alzheimer's disease and hypertension. Immunomodulatory and anti-inflammatory activities of the phenolic fraction of *A. theiformis* were reported by Hsoidrou *et al*., 2014. Mangiferin, tormentic acid ester glucoside, 23-hydroxytormentic acid ester glucoside, and 6β-hydroxytormentic acid ester glucoside (Gopalsamy, Vargas, Guého, Ricaud and Hostettmann, 1988) were previously identified in *A. theiformis*; however, the main benefit of *A. theiformis* seems to be stem from its richness in mangiferin. Mangiferin (Fig 2.2.), a natural xanthone glycoside, is also found in various parts of plants belonging to Mangiferaceae, Zingiberaceae, Celastraceae and Gentianaceae plant families (Rauf, Imranb and Patel, 2017).

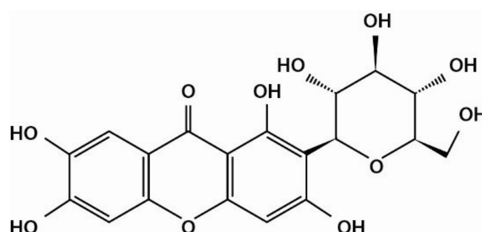


Fig. 2.2. Mangiferin

Mangiferin demonstrates various bioactivities such as anti-aging (Ochocka, Hering, Stefanowicz-Hajduk, Cal and Barańska, 2017), anticancer (Gold-Smith, Fernandez and Bishop, 2016) properties, antioxidant (Saha, Sadhukhan, Sinha, Agarwal and Sil, 2016), antidepressant (Dimitrov, Nikolova, Benbasat, Kitanov and Danchev, 2011). With regard to this, mangiferin has been a constituent of folk medicines without any known adverse effects. A commercialy used extract of mango (*Mangifera indica*) bark *Vimang* is commonly used as a traditional medicine in Cuba in colorectal cancer therapy, and it also shows excellent results in the improvement of the quality of life of terminally ill patients with HIV/ systemic lupus, and infertility in women. Moreover, many patents which focused on the therapeutic or cosmetic application of mangiferin and its derivatives have been issued in recent years (Telang, Dhulap, Mandhare and Hirwani, 2013).

2.3. Plants as natural sources of bioactive compounds and antioxidants

The plant kingdom offers a rich source of a wide range of structural biodiversity of natural secondary metabolites which have no fundamental role in the maintenance of life processes in the plants, but they are still important for the plant to interact with its environment for adaptation and defense. Production of secondary metabolites is stimulated by various environmental stresses, such as UV, drought,

changes in temperature conditions, pollutants and others (Ramakrishna and Ravishankar, 2011). Based on their biosynthetic origin, plants secondary metabolites can be divided into three major groups: terpenoids, alkaloids and phenolic compounds. Terpenoids represent the most abundant and structurally diverse group of plant secondary metabolites. Many terpenoids are of great medicinal value. For example, sesquiterpene lactone artemisinin and diterpene alkaloid taxol are drugs used for the treatment of malaria and cancer, respectively. Alkaloids are low molecular weight nitrogen containing compounds with at least one ring with nitrogen atoms which are usually present in the rings. They play an important role in plant defense systems (Fang *et al*., 2011).

The majority of natural antioxidants are phenolic compounds, therefore, most attention in this thesis shall be focused on them. Although synthetic antioxidants, such as butylated hydroxyanisole and butylated hydroxytoluene, have been developed, but their uses are limited due to their toxicity. Phenolic compounds of plants possess the ideal structure chemistry for free radical scavenging activities because of their phenolic hydroxyl groups that are prone to donating a hydrogen atom or an electron to a free radical and extended conjugated aromatic system to delocalize an unpaired electron and are preferable due to their low cytotoxicity (Dai and Mumper, 2010). According to their structure, phenolic compounds can be classified into several groups, such as phenolic acids, flavonoids, stilbenes, coumarins, lignins and tannins. A more comprehensive classification is shown in Fig. 2.3.

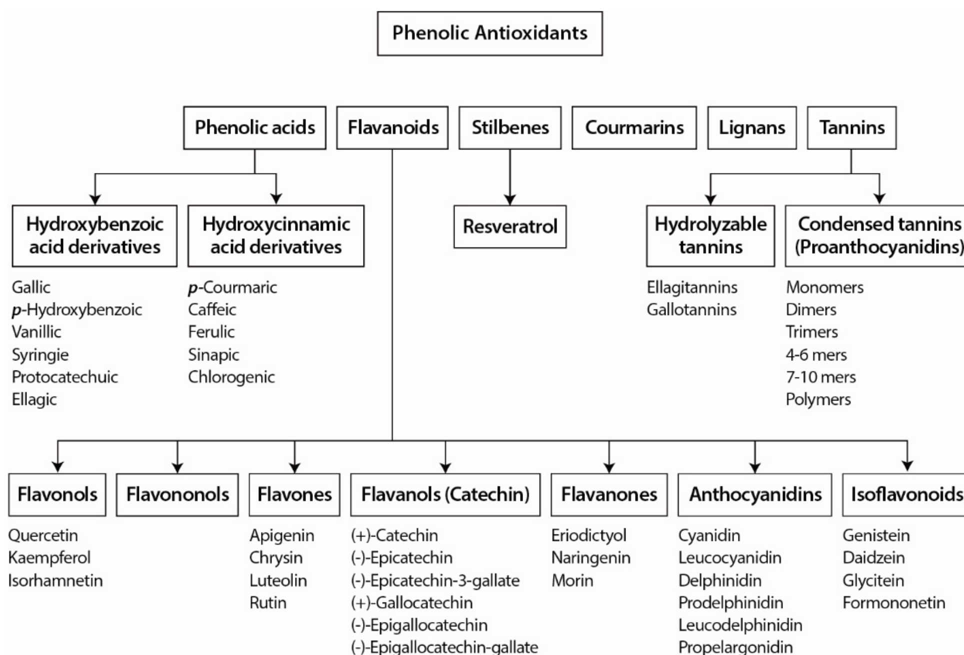


Fig. 2.3. Classification of phenolic compounds (Shahidi and Ambigaipalan, 2015)

Phenolic acids are comprised of derivatives of cinnamic acid (e.g., p-coumaric, caffeic, and ferulic acids) and derivatives of benzoic acid (e.g., gallic acid and

hydroxybenzoic acids). Fruits, such as blueberries, kiwis, plums, cherries and apples, are found to be rich in hydroxycinnamic acids, mainly caffeic acid, which account for 75–100% of the total hydroxycinnamic acid content in many fruits, whereas ferulic acid is the most abundant phenolic acid in cereal grains and accounts for about 90% of the total polyphenol content of wheat grain (Xu *et al.*, 2017).

Flavonoids, which are the most numerous phenolic compound group of dietary phenols, are subdivided into several subclasses on the basis of the structural features of the C ring (Fig 2.4). Fruits, vegetables, grains, bark, roots, stems, flowers, tea and wine are great sources of flavonoids. Examples of some naturally occurring flavonoids are as follows: flavonols – quercetin, kaempferol, rutin is present in onions, leeks, broccoli, tomato; flavanols – (+)-catechin, (+)-gallocatechin – are present in tea; flavonones – naringin, hesperidin, naringenin – are present in citruses, oranges; isoflavones – genistein and daidzin – are present in the soybean (Shahidi and Ambigaipalan, 2015).

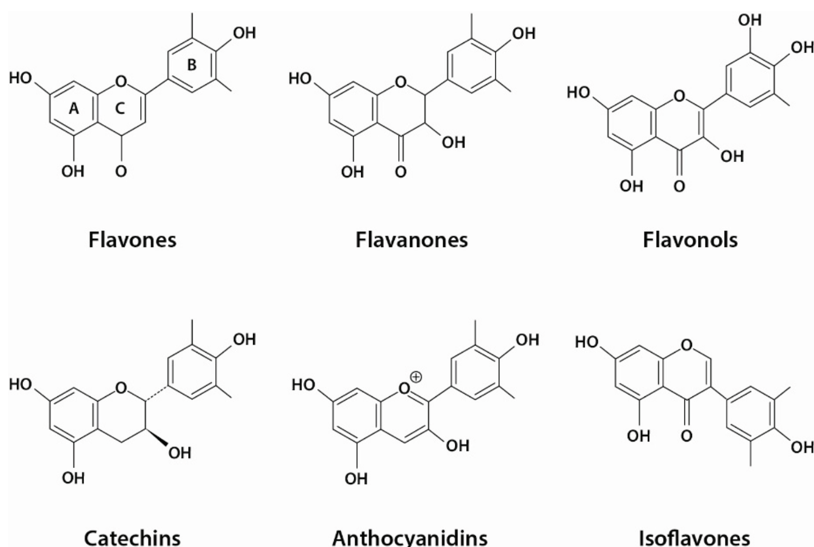


Fig. 2.4. Subclasses of flavonoids (Hollman, 2004)

Tannins are natural polyphenols with a molecular weight ranging from 500 to 5000 Dalton; they are usually classified into condensed tannins (proanthocyanidins) and hydrolyzable tannins (gallo- and ellagi- tannins) (Arapitsas, 2012). What regards their chemical structure, gallotannins consist of gallic acid esterified to polyol, while ellagitannins are formed from oxidative coupling of two galloylgroups in gallotannin to form hexahydroxydiphenic acid (HHDP). Proanthocyanidins are dimers, trimers or polymers of catechin and epicatechin. Complex tannins are built from gallotannins or ellagitannins together with catechin or another minor polyphenol (Fig. 2.5) (Møller, Hansen and Cornett, 2009). Hydrolyzable tannins are widespread in fruit, vegetables and other types of food. In particular, they are frequently found in large amounts in Rosidae (raspberries, blackberries, cloudberries, arctic raspberries, strawberries, etc.), Fragales (oak, carob, nuts, tonoak, etc.), Brassicales (capparis), Myrtales (myrtle, pomegranate, etc.) and Sapindales (Chinese olives,

mango, etc.) plants. Here are some examples of these compounds: castalgin and grandinin in oak-aged wine; lambertianin C and sanguiin H-6 in the raspberry and the strawberry; glansreginin A, glansreginin B in walnuts (Arapitsas, 2012).

In plant cells, most phenolic compounds are coupled to sugars in order to reduce their endogenous toxicity (Hounsome, Hounsome, Tomos and Edwards-Jones, 2008).

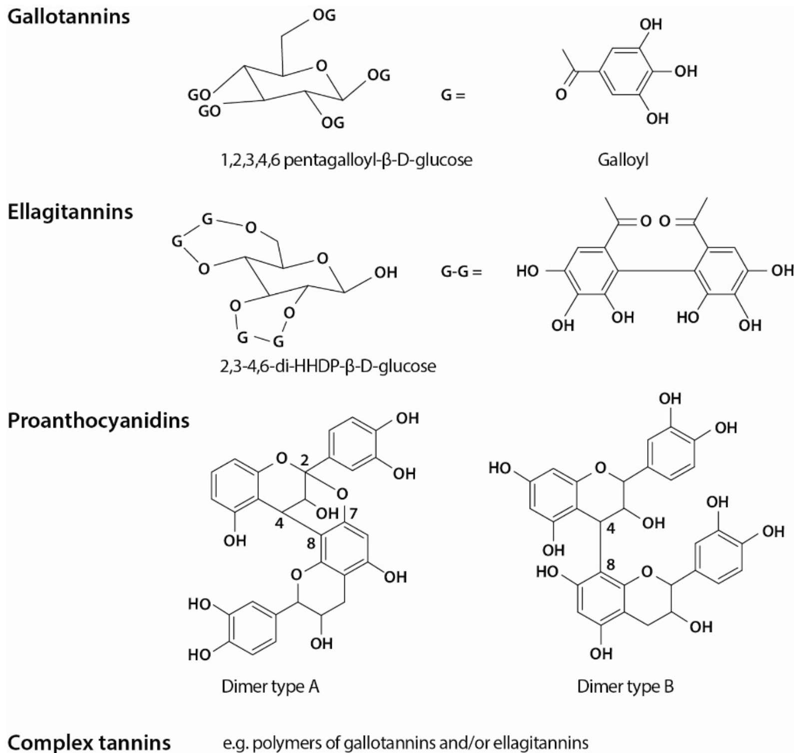


Fig. 2.5. The structure of gallotannins, ellagitannins and proanthocyanidins (Møller, Hansen and Cornett, 2009)

It has been determined that phenolic compounds exert their biological action through several mechanisms which can be divided into antioxidant and non-antioxidant related actions.

Antioxidant related actions can act at:

ROS-removing level by

- ROS-scavenging (electron/hydrogen transfer);
- Induction of ROS-removing enzymes (e.g., SOD, CAT, GPx);
- Induction of endogenous antioxidant-synthesizing enzymes (e.g., glutathione synthase).

ROS-formation level by

- Metal chelation (iron and copper);
- Inhibition or repression of ROS-forming enzymes (e.g., XO, NOX, LOX, MAO, iNOS).

Polyphenols exert their non-antioxidant related actions at the chemopreventive level (antimutagenic, antiproliferative, proapoptotic, antiangiogenic), the immunological level (immunomodulatory, antiallergic), the gastrointestinal level (interfering metal absorption, carbohydrate and lipid digestion/absorption) and others (Sandoval-Acuña, Ferreira and Speisky, 2014).

2.4. Biosynthetic pathway of phenolic compounds

The majority of phenolic compounds in plants are formed through the shikimic acid pathway and phenylpropanoid metabolism. The shikimic acid pathway is the main one responsible for the production of the precursors of the phenolic compounds of most plants and is a major link between the primary and secondary metabolism in higher plants. Then, phenylpropanoid metabolism leads to the production of numerous molecules, such as hydroxycinnamic acids, hydroxybenzoic acids, flavonoids, and lignin (Rosa, Moreno-Escamilla, Rodrigo-García and Alvarez-Parrilla, 2019). Biosynthetic pathways of some phenolic compounds are demonstrated in Fig. 2.6.

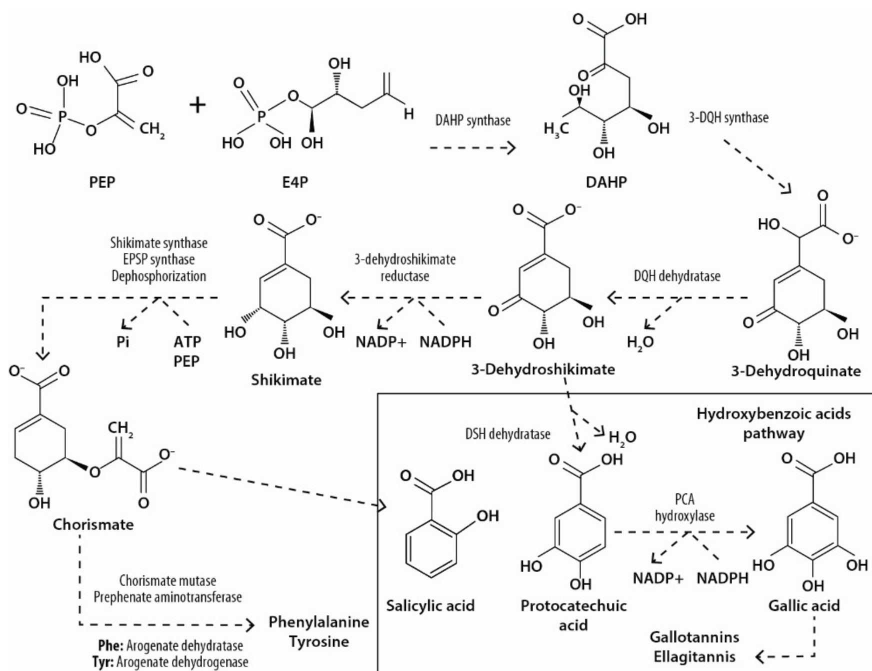


Fig. 2.6. Biosynthesis of shikimic and hydroxybenzoic acids. DAHP, 3-deoxy-D-arabinoheptulosonate-7- phosphate; DHS, 3,5-didehydroshikimate; DQH, dehydroquinate; PEP, phosphoenolpyruvate; E4P, eristrose-4-phosphate; PCA, protocatechuic acid (Rosa, Moreno-Escamilla, Rodrigo-García and Alvarez-Parrilla, 2019)

The shikimic acid pathway shall be discussed in more detail. The shikimic acid pathway requires two starting materials: phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P). These two compounds are generated from metabolism of glucose or another carbohydrate. PEP is produced from glucose through the

glycolysis pathway, while E4P is produced through the pentose phosphate pathway. When the starting materials are generated, they are condensated through the action of enzyme 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase to produce DAHP (Ghosh, Chisti and Banerjee, 2012). Next, phosphate and water are removed in two successive enzymatic reactions to form 3-dehydroshikimic acid (3-DHS), the precursor of gallic acid, gallotannins, and ellagitannins. Shikimic acid is then obtained via a hydrogenation reaction catalyzed by shikimate dehydrogenase. Chorismate is the next key intermediate in the shikimic acid pathway, the rearrangement of its aliphatic chain, transamination and dehydration, which leads to the production of phenylalanine and tyrosine which are the main subtrates for the synthesis of phenolic compounds through the phenylpropanoid pathway (Rosa, Moreno-Escamilla, Rodrigo-García and Alvarez-Parrilla, 2019).

Phenylalanine is the primary substrate for the synthesis of phenolic compounds through the phenylpropanoid pathway in most plants. Normally, in the first enzymatic step of the phenylpropanoid pathway, phenylalanine is deaminated by phenylalanine ammonia lyase (PAL) to yield cinnamic acid which is then hydroxylated and transformed to p-coumaric acid under the catalysis of cinnamate 4-hydroxylase (C4H) in the second enzymatic step (Fig. 2.7).

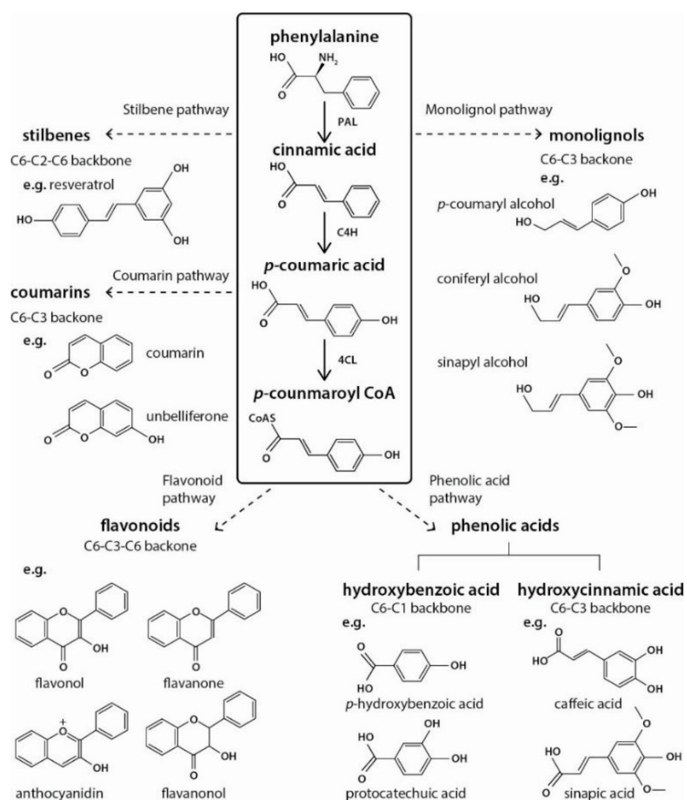


Fig. 2.7. A schematic view of biosynthetic pathways of phenylpropanoids and representative chemical structures. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl CoA ligase (Deng and Lu, 2017)

Following this, 4-coumaroyl CoA ligase (4CL) catalyzes the conversion of p-coumaric acid into p-coumaroyl-CoA, which is an important branch point leading to the generation of various phenylpropanoid compounds (Deng and Lu, 2017). Natural biosynthesis of these metabolites depends on the physiology and developmental stage of the plant (Xu, Wang, Pu, Tao and Zhang, 2017; González-Aguilar *et al*., 2013).

2.5. Extraction of phytochemical compounds

Plants contain a wide variety of chemical constituents, whereas the presence of the target secondary metabolites is usually low, which presents a great challenge in their recovery (Bourgaud, Gravot, Milesi and Gontier, 2001). The high diversity of phenolic compounds requires using suitable approaches for their extraction. Extraction efficiency is one of the most important steps in the practical application of plant-derived products. The extraction process allows isolating and characterizing the desired components of a plant material (Sasidharan, Chen, Saravanan, Sundram and Latha, 2011). The main objective of the extraction process should be to give the maximum yield of bioactive compounds with the best properties (Spigno, Tramelli and De Faveri, 2007).

Today, a large number of various solvents and extraction techniques are used for the isolation and separation of bioactive compounds from plants. Usually, the most commonly used strategies employ solid–liquid extraction which can be defined as a mass transport phenomenon in which solids contained in a solid matrix migrate into a solvent in contact with the matrix (Ignat, Volf and Popa, 2011). There are many different traditional extraction methods, such as Soxhlet, heat reflux, and maceration. Nevertheless, due to several disadvantages, they are being replaced by advanced extraction techniques including supercritical fluid (SFE), microwave assisted, ultrasound assisted and pressurized liquid (PLE) extractions which are faster and more environmentally friendly (Brusotti, Cesari, Dentamaro, Caccialanza and Massolini, 2014). The use of novel technologies allows enhancing the mass transfer rates, increasing cell permeability as well as increasing secondary metabolite diffusion, which leads to higher extraction yields, fewer impurities in the final extract, extractions at room temperature with the preservation of thermo-sensitive structures, use of various non-organic solvents, low energy consumption, short operation time, and they have no significant effect or only show a lower effect on the structure of bioactive compounds (Moreira, Alexandre, Pintado and Saraiva, 2019).

The advantages of SFE over the traditional extraction techniques are reduced pollution, increased sample throughput, production of solvent-free extracts and reduced risks of the thermal degradation of target compounds due to moderate process temperatures which are usually applied (Bampouli *et al*., 2014; Leitão, Prado, Veggi, Meireles and Pereira, 2013). In SFE, a supercritical fluid is present, where the temperature and pressure are above its critical point, thus forming a homogenous phase with both liquid-like and gas-like properties – a mesophase (Khaw, Parat, Shaw and Falconer, 2017). Among supercritical fluids, carbon dioxide (CO_2) is most commonly used because it features modest critical conditions, it

readily achieves separation from solutes, is environmental friendly, non-flammable and inexpensive (Bleve *et al.*, 2008). On top of that, supercritical CO_2 is nonpolar; thus it is an excellent solvent for the extraction of non-polar compounds; however, it is limited to the recovery of un-polar compounds. In this case, a small concentration of ethanol or other alcohols can be incorporated to the extraction system in order to increase the efficiency of extraction. SFE extraction consists basically of two major steps: extraction of the soluble substances from the solid matrix by CO_2 at a high pressure and separation of these compounds from the supercritical solvent after the expansion. The extraction process is illustrated in Fig. 2.8. During the extraction, the solvent flows through the solid sample loaded extractor thus forming a fixed bed and dissolving the soluble compounds. The solute-solvent mixture separates by rapidly reducing either the pressure and/or increasing the temperature (Pereira and Meireles, 2010).

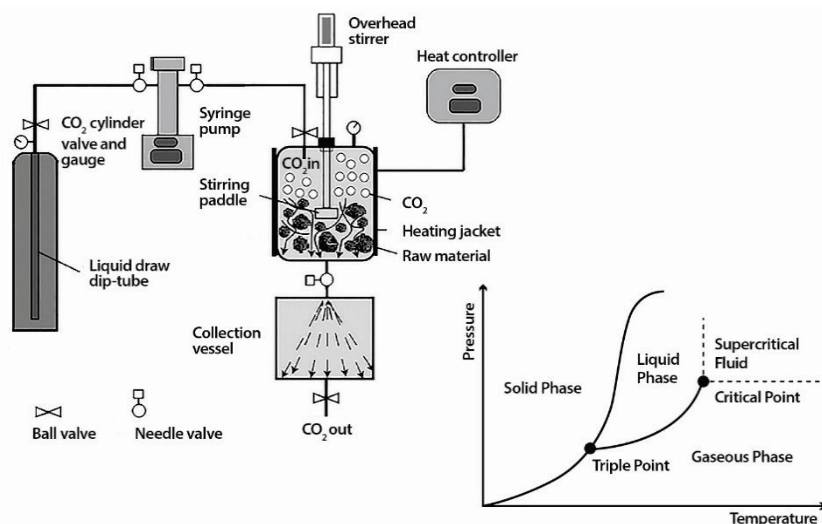


Fig. 2.8. A typical supercritical fluid extraction system (Khaw, Parat, Shaw and Falconer, 2017) and phase diagram

Meanwhile, PLE is an automated time and solvents saving technique which combines the elevated temperature and pressure with liquid solvents in order to achieve fast and efficient extraction of the analytes from the solid matrix (Carabias-Martínez, Rodríguez-Gonzalo, Revilla-Ruiz and Hernández-Méndez, 2005). In PLE, the high pressure helps the extraction cells to be filled faster and thus forces the liquid into the solid matrix. This extraction technique is limited for thermolabile compounds as the high temperature can have deleterious effects on their structure and functional activity (González-Aguilar *et al.*, 2013).

A schematic diagram of the PLE system is presented in Fig. 2.9. Firstly, the sample alone or mixed with diatomaceous earth is loaded into the stainless steel extraction cell and capped with two filtration end fittings which are tightly fitted for the high-pressure closure. Inert diatomaceous earth is a dispersing/drying agent which allows to absorb 2 mL of water per one gram of hydromatrix and thus reduces particle clumping and solvent channeling in the extraction cell. Then, the extraction

cell is placed in the oven of the extractor where the cell is heated (up to 200 °C) while maintaining the pressure (up to 3000 psi). The solvent is pumped from the solvent bottles into the cell, which is pressurized. PLE can be performed in the static or dynamic mode, or in a combination of both. In the static mode, the solvent is replaced between cycles, while, in the dynamic mode, the fresh solvent is continuously pumped through the sample thus accelerating the mass transfer rate. The extraction efficiency of the dynamic mode is equal or even higher than that of static extraction, however, the dynamic mode requires a larger volume of the solvent compared to static extraction. More than one extraction cycle can be used to achieve exhaustive extraction. After the extraction phase has been finished, the solvent is allowed to flow to the collection vial. Finally, at the end of extraction, the sample is purged with N_2 gas in order to recover the final solvent residues (Lundstedt, 2003; Picó, 2017).

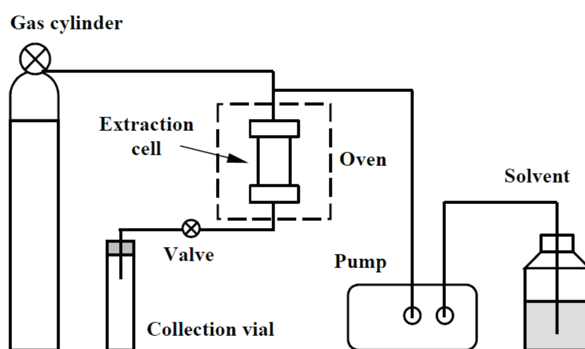


Fig. 2.9. Schematic diagram of PLE system (Lundstedt, 2003)

The selection of the most suitable solvent or solvent system is based on the properties of the targeted phenols which are present in the plant material. The extraction efficiency is greatly impacted by the nature of the solvent relative to the types of phenols which are present in the plant material (Rosa, Moreno-Escamilla, Rodrigo-García and Alvarez-Parrilla, 2019). The solvents used for the extraction of biomolecules from plants are chosen based on the polarity of the solute of interest: a solvent of similar polarity to the solute will properly dissolve the solute (Watson, Baharlouei, Altemimi, Lightfoot and Lakhssassi, 2017). Solvents that have been used in the extraction of plant-based phenols include water, alcohols (n-propanol, isopropanol, ethanol, methanol), ethers (diethyl ether, methyl tert-butyl ether), ethyl acetate, ketone solvents (methyl ethyl ketone, acetone, methyl isobutyl ketone), and compatible mixtures of these solvents (Rosa, Moreno-Escamilla, Rodrigo-García and Alvarez-Parrilla, 2019).

Different approaches may be applied for extract preparation, namely, single step or sequential extraction. Often, the single step extraction is not efficient enough to the recovery of all the desired compounds due to the limitation in polarity of the solvent in use. In sequential extraction, phenolic compounds are systematically released from the plant matrix. This allows obtaining differentiated extracts according to their polarity. Usually, sequential extraction from the same raw material provides a higher total extraction yield and a higher concentration of

phenolic compounds than the single step extraction (Garmus, Paviani, Queiroga, Magalhães and Cabral, 2014).

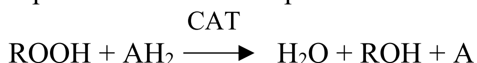
After the solid–liquid extraction, the next step in the sample preparation is the purification of the raw extract in order to obtain more concentrated polyphenol rich fractions. The classical liquid–liquid extraction is one of the most used sample pre-treatment methods in order to purify a wide range of compounds. Liquid–liquid extraction is a mass transfer operation, in which, a liquid solution initially containing one or more solutes is thoroughly mixed with an immiscible or a nearly immiscible liquid (solvent) (Ignat, Volf and Popa, 2011).

2.6. Oxidative stress and antioxidant defenses

Although oxygen is essential for aerobic life due to oxidative metabolism which represents a principal source of energy, however, partial reduction of molecular oxygen results in the formation of potentially dangerous reactive oxygen species (ROS). The biologically most important ROS are superoxide radical anions ($O_2^{\bullet-}$), hydroxyl ($^{\bullet}OH$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$) radicals, hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), which are constantly produced in cells and are also provided by exogenous sources, such as ionizing radiation and xenobiotics (Pourova, Kottova, Voprsalova and Pour, 2010; Klaunig, Wang, Pu and Zhou, 2011). ROS play dual roles within aerobic cells: at a low level, they exert beneficial effects on the cellular responses in serving as the signaling molecules in a wide range of normal cellular processes; at a high level, ROS act as oxidizing agents that may do damage which is important to the cell structures of such biomolecules as DNA, lipids and proteins, thereby being a main contributor of chronic diseases (Di Meo, Reed, Venditti and Victor, 2016; Rowe, Degtyareva and Doetsch, 2008). With regard to this, the balance between oxidation and reduction plays an important role in maintaining a healthy biological system (Shen, Li, Qiu, Liu and Zeng, 2016).

Aerobic organisms feature integrated antioxidant systems which include enzymatic and non-enzymatic antioxidants that are usually effective in blocking the harmful effects of ROS (Valko *et al*., 2007; Poljsak, Šuput and Milisav, 2013) (Fig. 2.10). In the living systems, antioxidants act at different levels, and, on the basis of the line of defense, antioxidants can be categorized as first, second and third line defense antioxidants (Ighodaro and Akinloye, 2017). Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are key enzymes acting as the first line defense antioxidants in the suppression or prevention of the formation of free radicals or reactive species in cells. SOD destroys the free radical superoxide anion by converting it to the less-reactive species H_2O_2 that can in turn be destroyed by CAT or GPx reactions (Fig. 2.11). In humans, there are three forms of SOD: SOD1, SOD2 and SOD3. SOD1 is a cytoplasmic antioxidant enzyme containing zinc and copper in its reactive centre. SOD2 is a mitochondrial matrix enzyme containing one manganese atom in its reactive centre that scavenges oxygen radicals produced by the extensive oxidation-reduction and electron transport reactions that occur in mitochondria. SOD3 is extracellular SOD found in the plasma, lymph and synovial fluid. H_2O_2 is further enzymically catabolized by CAT and GPx. CAT reacts with H_2O_2 to form water and molecular oxygen (Mates, 2007; Fukai and Ushio-Fukai,

2011). Moreover, CAT reacts with H donors (methanol, ethanol, formic acid, phenol) by using 1 mole of peroxide in a kind of peroxidase activity:



$$ROOH + AH_2 \xrightarrow{CAT} H_2O + ROH + A$$

GPx are tetrameric proteins in which each monomer contains one atom of selenium at the catalytic site. GPx enzyme removes H_2O_2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG). Glutathione reductase (GRx) regenerates GSH from GSSG, with NADPH as a source of reducing power (Shen, Li, Qiu, Liu and Zeng, 2016).

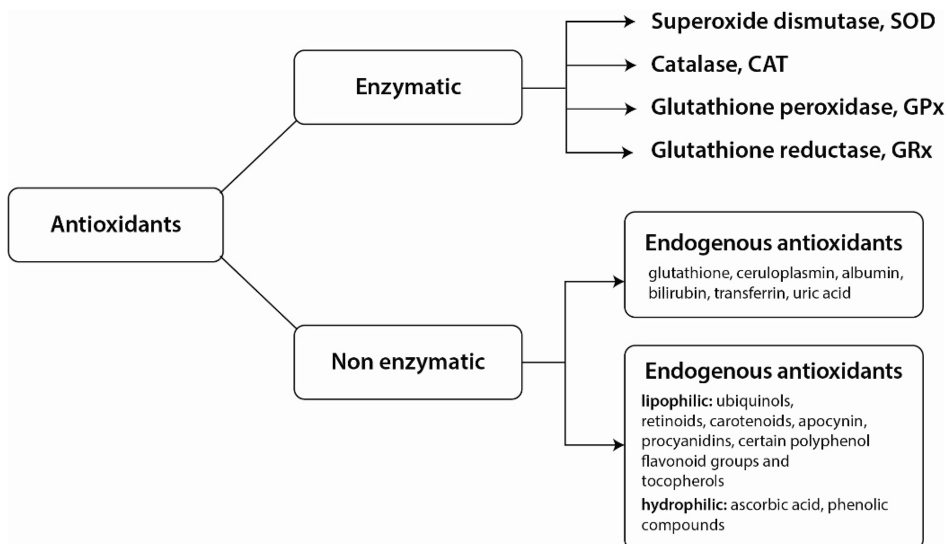


Fig. 2.10. Enzymatic and non-enzymatic classification of antioxidants, adapted from (Shen, Li, Qiu, Liu and Zeng, 2016)

Metal ion binding proteins, such as transferrin and caeruloplasmin, which chelate or sequester iron and copper, respectively, and consequently preventing them from free radical formation, also belong to the class of the first line defense antioxidants (Ighodaro and Akinloye, 2017).

Exogenous antioxidants which are present in fruits, vegetables, plants, as well as in uric acid and glutathione, belong to the second line defense antioxidants. They scavenge free radicals by donating an electron or a hydrogen atom to them, and, in the process, they become free radicals themselves, but without the damaging effects (Ighodaro and Akinloye, 2017). Third line defense antioxidants act after free radical damage has occurred. They are a group of enzymes serving for the repair of damaged DNA (polymerases, glycosylases and nucleases), proteins and lipids (proteinases, proteases and peptidases) and are located both in cytosol and mitochondria of mammalian cells.

Oxidative stress (OS) occurs when a natural antioxidant system is not sufficient to keep the balance between the production and inactivation of ROS. OS contributes to many pathological conditions and diseases, including aging, cancer,

neurological disorders, atherosclerosis, hypertension, chronic obstructive pulmonary disease, asthma (Ighodaro and Akinloye, 2017).

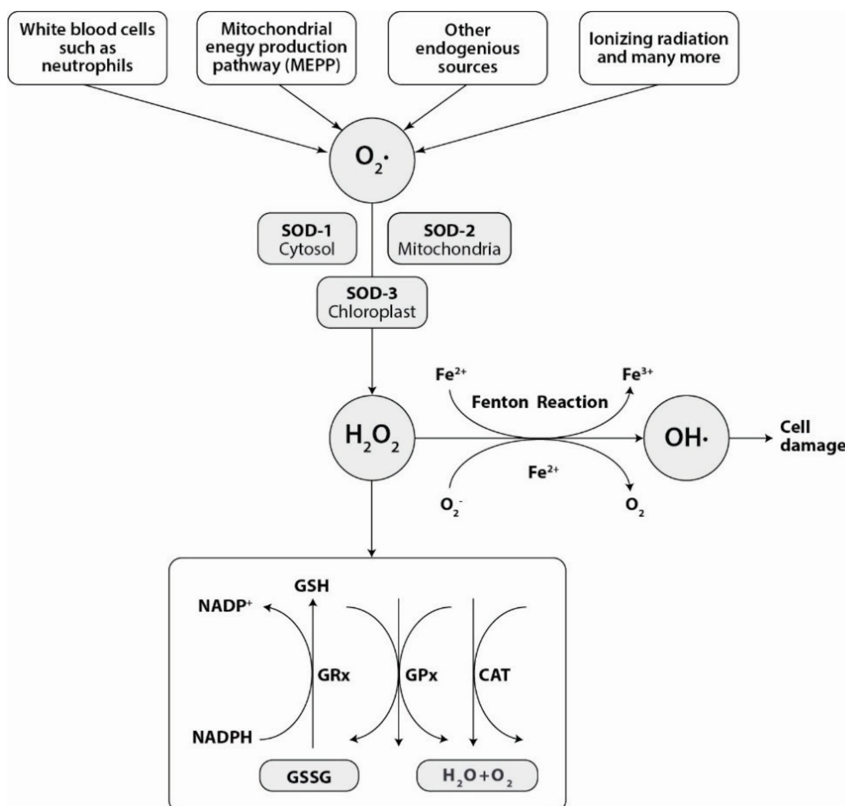


Fig. 2.11. First line antioxidant defence against ROS, modified by Shen, Li, Qiu, Liu and Zeng, 2016; Ighodaro and Akinloye, 2017

2.7. Evaluation of antioxidant activity

Evaluation of antioxidant activity is a fairly simple and cheap screening method which provides valuable data for better understanding of the further application of plant extracts. Generally, extracts possessing good antioxidant activity may also be characterized as good sources of bioactive compounds as well as promising agents for the development of new ingredients of functional foods and nutraceuticals. The effectiveness of antioxidants can be influenced by a number of various factors, such as their structural features, concentration, temperature, type of oxidation substrate, and the physical state of the system as well as the presence of pro-oxidants and synergists (Pokorny, Yanishlieva, Gordon and Yanishlieva-Maslarova, 2010).

In vitro

Numerous methods using a variety of systems and approaches can be used for the evaluation of the antioxidant activity of plant-derived products. Many chemistry *in vitro* methods have been developed for quick, easy, cheap and reproducible

evaluation of samples. Antioxidant activity can be monitored by a variety of assays with different mechanisms, including single electron transfer (SET), hydrogen atom transfer (HAT), reducing power, and metal chelation, among others (Shahidi and Zhong, 2015). SET-based assays measure the antioxidant reducing capacity when a phenolic antioxidant transfers one electron to reduce an oxidizable probe (Fig. 2.12). In SET-based assays, an oxidant changes its color when it is reduced, and the degree of changes in the color is correlated with the sample's antioxidant concentrations (Zulueta, Esteve and Frígola, 2009). Many common antioxidant assays are based on this mechanism, including total phenolic content (TPC), $DPPH^{\bullet}$ and $ABTS^{\bullet +}$ free radical scavenging activity, FRAP (ferric reducing antioxidant power), and copper reduction assays (Huang, Boxin and Prior, 2005; Shahidi and Zhong, 2015).

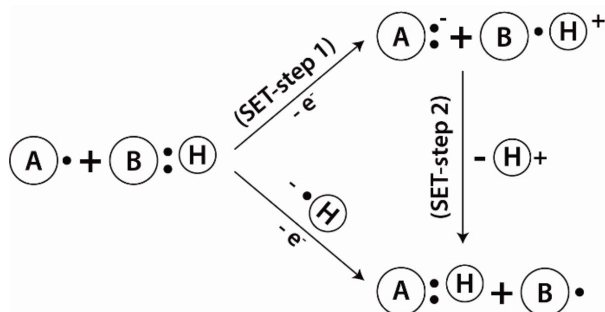


Fig. 2.12. Mechanisms of an antioxidant reacting with a free radical: single electron transfer (SET) and hydrogen atom transfer (HAT) (Liang and Kitts, 2014)

HAT-based assays quantify hydrogen atom donating capacity and measure antioxidant radical chain-breaking capacity. The oxygen radical absorbance capacity (ORAC) assay is one of the most common HAT-based methods for assessing the scavenging capacity of peroxyl radicals ($ROO^{\bullet}$). $ROO^{\bullet}$ are commonly found in food and biological samples, and they are formed via lipid oxidation chain reactions. They have harmful effects on health and are also associated with the quality deterioration of foods (Magalhães, Segundo, Reis and Lima, 2008). In the ORAC assay, water-soluble 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH) acts as the peroxyl radical generator, while the presence of antioxidant compounds inhibits or retards the oxidation of the target/probe induced by peroxyl radicals. A schematic representation of chemical reactions occuring in this assay is represented in Fig. 2.13. The principle of this assay is based on the intensity of the fluorescence decrease of the target/probe along time under reproducible and constant flux of peroxyl radicals generated from the thermal decomposition of AAPH in an aqueous buffer. In the presence of a sample that contains chain-breaking antioxidants, the decay of fluorescence is inhibited (Magalhães, Segundo, Reis and Lima, 2008).

Extraction is a good way to isolate bioactive antioxidants from the plant material; however, some insoluble components cannot be solubilized without altering their molecular nature by chemical or enzymatic treatment. Due to this, some compounds remain undervalued regarding their antioxidant activity. The *QUENCHER* procedure is suggested to overcome this problem because it enables the measurement of the antioxidant potential of the whole plant material including

its insoluble fraction. The *QUENCHER* procedure is based on the surface reaction phenomenon between the solid (bound antioxidant compounds) and the liquid (soluble free radicals derived from reagents) (Serpen, Gökmen and Fogliano, 2012).

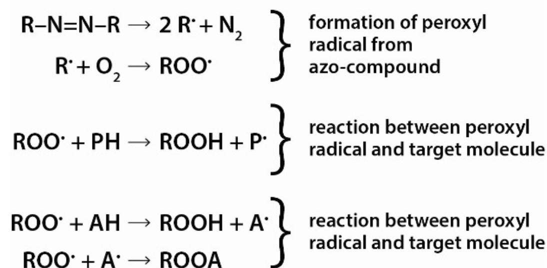


Fig. 2.13. Schematic representation of ORAC assay (Magalhães, Segundo, Reis and Lima, 2008)

Cell-based

Although *in vitro* methods are widely used for the antioxidant activity evaluation of plant samples, however, they still have some limitations because they do not take into account the bioavailability, uptake and metabolism of antioxidant compounds. It is not an easy approach to measure antioxidant effectiveness directly in animals or humans because these studies are expensive and time-consuming, therefore, they are not suitable for the initial antioxidant screening. In terms of this issue, it is suggested to use the cellular model which considers the distribution and metabolism of the antioxidants and a better correlation level with their performance *in vivo* (Kelly and Wolfe, 2007; Shahidi and Zhong, 2015). The most commonly used cell-based assay is the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay (Fig. 2.14) where the nonpolar and nonionic DCFH-DA probe passively diffuses into cells and is hydrolyzed by intracellular esterases to form nonfluorescent 2',7'-dichlorofluorescin (DCFH). In the presence of ROS, intracellular oxidases and oxidants, DCFH is oxidized to fluorescent 2',7'-dichlorofluorescein (DCF) which is trapped inside the cells (Girard-Lalancette, Pichette and Legault, 2009). When ROS production is not compensated by the cellular antioxidant defence system, oxidative stress occurs (Marimoutou *et al.*, 2015). Bioactive compounds can quench this reaction and prevent the generation of DCF. This can be accomplished on the surface of the cell membrane or within the cell following the uptake of antioxidant compounds (Chen *et al.*, 2017).

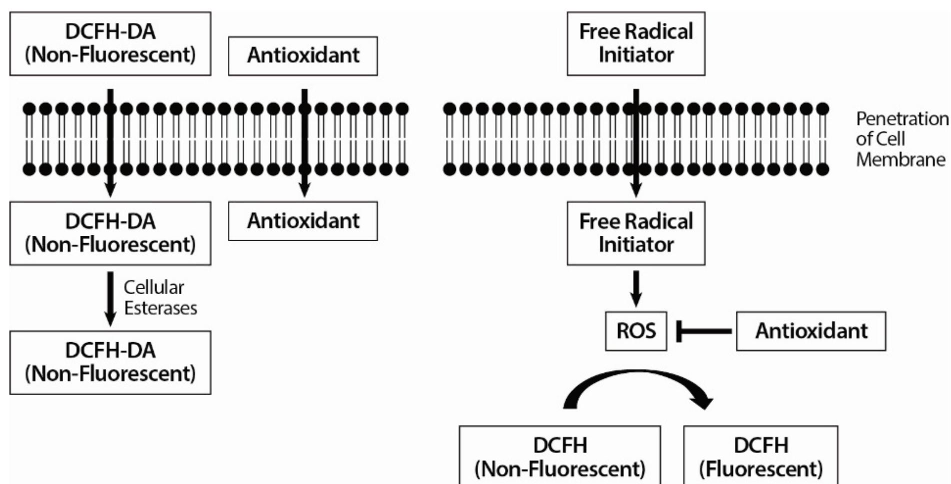


Fig. 2.14. Mechanism of DCFH-DA assay

2.8. Gene expression

There is an increasing number of articles in the scientific literature claiming that bioactive compounds without their direct action can also act indirectly as signaling molecules in the regulation of certain biological pathways. Some studies demonstrated that bioactive compounds can regulate the gene expression of antioxidative enzymes (Yeh, Ching and Yen, 2009; Wen *et al.*, 2015).

The quantitative real time polymerase chain reaction (RT-QPCR) assay is widely used to estimate the expression level of the genes of interest. The scheme of the QPCR assay is demonstrated in Fig. 2.15. The first step in the analysis of gene expression is RNA extraction. Purification of RNA by using a TRIzol reagent is one of the methods for the extraction of RNA. TRIzol is a monophasic solution of phenol and guanidinium isothiocyanate that simultaneously solubilizes biological material and denatures protein. After solubilization, the addition of chloroform causes phase separation, where protein is extracted to the organic phase, DNA resolves at the interface, and RNA remains in the aqueous phase. Therefore, RNA, DNA, and protein can be purified from a single sample (hence, the name TRIzol). The quality of RNA plays the crucial role in order to obtain accurate and reliable data of gene expression. After the extraction, the yield and purity of RNA is determined by measuring its absorbance at 260 nm and at 280 nm. Absorbance at 260 nm is used to measure the amount of the nucleic acid which is present in the sample, while at 280 nm we measure the amount of protein in the sample. It is considered that if the A260/280 ratio ranges between 1.8–2.0, the purity of RNA is acceptable. The second step in quantitative RT-PCR is the production of a single-stranded complementary DNA copy (cDNA) of RNA through the reverse transcriptase. This process is called reverse transcription. The third step is the amplification of specific DNA targets by using the polymerase chain reaction (Pfaffi, 2004; Rio, Jr, Hannon and Nilsen, 2019).

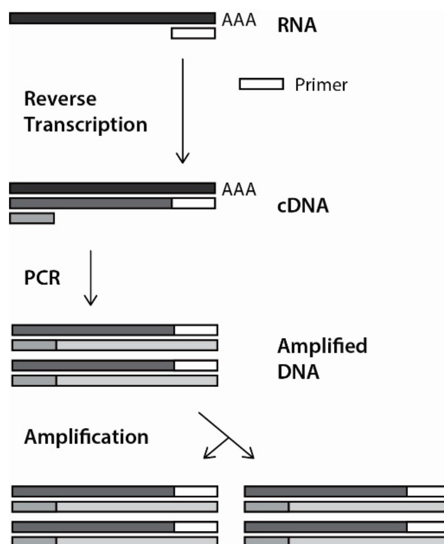


Fig. 2.15. Flowchart of RT-QPCR

2.9. Cytotoxicity

It is believed that natural products are safe and free from side effects, however, this idea is inherently false. Plants contain a diversity of constituents, and some of them are highly toxic. Good examples are the most cytotoxic anticancer-plant derived drugs, such as digitalis and pyrrolizidine alkaloids (Calixto, 2000). Thus, the evaluation of cytotoxicity plays a crucial role in the further application of plant-derived products. Cytotoxicity evaluation usually is the first step in cellular assays because it is important to perform assays with non-cytotoxic concentrations of samples. MTT and trypan blue exclusion assays are often used for cytotoxicity evaluation.

MTT cell viability assay was described for the first time by Mosmann, 1983. This colorimetric assay measures the cellular metabolic activity. Viable cells contain NADPH-dependent oxidoreductase enzymes which reduce the MTT reagent to formazan, an insoluble crystalline product with a deep purple color (Fig. 2.16). In trypan, the blue exclusion assay is used as the blue color trypan blue dye which can be absorbed by the dead cells; thus they have a blue cytoplasm, while in the live cells, the cell membranes are not colored (Soltanian, Sheikhbahaei and Mohamadi, 2017).

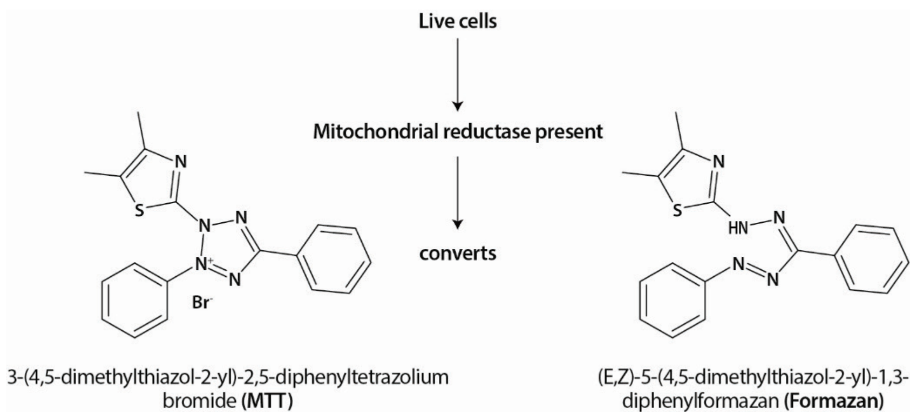


Fig. 2.16. Mechanism of MTT cell viability assay

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Research objects

The aerial parts of *Phyllanthus phillyreifolius* and *Aphloia theiformis* were collected in the south-west of Reunion Island in November 2013. The plants were dried at 37 °C during 2 days. Voucher specimens No. UR-PP2013/1 and JF874, respectively, were deposited in the herbarium of the University of Reunion by prof. Dominique Strasberg. Before extraction, the plants were ground in the laboratory mill *Retsch ZM200* (*Retch GmbH*, Haan, Germany) to the particle size of 1 mm.

Human Embryonic Kidney 293 (HEK293) (*ATCC CRL-1573*, Manassas, VA, USA) cell line was cultured at 37 °C with 5% CO_2 in Dulbecco's modified eagle medium (DMEM) (*Gibco/Invitrogen*, Carlsbad, CA, USA), supplemented with 10% heat inactivated fetal bovine serum (*Dutscher*, Brumath, France) and 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 100 U/mL of penicillin, 0.1 mg/mL of streptomycin and 0.5 µg/mL of fungizone (*PAN Biotech*, Germany). Along the experiments, the cells were monitored by microscopic observation in order to detect any morphological changes.

3.1.2. Chemicals and reagents

2,2-Diphenyl-1-picryhydrazy ($DPPH^{\bullet}$, 98%), 2,2-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS, 98%), fluorescein (FL), 2,2-azobis(2-amidinopropane) dihydrochloride AAPH, gallic acid, 6-hydroxy-2-5-7-8- tetramethylchroman-2-carboxylic acid (Trolox, 97%), 2,7-dichlorofluorescin diacetate, 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue, hydrogen peroxide (H_2O_2) were purchased from *Sigma-Aldrich* (Steinheim, Germany). 2.0 M Folin–Ciocalteu reagent (FCR), KCl, Na_2HPO_4, $K_2S_2O_8$, NaCl, Na_2CO_3 and HPLC grade acetonitrile were purchased from *Merck* (Darmstadt, Germany). KH_2PO_4 was sourced from *Jansen Chimica* (Beerse, Belgium). 2,4,6-tripyridyl-s-triazine (TPTZ) was obtained from *Fluka Chemicals* (Steinheim, Switzerland). Dimethyl sulfoxide (DMSO), n-butanol and ethyl acetate were purchased from *Sigma-Aldrich* (Saint-Quentin-Fallavier, France). Reference compounds, gallic acid, rutin and quercitrin hydrate were purchased from *Sigma Chemical Co*. (St. Louis, MO, USA), ellagic acid was sourced from *Fluka Biochemica* (Buchs, Switzerland), geraniin was bought from *ALB Technology* (Mongkok Kowloon, Hong Kong, China). Analytical grade solvents, hexane, acetone, methanol and acetic acid were sourced from *StanLab* (Lublin, Poland); ethanol of the agricultural origin (96.6%) was obtained from *Stumbras* (Kaunas, Lithuania). HPLC grade solvents for chromatographic analyses were purchased from *Sigma-Aldrich Chemie* (Steinheim, Germany). Ultrapure water was produced by using a *Simplicity 185* system (*Millipore*, MA, USA). All the chemicals and reagents used in this study were of the analytical and HPLC grade.

3.2. Preparation of extracts and fractions

The extraction schemes as well as the research plan are presented in Fig. 3.1 and 3.2. After extractions, the solvents were removed in a rotary evaporator (*Büchi*, Flawil, Switzerland) at 42 °C, while the residual water was freeze-dried. The amount of extracts and fractions was determined gravimetrically (±0.001 g). The extracts are further marked with a lowercase letter depending on the extraction method: s – single, c – consecutive, and p – pressurized. Dry extracts were stored in a freezer prior to further analysis. The solid residues after each extraction of *P. phillyreifolius* were dried at 30 °C and kept in a dry, well-ventilated and dark place prior to the *QUENCHER* measurements.

3.2.1. Soxhlet extraction

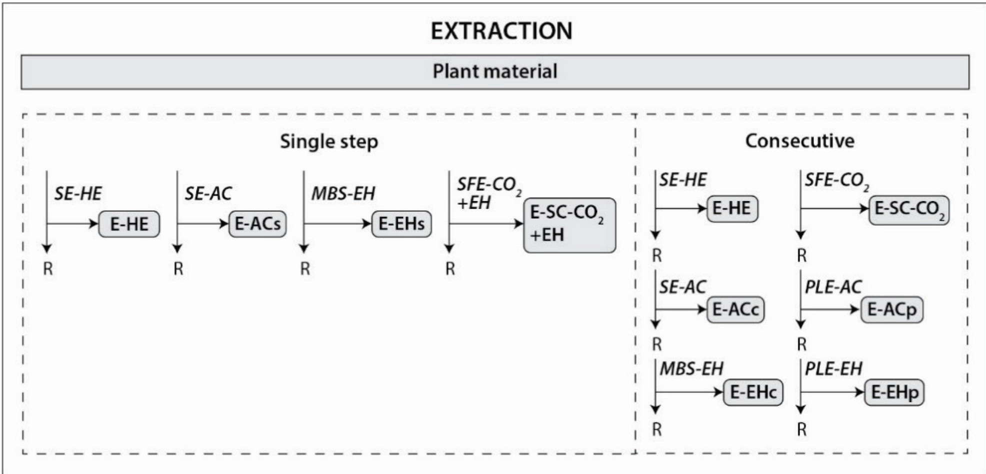
The sample (12 g) was placed into a cellulose thimble and extracted with hexane and acetone for 3 h in a Soxhlet extractor (*Behr Labor-Technik*, Düsseldorf, Germany). In sequential extractions, ground plants were firstly extracted with hexane, and then the residue was re-extracted with acetone.

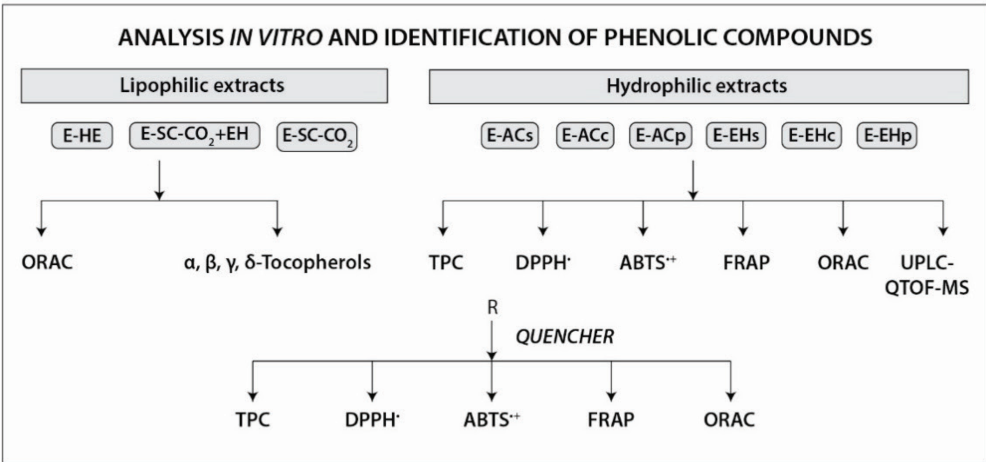
3.2.2. Maceration by stirring

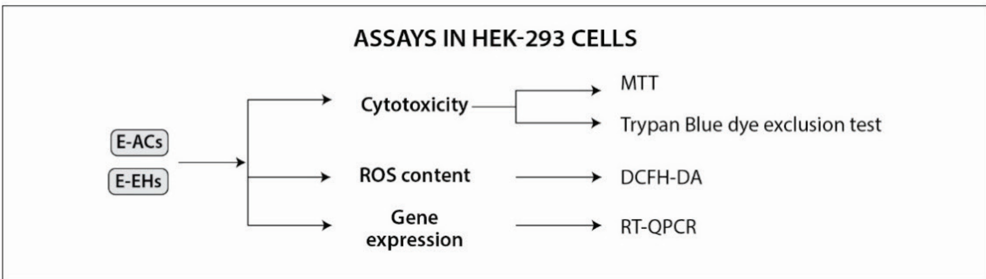
For hydroethanolic (EH) extracts, 5 g of plant material and residue after acetone extraction (in the consecutive extraction mode) was extracted 2 times by stirring with 100 mL 70% ethanol at room temperature for 1 h; the extracts were filtered through Whatman No.1 filter paper and combined.

3.2.3. Supercritical fluid extraction (SFE-CO_2)

SFE-CO_2 was carried out in a supercritical fluid extractor *Helix* (*Applied Separation*, USA). 15 g of plant material was loaded into 320 mm length×14 mm internal diameter extractor, its ends were plugged with cotton wool to avoid particle clogging in the system and to eliminate the vessel's dead volume. The conditions for the extraction with pure CO_2 and 2%, 5%, 10%, v/v of co-solvent (ethanol) were set as follows: time 120 min (including 10 min of static extraction time), pressure 47.5 MPa, temperature 45 °C, flow rate of CO_2 2–3 L/min. The volume of the consumed CO_2 was measured with a ball float rotameter and a digital mass flow meter in standard liters per minute (SL/min) at the standard state (P CO_2 = 100 kPa, T CO_2 = 20 °C, q CO_2 = 0.0018 g/mL). The flow rate of CO_2 in the system was controlled manually with a micro-metering valve (a back-pressure regulator) and kept constant during all experiments. CO_2 extracts were collected in glass vials, and, when the extraction was completed, the vials were kept until constant weight to avoid CO_2 residues.

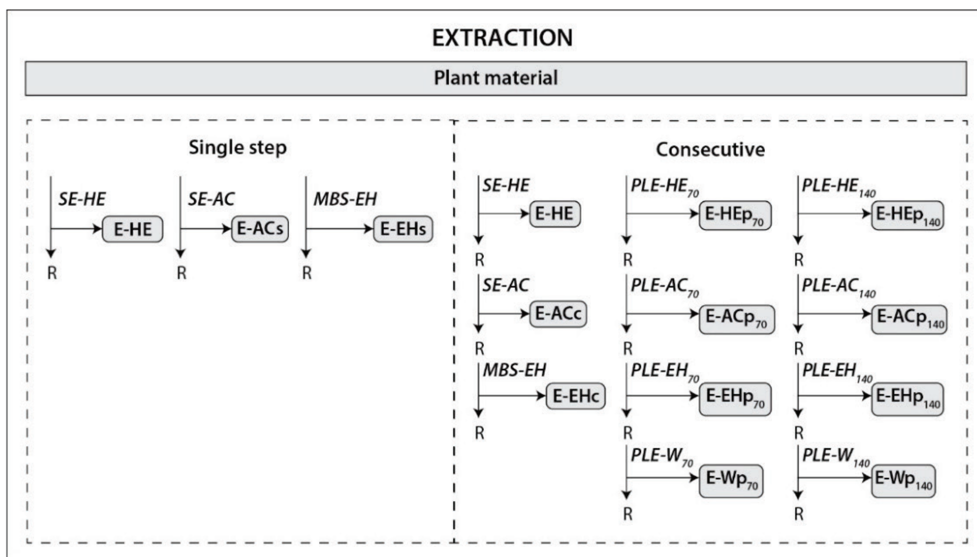


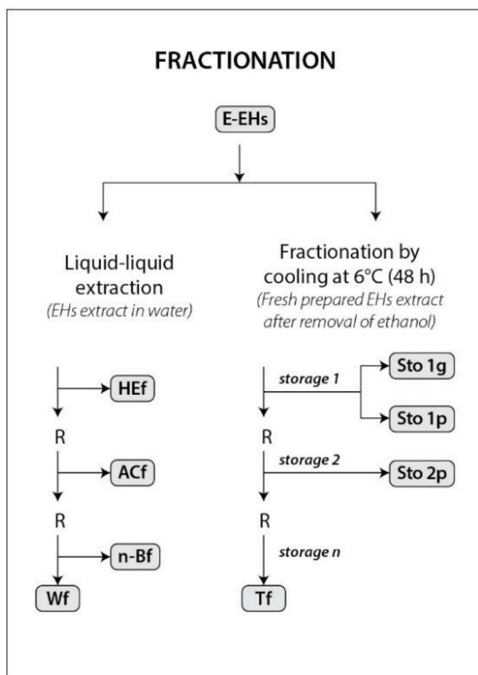


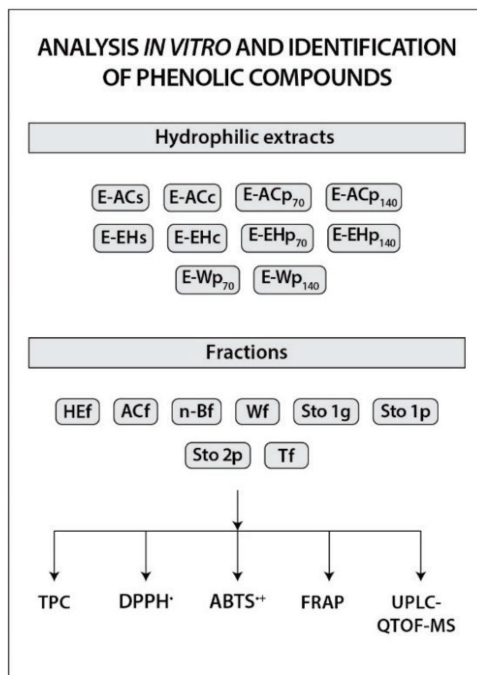


E – extract; **SE** – Soxhlet extraction; **MBS** – Maceration by stirring; **SC-CO_2** – Supercritical carbon dioxide (CO_2); **SFE-CO_2** – Supercritical CO_2 extraction; **PLE** – Pressurized liquid extraction; **HE** – hexane; **AC** – acetone; **EH** – ethanol:water (70:30); **R** – residue; **s** – single; **c** – consecutive; **p** – pressurized.

Fig. 3.1. Research scheme of *P. phillyreifolius*







E – extract; **SE** – Soxhlet extraction; **MBS** – Maceration by stirring; **PLE** – Pressurized liquid extraction; **HE** – hexane; **AC** – acetone; **EH** – ethanol:water (70:30); **W** – water; **ECf** – ethyl acetate; **n-Bf** – n-butanol; **R** – residue; **Sto1g** – green fraction (storage 1); **Sto1p** – precipitated fraction (storage 1); **Sto2p** – precipitated fraction (storage 2); **Tf** – transparent fraction; **s** – single; **c** – consecutive; **p** – pressurized.

Fig. 3.2. Research scheme of *A. theiformis*

3.2.4. Pressurized liquid extraction (PLE)

Pressurized solvent extraction (PLE) was performed on a *Dionex ASE 350* system (*Dionex*, Sunnyvale, CA, USA). 10 g of *P. phillyreifolius* plant material was placed in a 34 ml *Dionex* stainless-steel extraction cell (2.9 cm diameter) containing a cellulose filter at the ends to avoid solid particles in the collection vial. Before PLE extraction, 3.5 g of *A. theiformis* samples was mixed with diatomaceous earth (1:1) and placed in the extraction cell. The cells were preheated for 5 min to ensure that the samples reached thermal equilibrium at 10 MPa and a 70 °C or 140 °C temperature before the 3 extraction cycles taking 5 min each (for a total time of 15 min). Afterwards, the cell was purged for 60 s with nitrogen to collect the extract in the collection vial.

3.2.5. Liquid-liquid extraction

For liquid-liquid extraction, 0.5 g of dried EHs of *A. theiformis* was dissolved in 50 ml of distilled water and then partitioned sequentially with hexane, ethyl acetate and n-butanol in a separating funnel. Several successive extractions with every solvent were made, while using 100 ml of the solvent every time. In total, 500–600 ml of each solvent was used. Organic solvents were removed in a rotary evaporator, and the water was freeze-dried.

3.2.6. Fractionation by cooling

After maceration by stirring of *A. theiformis* with 70% ethanol, ethanol was removed in a rotary evaporator, and the extract was stored in a refrigerator at 6 °C for 48 h. The resulting precipitate was separated from the extract by centrifugation at 4500 rpm/min for 5 min and by pouring the transparent extract part to another test tube, while the precipitated fraction was removed from centrifuge tubes by adding water. After the first storage, the precipitated part of the extract consisted of two layers – green and white-yellow, therefore, they were further separated by centrifugation thus obtaining two fractions: the green fraction (Sto1g) and the precipitated fraction (Sto1p) (Fig. 3.3).

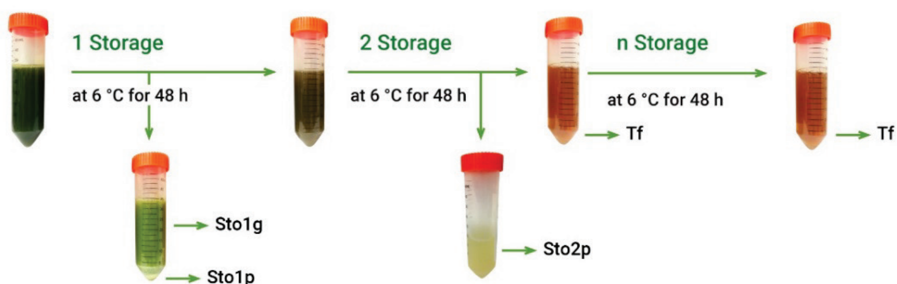


Fig. 3.3. Fractionation scheme of hydroethanolic extract (EHs) of *A. theiformis*

The transparent extract was further stored in a refrigerator at 6 °C for 48 h, and the resulting precipitate was separated by centrifugation thus obtaining the

precipitated fraction (Sto2p). This procedure was repeated until the complete absence of the precipitate. In most cases, the second storage was the last one giving precipitates, however, in some cases, a low amount of a yellow-white-colored precipitate was also obtained after the third and fourth storages. Due to the low amount of these precipitates and the same visual appearance as Sto2p, these precipitates were combined and analyzed together. The part of the extract which does not precipitate was assigned to the transparent fraction (Tf). After the fractionation, the water was freeze-dried.

3.3. Antioxidant activity evaluation *in vitro*

A working solution of *P. phillyreifolius* was prepared by dissolving extracts in methanol at a concentration of 10 mg/mL, except for lipophilic extracts which were diluted in acetone:methanol (1:9, v/v), while *A. theiformis* extracts were dissolved in DMSO and methanol (1:9, v/v). Methanol was used for further dilutions needed for every individual assay. Not fully dissolved extracts were treated in the ultrasonic bath *ASTRA-SONTM*, model *9H* (*Heat Systems Ultrasonics*, NY, USA) and filtered. The samples of *P. phillyreifolius* for the *QUENCHER* antioxidant activity assessment were prepared as described by Serpen *et al*. (2007) with some modifications. Plant materials (before and after extractions) were additionally ground to a particle size of 0.2 mm, mixed with microcrystalline cellulose at a concentration of 500 µg/mg and vortexed for 30 s. The further solid dilution step was performed according to antioxidant activity measurements: when the samples exerted too high antioxidant activity, they were diluted with microcrystalline cellulose. In each solid cellulose dilution step, a certain part of the previous dilution was mixed with cellulose thus obtaining dilution of the required concentration. The absorbances were measured with a *Spectronic Genesys 8* spectrophotometer (*Thermo Spectronic*, Rochester, NY) in semi-micro cuvets (*Ratiolab GmbH*, Dreieich, Germany). All the experiments were replicated at least 3 times.

3.3.1. Analysis of the total phenolic content (TPC) by Folin-Ciocalteu assay

TPC was determined spectrophotometrically with the Folin-Ciocalteu reagent (FCR) (Singleton, Orthofer and Lamuela-Raventós, 1998). Briefly, 150 µL of extract solution was mixed with 750 µL of FCR (previously diluted with distilled water at a ratio 1:9 (v/v)) and 600 µL of 7.5% Na_2CO_3 solution and left in the dark for 2 hours. For the *QUENCHER* assay, 10 mg of the sample or cellulose (blank) were transferred in an eppendorf, mixed with 150 µL of distilled water, 750 µL of FCR and 600 µL of 7.5% Na_2CO_3 solution, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark and centrifugated (4500 rpm, 5 min). Afterwards, the absorbance was measured at 760 nm. TPC was calculated from the calibration curve when using 150 µL gallic acid (GA) solutions (0–80 mg GA/mL ethanol) as a standard, and the results were expressed as mg GA equivalents per gram of the extract or fraction (mg GAE/g DWE or DWF) and the dry plant material (mg GAE/g DWP).

3.3.2. DPPH$^{\bullet}$ scavenging assay

The radical scavenging capacity (RSC) of extracts against DPPH$^{\bullet}$ was measured by the method of Brand-Williams, Cuveliera and Berset (1995) with some modifications. DPPH$^{\bullet}$ methanolic solution (~90 μM, final absorption 0.80 ± 0.03) was prepared daily before measurements; 1000 μL of DPPH$^{\bullet}$ solutions was mixed with 500 μL of extract solution or MeOH (blank) and left in the dark for 2 hours. For the *QUENCHER* aassay, 10 mg of the sample or cellulose (blank) were transferred to an eppendorf, mixed with 500 μL of MeOH and 1000 μL of DPPH$^{\bullet}$ solutions, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark, and centrifugated (4500 rpm, 5 min). The decrease in the absorbance value was measured at 517 nm. The RSC value was calculated from the calibration curve by using 500 μL Trolox solutions (0–50 μM TE in MeOH), and the results were expressed in μM of Trolox equivalents (μM TE/g DWE or DWF and DWP).

3.3.3. ABTS$^{\bullet+}$ scavenging assay

The ABTS$^{\bullet+}$ scavenging assay was obtained by the method described by Re *et al*. (1999) with slight modifications. Firstly, a phosphate buffered saline (PBS) solution (75 mM/L; pH 7.4) was prepared by dissolving 8.18 g NaCl, 0.27 g, KH_2PO_4, 3.58, Na_2HPO_4 ×12 H_2O and 0.15 g KCl in 1 L of distilled water. Stock ABTS$^{\bullet+}$ solution was prepared by mixing 50 mL ABTS (2 mM PBS) with 200 μL $K_2S_2O_8$ (70 mM H2O) and keeping for 12–16 h at room temperature in the dark. Before each assay, stock ABTS$^{\bullet+}$ solution was diluted with PBS to obtain the working ABTS$^{\bullet+}$ solution with absorbance of 0.80 ± 0.03 at 734 nm. For the analysis, 25 μL of sample or MeOH (blank) were mixed with 1500 μL of working ABTS$^{\bullet+}$ solution and left in the dark for 2 hours. For the *QUENCHER* assay, 10 mg of the sample or cellulose (blank) was transferred to an eppendorf, mixed with 25 μL of MeOH and 1500 μL of ABTS$^{\bullet+}$ solutions, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark, and centrifugated (4500 rpm, 5 min). The decrease in the absorbance value was measured at 734 nm. The radical scavenging capacity (RSC) was calculated from the calibration curve by using 25 μL Trolox solutions (0−1800 μM TE in MeOH), and the results were expressed in μM TE/g DWE or DWF and DWP.

3.3.4. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was obtained by employing the method of Benzie and Strain (1996) with some modifications. FRAP reagent was prepared by mixing a 10 mM TPTZ solution in 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$ and an acetate buffer (300 mM, pH 3.6) at 1:1:10 (v/v/v). For the measurement, 50 μL of the sample or MeOH (blank) was mixed with 150 μL of distilled H_2O and 1500 μL of a freshly prepared FRAP reagent. For the *QUENCHER* assay, 10 mg of the sample or cellulose (blank) was transferred to an eppendorf, mixed with 50 μL of MeOH, 150 μL of distilled H_2O and 1500 μL of the FRAP reagent, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark, and centrifugated (4500 rpm, 5 min). After 2 h incubation in the dark at 37 °C, the decrease in the absorbance was read at 593 nm. A series of Trolox

solutions in the concentration ranges of 0–800 μM TE in MeOH were used for the calibration, and the results were expressed in μM TE/g DWE or DWF and DWP.

3.3.5. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay obtained as described by Prior *et al.* (2003) by using fluorescein as a fluorescent probe. Briefly, 25 μL of the sample or MeOH (blank) was mixed with 150 μL of fluorescein solution (14 μM in PBS) in a clear-bottom 96-well black opaque microplate. The mixture was preincubated for 15 min at 37 °C and 25 μL of AAPH solution (240 mM in PBS) as a peroxyl radical generator was immediately added by using a multichannel pipet. The fluorescence was recorded for 120 cycles (every cycle 1 min × 1.1) at 485 excitation and 520 emission wavelengths at 37 °C in a *FLUOstar Omega* reader (*BMG Labtech*, Offenburg, Germany). For the *QUENCHER* assay, 10 mg of the sample or cellulose (blank) was mixed with 150 μL of PBS solution and 900 μL of fluorescein solution, vortexed for 15 s, shaken at 250 rpm for 120 min in the dark, and centrifuged (4500 rpm, 5 min). The fluorescence was recorded for 120 cycles, followed by rapid addition of AAPH. Trolox solutions (0–250 μM in PBS) were used for calibration. The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area under the curve (AUC) as follows: $AUC = (1+f_1/f_0+f_2/f_0 \ldots f_i/f_0)$, where f_0 is the initial fluorescence reading at time 0 min and f_i is the fluorescence reading at time i, which were expressed in μM TE/g DWE or DWF and DWP.

3.4. Determination of tocopherols by high performance liquid chromatography (HPLC)

Quantitative determination of tocopherols (α, β, λ and γ) in hexane and SC-CO_2 extracts of *P. phillyreifolius* was performed in an HPLC system as described by Kraujalis and Venskutonis, 2013. A *Perkin Elmer Series HPLC* system was equipped with a *C30* reverse–phase column (5 μm, 250 mm×4.6 mm), thermostated at 30°C while applying isocratic elution with acetonitrile:methanol:dichlormethane (72/22/6, v/v/v). For the analysis, extracts and standards were dissolved in a mobile phase at a final concentration of 0.1 mg/mL and 0–10 μg/mL, respectively. The injection volume was 20 μL, and the flow rate was set at 1 mL/min. A fluorescence detector at 290 nm excitation and 330 nm emission was used for detection. Tocopherols were identified by comparing the retention time of the peaks to those of the pure standard solutions. The quantity of tocopherols in the samples was determined by using the calibration curves (the peak area versus the injected amount).

3.5. Identification and quantification of phenolic compounds by UPLC-QTOF-MS analysis

The chromatographic separation of analytes was carried out on an *Acquity UPLC* (*Waters*, Milford, MA, USA) system equipped with a binary pump, an autosampler, a photodiode array (PDA) detector, a column manager, with the data station running the *Compass* acquisition and data software.

Compounds of *P. phillyreifolius* were separated on an *Acquity BEH*, *C18* column (100 mm × 2.1 mm, 1.7 µm) maintained at 40 °C. The eluent system consisted of solvents A (0.1% formic acid in ultra pure water) and B (100% acetonitrile) with a linear gradient programmed as follows: 0–14 min, 5% B; 15–17 min, 100% B; 18 min, 5% B. The flow rate was 0.4 mL/min, the temperature of the sample was 12 °C, and the sample injection volume was 1 µL. The separation of *A. theiformis* compounds was performed on *Acquity BEH*, *C18* column (50 mm × 2.1 mm, 1.7 µm) maintained at 30 °C. The eluent system consisted of solvents A (0.4% formic acid in ultra pure water) and B (100% acetonitrile) with a linear gradient programmed as follows: 0–14 min, 5% B; 15–17 min, 100% B; 18 min, 5% B.

The flow rate was 0.4 mL/min, the temperature of the sample was 10 °C, and the sample injection volume was 1 µL. The effluents from the PDA detector were introduced directly into the quadrupole-time of a flight mass spectrometer (Q-TOF) equipped with an electrospray ionization source controlled by *HyStar 3.2 SR2* software (*Bruker Daltonic*, Bremen, Germany). All the MS data was recorded in the ESI negative ionization mode in a range of 80–1200 m/z, and the capillary voltage was maintained at +4000 V. Nitrogen was used as a nebulizer gas at 2.5 bar and a drying gas at a flow rate of 10 L/min. The peaks were identified by comparing their retention times and parent ions with external standards, references and commercial databases.

Selected phenolics were quantified by using an *Acquity UPLCMS H-Class* equipped with *Xevo TQ-S* tandem quadrupole mass spectrometer (*Waters*, Milford, MA) operating in the negative electrospray ionization (ESI) mode, the capillary voltage was set to 1500 V, the cone voltage was –20 V, the source offset was –50 V. The desolvation temperature was 500 °C, the desolvation gas flow was –1000 L/h, the cone gas flow was –150 L/h, and the nebulizer pressure was set to 7 bar.

The chromatographic separation of *P. phillyreifolius* samples was performed while using the same column and solvents as described above with a linear gradient programmed as: 0.0–7 min, 5% B; 8–9 min, 50,7% B; 10–11 min, 100% B; 12–20 min, 5% B. The flow rate was 0.4 mL/min, and the sample injection volume was 5 µL. MS detection was achieved in the single-ion-monitoring (SIM) mode. The m/z values and the dwell times of components were set as follows: 169.1595m/z and 0.1 s (gallic acid), 301.0957 m/z at 0.025 s (ellagic acid), 447.1595 m/z at 0.025 s (quercitrin), 609.2233 m/z at 0.025 s (rutin), 951.1957 m/z at 0.050 s (geraniin), 991.1000 m/z at 0.025 s (phyllanthusiin D), 1109.1000 m/z at 0.050 s (elaeocarpusin). The concentrations of phytochemicals were calculated from the calibration curves prepared by using concentrations of 0.05–50 µg/mL of different standard compounds: gallic acid (y=34937x-16.54; R^2=0.9937), ellagic acid (y=6142.6x+16634; R^2=0.9954), geraniin (y=6158x+4127; R^2=0.9986), rutin (y=45440x+28682; R^2=0.9953) and quercitrin (y=65477x+19631; R^2=0.9953). For the determination of the fragmentation patterns of some compounds, direct infusion was made to a *Waters TQ-S* system by deploying collision induced dissociation (CID) using argon as a collision gas at 25 eV and a flow rate of 0.11 mL/min.

The chromatographic separation of *A. theiformis* samples was performed by using the same column and solvents as described above with a linear gradient

programmed as: 0–9 min, 100% A; 9–10 min, 100% B; 10–14 min, 100% A. The flow rate was 0.4 mL/min, and the sample injection volume was 2 µL. MS detection was achieved in the multiple reaction monitoring (MRM) mode. The m/z values and dwell times of the components were set as follows: 421.0778m/z and 0.002 s (mangiferin), 487.3430 m/z at 0.05 s (tormentic acid), 503.3377 m/z at 0.05 s (hydroxytormentic acid). The concentrations of phytochemicals were calculated from the calibration curves prepared by using concentrations of 0.02–40 µg/mL of different standard compounds: mangiferin (y=33.948x+83711; R^2=0.9949), tormentic acid (y=5.0611x+270.28; R^2=0.9917), and hydroxytormentic acid (y=4.0672x+5823; R^2=0.9916). For the determination of the fragmentation patterns of some compounds, the scan wave DS (daughter scan) function was applied.

MassLynx 4.1 software was used for instrument control and data collection. All the samples were run in triplicates. The results were expressed in the dry weight of extracts (DWE) or fractions (DWF). The amount of quantified compounds in the extracts was also recalculated to the dry weight of the whole plant material (DWP).

3.6. Assays in cells

3.6.1. MTT cell viability assay

Cytotoxicity was evaluated by spectrophotometric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay as previously described by Frumence *et al*. (2016) with some modifications. Stock solutions of acetonic (ACs) and hydroethanolic (EHs) extracts of *P. phillyreifolius* were prepared by dilution extracts in DMEM at a concentration of 1000 µg/mL. Briefly, serial dilutions of extracts in the DMEM medium were added to 1 x 10^4 cells/well in a 96-well plate and incubated for 24 h. The supernatant was discarded, and a solution containing MTT (5 mg/mL) was applied for 1 h. After the incubation, the supernatant was removed, and the formazan crystals were solubilized with 100 µL of DMSO. The optical density (OD) was measured at 570 nm with a background subtraction at 690 nm. Data from three independent experiments was normalized by using the following equation: cell viability (%) = (OD sample value)/(OD cell control) x 100. The concentration that inhibited the viability in 50% of cells (IC_{50}) was obtained by performing nonlinear regression followed by the construction of a sigmoidal concentration-response curve (variable slope; *Graphpad Prism 5*).

3.6.2. Trypan blue exclusion assay

Nontoxic concentrations of extracts determined by the MTT assay was confirmed by the trypan blue exclusion assay as decribed by Poompachee and Chudapongse (2012) with some modifications. Briefly, 3 ml of HEK293 cells were preincubated overnight in 6-well microplates at 1x10^6 cells per well. After the incubation, the growing media was removed, and the cells were treated with samples at 0–1000 µg/mL for 24 h. Then, the supernatant was removed, the cells were washed with physiological water, and the cells were also trypsinized. The numbers of viable (unstained) cells were counted with a hemacytometer under a light microscope, and the results were calculated and expressed as the percentage of the live cells compared to total number of cells (control sample).

3.6.3. Cellular antioxidant activity (CAA)

The intracellular formation of ROS was evaluated by using oxidation sensitive DCFH-DA probes (Baret *et al.*, 2013). Briefly, HEK293 cells were cultivated overnight in black 96-well microplates at 1×10^4 cells per well. After the treatment with various concentrations of extracts (31.25÷250 μg/mL) for 3 h or 24 h, the cells were washed with 50 μL of PBS and incubated for 45 min with 100 μL of DCFH-DA (10 μM) in the dark. To assess the antioxidant activity, the supernatant was removed, and the cells were incubated for 1 h with or without 20 μL of H_2O_2 (100 μM). The fluorescence was measured on the plate reader (*BMG-Labtech*, Offenburg, Germany) while using 485 nm excitation and 530 nm emission wavelengths.

3.6.4. RNA isolation, reverse transcription and quantitative real time PCR (QPCR)

The total RNA was extracted from cells with an *RNeasy* kit (*Qiagen*, Courtaboeuf, France), and reverse transcription was performed by using 500 ng of the total RNA. Quantificative PCR was performed on an *ABI7500 Real-Time PCR System* (*Applied Biosystems*, *Life Technologies*, Villebon-sur-Yvette, France). Briefly, 10 ng cDNA was amplified by using 0.2 μM of each primer and 1X GoTaq Master Mix (*Promega*, Charbonnières-les-Bains, France). The data was normalized to the internal standard *GAPDH*. For each single-well amplification reaction, a threshold cycle (Ct) was calculated by using the *ABI7500* program (*Applied Biosystems*) in the exponential phase of amplification. Relative changes in the gene expression were determined by using the ΔΔCt method and reported relative to the control values. The primers used in this study are listed in Table 3.1.

Table 3.1. List of primers used for RT-QPCR

Gene	Sense primer (5′-3′)	Antisense primer (3′-5′)
SOD1	ACCAGTGTGCGGCCAATGATG	GCTGTACCAGTGCAGGTCCTCA
SOD2	AAGCTGACGGCTGCATCTGTTG	CGTGCTCCCACACATCAATCCC
GPx	AGTTCGGACATCAGGAGAA	AGGGCTTCTATATCGGGTTC
CAT	AGTCTCGCCGCATCTTCAACAG	TCTGGGACTTCTGGAGCCTACG
GAPDH	GGGAGCCAAAAGGGTCATCA	TGATGGCATGGACTGTGGTC

3.7. Statistical analysis

Statistical analysis was performed by using *Graphpad Prism* software (version *5.0*; *Graphpad software*, La Jola, CA, USA). The results were subjected to analysis of variance (one-way *ANOVA*), and significant differences among the means were determined by using Tukey's multiple comparison test. The differences were considered to be significant when the p-values were below 0.05 ($p < 0.05$). All the data was expressed as mean ± standard deviation (SD). Pearson's correlation coefficient for mangiferin content/TPC and the antioxidant activity assays was calculated by using *MS Excel 2010*.

4. RESULTS AND DISCUSSION

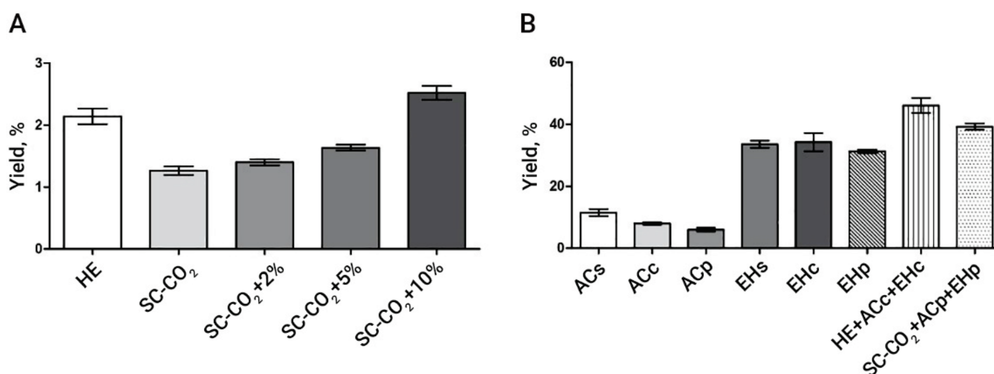
4.1. Valorisation of *P. phillyreifolius* by different extraction methods and evaluation of antioxidant properties and phytochemical composition[1]

4.1.1. The effects of different extraction methods on the yield of extracts

The yield of a herbal extract is an important parameter which may be used as a general indicator of the extraction efficiency and usually depends on the solvent polarity, the extraction time and temperature, the sample-to-solvent ratio, as well as on the chemical composition and physical characteristics of the samples (Šliumpaitė, Venskutonis, Murkovic and Ragažinskienė, 2013; Dai and Mumper, 2010). The yields of *P. phillyreifollius* extracts obtained by various solvents and methods are summarized in Fig. 4.1. The variation of the yields of various extracts is attributed to the polarities of different compounds which are present in *P. phillyreifollius* leaves. The lipophilic fractions of *P. phillyreifollius* which were isolated with hexane and SFE-CO_2 constituted only 2.14% and 1.26%, respectively. That is related to the low levels of non-polar substances in the plant. Kraujalienė, Pukalskas, Kraujalis and Venskutonis (2016) also reported that SFE-CO_2 of the herbal material gave a lower yield than hexane. In order to increase the yield of SC-CO_2 extracts, different concentrations (2%, 5% and 10%) of ethanol as a co-solvent were added in the SFE extraction system. Thus, 10% of ethanol increased the yield almost two times compared to the extract obtained with pure CO_2. Further extraction steps with acetone and 70% ethanol were applied for separating higher polarity hydrophilic fractions. Although acetone was quite an effective solvent, and the yields of this fraction ranged from 5.98% (ACp) to 11.5% (ACs), the highest recovery of soluble substances was achieved by the polar protic solvent, 70% ethanol; the yield of hydroethanolic extracts ranged from 31.3% (ETp) to 34.3% (ETc) thus demonstrating that high polarity soluble substances are dominant in *P. phillyreifollius*.

The total yield in the three steps of consecutive extractions while using consecutive extraction methods (44.4%) was significantly higher than the highest yield obtained in one-step extraction with 70% ethanol (38.6%). This effect could be related to the solubility of different classes of phenolic compounds, which depends on the solvent polarity and their interactions with other plant constituents (Garmus, Paviani, Queiroga and Cabral, 2015); therefore, in terms of the total yield, consecutive extraction was superior to one step extraction. Consecutive extraction was also perfomed by using high pressure extraction methods, SFE and PLE; in this case, the total yield was by 14.8% lower comparing to the conventional extraction.

[1] This section was prepared with reference to Grauzdytė, Pukalskas, El Kalamouni and Venskutonis (2018)



4.1 Fig. Yields of *P. phillyreifolius* lipophilic (A) and hydrophilic (B) extracts obtained by various extraction methods and solvents

4.1.2. Tocopherol content and antioxidant properties of lipophilic fractions

Antioxidant properties of lipophilic fractions

In this study, lipophilic extracts were characterized by the content of tocopherol and ORAC. Tocopherols are well-known lipophilic constituents possessing vitamin E activity. They are recognized as potent lipophilic antioxidants providing protection against lipid peroxidation in biological tissues (Hounsome, Hounsome, Tomos and Edwards-Jones, 2008). In the nature, vitamin E is present in eight different forms, α-, β-, δ-, and γ-tocopherols (T) and α-, β-, δ-, and γ–tocotrienols determined by the numbers and the positions of methyl groups on the chromanol ring (Zingg, 2007; Saini and Keum, 2016). With respect to the isomer composition, γ-T is the most abundant tocopherol in seed oils, major dietary sources of tocopherols, while α-T is the most abundant form in the leaves and is the predominant form of vitamin E in human blood and tissues (Szymańska and Kruk, 2008). Additionally, among the four known homologous of tocopherols, α-T demonstrates the highest activity, followed by γ, δ and β isomers (Zingg, 2007).

Three isomers of tocopherols were detected in the tested extracts by HPLC, with α-T being the predominant form (approximately 87% of the total identified tocopherols), while other β- and γ-T were found at a lower content, 9.86–12.7% and 2.11–2.87%, respectively. The total amount of the extracted tocopherols ranged between 13.4–20.6 mg/g extract and 0.30–0.44 mg/g DWP (Table 4.1). It may be observed that the SC-CO_2 extract contained a higher amount of tocopherols than the hexane extract; however, due to a smaller yield, the total recovery of tocopherols expressed in mg/g DWP was higher in the case of hexane extraction. It should be noted that the content of the tocopherols extracted by SFE significantly increased by adding 2% of ethanol as a co-solvent. Kraujalis and Venskutonis (2013) also showed that the addition of a co-solvent in the SFE system improved the recovery of tocopherols. Meanwhile, the higher amount of a co-solvent (5% and 10% of ethanol) decreased the content of tocopherols in the extracts. That could be explained by the dilution of tocopherols with other more polar constituents which are extracted by increasing the co-solvent concentration (Quancheng, Guihua, Hong and Moucheng, 2004).

Table 4.1. Concentration of tocopherols (T) of lipophilic *P. phillyreifolius* extracts (in mg/g), recovery of T expressed as mg/g DWP

Sample	α-T		β-T		γ-T		Total tocopherols	
	DWE	DWP	DWE	DWP	DWE	DWP	DWE	DWP
HE	17.9±0.55[b]	0.38[d]	2.19±0.08[b]	0.05[c]	0.48±0.00[b]	0.01[c]	20.6±0.63[b]	0.44[d]
SC-CO_2	21.1±0.93[c]	0.27[a]	2.38±0.11[c]	0.03[a]	0.59±0.00[d]	0.007[a]	24.1±1.04[c]	0.30[a]
SC-CO_2 +2%	24.9±0.41[d]	0.35[c]	2.96±0.06[d]	0.04[b]	0.60±0.01[d]	0.008[a]	28.4±0.48[d]	0.40[c]
SC-CO_2 +5%	16.8±0.52[b]	0.28[a,b]	2.47±0.11[c]	0.04[b]	0.53±0.02[c]	0.009[b]	19.8±0.65[b]	0.32[a,b]
SC-CO_2 +10%	11.3±0.33[a]	0.29[b]	1.70±0.06[a]	0.06[d]	0.39±0.01[a]	0.01[c]	13.4±0.40[a]	0.34[b]

Values are represented as means ± standard deviation (n=3); the columns with different letters differ significantly for Tukey's test at $p < 0.05$; DWE – dry weight extract; DWP – dry weight initial plant

The antioxidant capacity of SC-CO_2 extracts evaluated by the $ORAC_{FL}$ assay increased by increasing the amount of the co-solvent, while the hexane extract containing more tocopherols than the extract obtained with SC-CO_2+10% ethanol demonstrated the lowest antioxidant activity (Fig. 4.2). These findings may be explained by the increasing recovery of higher polarity antioxidants in the case of modifying supercritical CO_2 with higher amounts of ethanol.

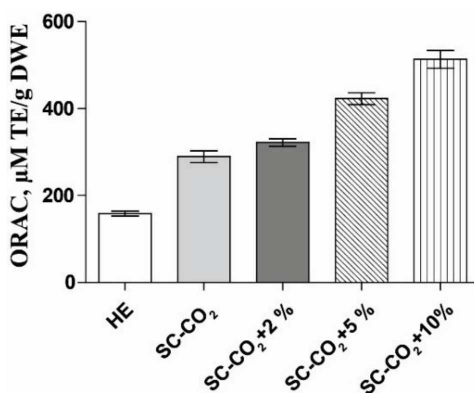


Fig. 4.2. Oxygen radical scavenging capacity (ORAC) of lipophilic *P. phillyreifolius* extracts (in µM TE/g DWE)

4.1.3. Total phenolic content and antioxidative properties of higher polarity fractions

Plant polyphenolics constitute one of the major groups of compounds acting as antioxidants due to their ability to donate electrons or hydrogen atoms; therefore, in this study, the TPC of *P. phillyreifolius* extracts was determined by using FCR, and the results were expressed as GAE in g of the extract. It is worth pointing out that FCR is not specific, and it detects all the phenolic groups found in the extract as well as other oxidation substrates (Everette *et al*., 2010). The TPC of the extracts obtained by using various solvents and the extraction methods ranged from 420±14.8 (ACp) to 510±24.0 (EHp) mg GAE/g DWE, however, only the hydroethanolic extract obtained by PLE (EHp) demonstrated significantly higher TPC values than the other extracts (Table 4.2). In comparison with the other, more widely studied, *Phyllanthus* species, the TPC of *P. phillyreifolius* was considerably higher than *P. amarus*, *P.urinaria* and *P. debilis*, 171±15.6; 325±17.8 and 380±22.8 mg GAE/g DWE, respectively (Kumaran and Karunakaran, 2006). Meanwhile, the results expressed in mg GAE/g DWP showed that 70% ethanol recovered a remarkably higher amount of phenolics than acetone.

Many phenolic compounds demonstrate free RSC under mechanisms based on a single electron transfer (SET) when the phenolic antioxidant transfers one electron to reduce an oxidisable probe, and/or on hydrogen atom donation (HAT) reactions (Niki, 2010; Huang *et al*., 2005). Therefore, several *in vitro* assays based on the chemical principle of SET including TPC, RSC of $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and FRAP as well as HAT based ORAC were applied for assessing the antioxidant properties of the other, nonlipophilic, *P. phillyreifolius* extracts. Trolox, a water soluble derivative of vitamin E, was used to quantify the antioxidant capacity of the extracts, and the results were expressed as trolox equivalents (TE) in 1 g of the extract (DWE). To evaluate the recovery of antioxidants from 1 g of the dried plant, the results were recalculated to g of the dry weight of the plant (DWP) (Table 4.2). Both values provide valuable information, whereas, sometimes, the extract may be a strong antioxidant, while its yield may be very low; and, on the contrary, less antioxidatively active but high yield extracts can recover a larger part of antioxidants from the plant matrix (Povilaitis, Šulniūtė, Venskutonis and Kraujalienė, 2015).

The RSC of plant extracts ranged from 3278±551 (EHc) to 4288±137 (EHp) μmol TE/g extract in the $DPPH^{\bullet}$ scavenging assay, while the $ABTS^{\bullet+}$ scavenging values ranged from 5804±170 (EHc) to 8190±319 (ACc) μmol TE/g DWE. In comparison, the other *Phyllanthus phillyreifolius* species endemic to Mauritius exhibited a lower radical scavenging activity than the values observed in this study. Among various extracts, the extract of *Phyllanthus phillyreifolius* var. *commersonii Müll.* As investigated in the previous study by Mahomoodally *et al*., 2018, the methanol extract possessed the best $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging properties (2.91 and 4.12 mmol TE/g, respectively). The ability of extracts to reduce ferric Fe (III) ions to their respective lower valency state as determined by the FRAP method varied from 5349±217 (EHp) to 6262±302 (ACp) μmol TE/g DWE.

Table 4. 2. Total phenolic content (TPC) (in mg GAE/g) and antioxidant capacity (in μM TE/g) of *P. phillyreifolius* extracts

Sample (*extraction method*)	TPC		DPPH$^{\bullet}$		ABTS$^{\bullet+}$		FRAP		ORAC	
	DWE	DWP	DWE	DWP	DWE	DWP	DWE	DWP	DWE	DWP
Single step extraction										
ACs *(SE)*	449±20.0^{a}	51.7^{c}	3395±121^{a}	391^{c}	7655±444^{c}	883^{c}	6062±212^{b}	699^{c}	3443±113^{c}	397^{c}
EHs (*MBS*)	454±13.5^{a}	153^{f}	3317±79.0^{a}	1116^{e}	6887±138^{b}	2316^{f}	5568±185^{a}	1907^{f}	3205±128^{b}	1077^{f}
Consecutive extraction										
ACc *(SE)*	429±1.10^{a}	33.5^{b}	3559±21.8^{a}	278^{b}	8190±319^{c}	640^{b}	5465±103^{a}	427^{b}	3669±142^{d}	287^{b}
EHc(*MBS*)	429±1.19^{a}	133^{d}	3278±55.1^{a}	1012^{d}	5804±170^{a}	1791^{d}	5649±210^{a}	1743^{e}	3259±127^{b}	1006^{e}
Σ		167		1290		2431		2170		1293
ACp (*PLE*)	420±14.8^{a}	24.6^{a}	4094±130^{b}	240^{a}	7963±241^{c}	466^{a}	6262±302^{b}	366^{a}	2834±96.6^{a}	166^{a}
EHp (*PLE*)	510±24.0^{b}	147^{e}	4288±137^{c}	1235^{f}	7311±269^{c}	2106^{e}	5349±217^{a}	1541^{d}	2918±94.1^{a}	841^{d}
Σ		172		1475		2572		1907		1007

Values are represented as mean ± standard deviation (n=3); columns with different letters differ significantly for Tukey's test at $p < 0.05$; DWE – dry weight extract; DWP – dry weight of the initial plant

The antioxidant capacity of extracts evaluated by the ORAC assay involving peroxyl radicals as the oxidants and therefore providing useful information on the antioxidant radical chain-breaking capacity (Huang, Boxin and Prior, 2005) varied in the range from 2834±96.6 (ACp) to 3669±142 (ACc) µmol TE/g DWE. For comparison, the ORAC values reported for well-known antioxidant compounds, such as caffeic acid, quercetin, or gallic acid, were 1528, 1335 and 6970 µmol TE/g, respectively (Villaño, Fernández-Pachón, Troncoso and García-Parrilla, 2005). These results indicate that, regardless of many variables involved in each extraction method and different responses in the applied assays, the analyzed extracts were strong antioxidants. However, the values of the antioxidant activity recalculated to g of DWP revealed a remarkably higher effectiveness of 70% ethanol in the recovery of antioxidants than acetone in all the assays. Nevertheless, the consecutive extraction gave significantly higher recovery of antioxidants in different assays than the one step extraction. This shows that the removal of the lipophilic material before extraction had positive influence on the recovery of antioxidants. Studies undertaken by Turkmen, Sari and Velioglu (2006) demonstrated that, in the extraction of polyphenols, the single step extraction compared to the multiple extraction procedure is not sufficient. Moreover, the sum of the recovered antioxidants in consecutive extraction showed the superiority of the innovative extraction methods against the conventional extraction in TPC, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays.

A strong positive correlation exists between TPC and antioxidant activity assays (0.975–0.994) (Fig 4.3). The highest correlation was observed between TPC and $ABTS^{\bullet+}$ assays. However, it should be noted that these correlations were observed only for the results expressed in mg GAE/g DWP.

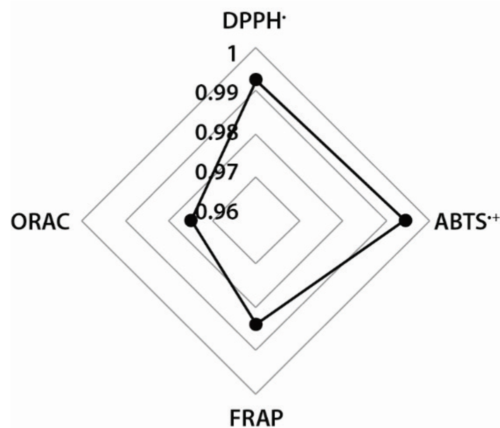


Fig. 4.3. Correlation coefficients between TPC and different antioxidant measurement assays

4.1.4. Antioxidant properties of solid *P. phillyreifolius* samples measured by *QUENCHER* method

Plant antioxidants may be strongly bound to other molecules and are not dissolved in extraction solvents; therefore, the evaluation of the residual antioxidant capacity of solids remaining after extraction is important for assessing the effectiveness of recovery of bioactive phytochemicals (Gökmen, Serpen, Pellegrini and Fogliano, 2009).

To obtain additional information on the antioxidant capacity of both soluble and insoluble parts and to evaluate the recovery of antioxidative constituents from *P. phillyreifolius*, all *in vitro* assays (DPPH$^{\bullet}$, ABTS$^{\bullet+}$, ORAC, FRAP and TPC) were applied to plant solids before and after extractions by employing the *QUENCHER* method which does not require the extraction step prior to the measurements and evaluates the antioxidant potential of the soluble and insoluble compounds due to the typical liquid–liquid interactions and the surface reaction occurring at the solid-liquid interface, respectively (Gökmen, Serpen, Pellegrini and Fogliano, 2009).

As presented in Table 4.3, the plant material before extraction demonstrated the highest antioxidant capacity in all the performed assays. The decrease in the antioxidant capacity after extraction occurs due to the gradual removal of active lipophilic and hidrophilic compounds during extractions (Šulniūtė, Ragažinskienė and Venskutonis, 2016). The results demonstrated that 70% of ethanol dissolved the highest amount of antioxidatively active constituents from *P. phillyreifolius* and thereby most significantly reduced its antioxidant activity; for example, RSC in the DPPH$^{\bullet}$ assay after extractions decreased from 1503±58.8 to 241±13.0 µM TE/g DWP. This is in agreement with the results obtained with the extracts when 70% ethanol demonstrated the highest recovery of antioxidants. Nonpolar solvents, such as n-hexane and supercritical CO_2, decreased the antioxidant activity of *P. phillyreifolius* approximately by 10%, while increasing the polarity of the solvents, whereas acetone and 70% ethanol decreased the antioxidant activity by approximately 20% and 70%, respectively.

Table 4.3. Antioxidant charachteristics of *P. phillyreifolius* samples before and after extraction measured by *QUENCHER* assay

Sample	TPC (mg GAE/g DWP)	DPPH$^{\bullet}$ (µM TE/g DWP)	ABTS$^{\bullet+}$ (µM TE/g DWP)	FRAP (µM TE/g DWP)	ORAC (µM TE/g DWP)
Before extraction:					
	172±4.46^{d,e}	1503±58.8^{d,f}	3025±104$^{d, e}$	2023±52.9$^{f, e}$	2132±105^{f}
After extraction:					
HE (*SE*)	154±2.31^{d}	1259 ±70.8^{c}	2820±113^{c,d}	1827±109^{d}	1834±106^{e}
SC-CO_2	164±9.31^{d,e}	1486±96.6^{d}	2990±85.5^{d}	1962±125^{e}	1905±126^{e}
SC-CO_2 +2 %	161±9.25^{d}	1444±76.0^{d}	2858±150^{c,d}	1885±87.7^{d,e}	1886±87.8^{e}
SC-CO_2 +5 %	155±10.5^{d}	1376±129^{c,d}	2840±109^{c,d}	1882±89.9^{d,e}	1787±135^{d}

Table 4.3 continued

Sample	TPC (mg GAE/g DWP)	DPPH$^{\bullet}$ (μM TE/g DWP)	ABTS$^{\bullet+}$ (μM TE/g DWP)	FRAP (μM TE/g DWP)	ORAC (μM TE/g DWP)
SC-CO_2 +10 %	161±10.6^{d}	1349±102^{c,d}	2907±129^{d}	1856±87.4^{d}	1730±129^{d}
ACs (*SE*)	156±10.4^{d}	962±64.0^{b}	2634±90.8^{c}	1778±85.2^{d}	1721±93.4^{c}
ACc (*SE*)	123±7.58^{c}	980±70.2^{b}	2694±121^{c}	1727±38.5^{d}	1661±98.6^{c}
ACp (*PLE*)	147±12.4^{d}	1101±44.0^{c}	2514±111^{c}	1793±39.9^{d}	1673±114^{c}
EHs (*MBS*)	45.4±1.96^{a}	241±13.0^{a}	728±39.0^{a}	345±18.6^{a}	711±48.5^{a,b}
EHc (*MBS*)	71.4±3.75^{b}	315±17.3^{a}	1114±29.1^{b}	603±39.8^{b}	606±30.8^{a}
EHp (*PLE*)	91.4±3.40^{c}	468±24.4^{b}	1293±63.8^{b}	765±38.6^{c}	865±38.4^{b}

Values are represented as mean ± standard deviation (n=3), columns with different letters differ significantly for Tukey's test at $p < 0.05$; DWP – dry weight initial plant

4.1.5. Characterization of phytochemicals by chromatography mass spectrometry

Based on the accurate mass, the chromatographic retention time, and on the comparison with the standards and literature data, 8 compounds of *P. phillyreifolius* extracts were identified by using UPLC-Q-TOF analysis (Tables 4.4 and 4.5). The representative chromatograms of the extracts and the structures of the main compounds quantified in *P. phillyreifolius* are presented in Figs. 4.4 and 4.5. Some of the identified compounds were further quantified by UPLC-TQ-S by using external standards and the total area under each peak. The quantity of phenolics is expressed both in mg/g extract DWE and in mg/g dry material (DWP) (Table 4.5).

The present data on the chemical composition indicated that *P. phillyreifolius* as well as other previously characterized species of the *Phyllanthus* genus (Markom, Hasan, Daud, Singh and Jahim, 2007; Sprenger and Cass, 2013) contains a considerable amount of hydrolysable tannins, mainly ellagitannins, which are characterized as polyphenolic compounds with various numbers of hexahydroxydiphenoyl (HHDP) units attached to the sugar moiety (Lu, Yang, Hsia, Wu and Yen, 2016). This family of bioactive polyphenols is abundant in some fruits, nuts and seeds, such as pomegranates, raspberries, strawberries, walnuts and almonds (Landete, 2011). It may be observed that ellagitannin geraniin with m/z =991.1062 corresponding to the molecular ion of $C_{41}H_{27}O_{27}$ is quantitatively a major constituent of *P. phillyreifolius*, and its structure was confirmed by comparison of the retention time with the commercial standard. Its concentration among the extracts ranged from 226 (ACp) to 327 (EHs) mg/g DWE, while the recovered amount varied from 13.2 (ACp) to 110 (EHs) mg/g DWP. Geraniin was first isolated from *Geranium thunbergii* and was described as a yellow-crystalline solid with no astringency on the tongue (Okuda, Yoshida and Nayeshiro, 1976). In Japan, due to

richness in geraniin, *G. thunbergi* is certified as an official antidiarrheal drug (Luger *et al*., 1998) and this supports the use of *P. phillyreifolius* in the traditional medicine for this purpose.

Two other high molecular weight compounds of *P. phillyreifolius* showed characteristic to ellagitannins chromatographic and spectral properties, such as the loss of the hexahydroxydiphenyl unit (HHDP; 302 amu) and the tendency to form double charged ions (Tuominen, Toivonen, Mutikainen and Salminen, 2013).

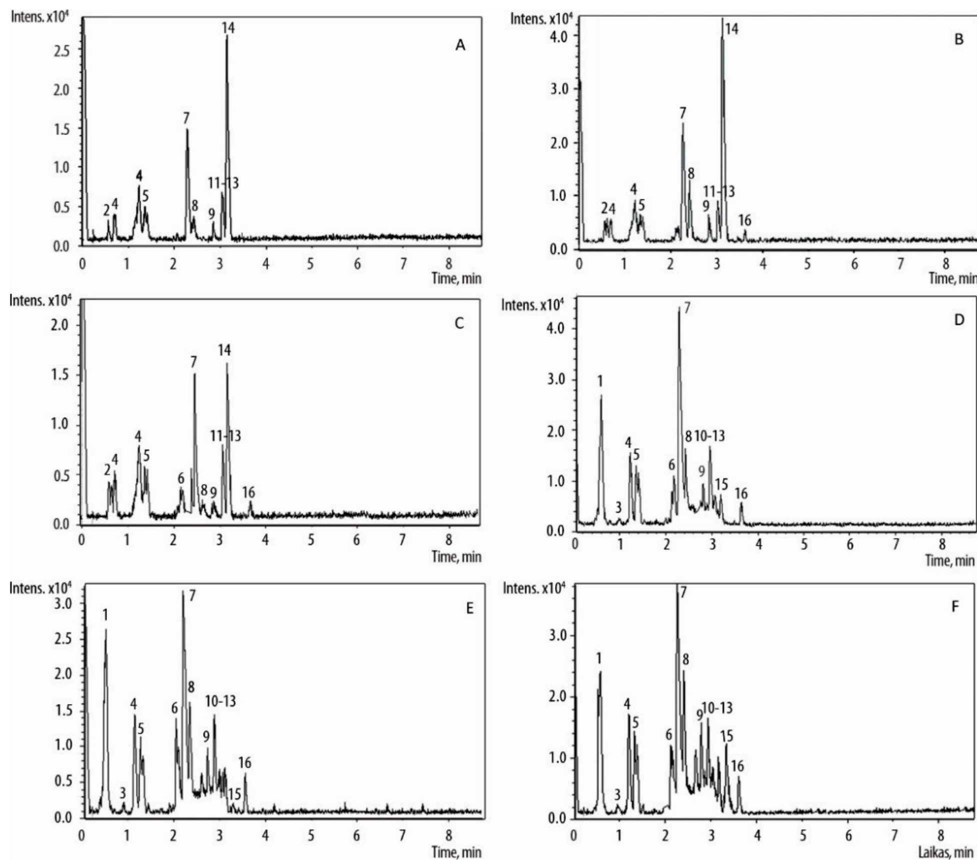


Fig. 4.4. Chromatograms of ACs (A), ACc (B), ACp (C), EHs (D), EHc (E) EHp (F) extracts obtained by UPLC-QTOF-MS. For peak numbers, see Table 4.4

In acetonic extracts, the compound with m/z =991.1062 corresponding to the molecular ion of $C_{44}H_{31}O_{27}$ was assigned to phyllanthusiin D which was previously isolated from the aqueous acetonic extract of *P. amarus* (Yeap Foo and Wong, 1992) and is regarded as an artifact condensate of geraniin with acetone produced during the extraction (Okuda and Ito, 2011). Another condensation product derived from geraniin and ascorbic acid with m/z=1109.0943 corresponding $C_{47}H_{33}O_{32}$ ion was also tentatively assigned to elaeocarpusin in all the extracts. This compound was previously reported in some *Acer*, *Rhus*, *Elaeocarpus* and *Cercidiphyllum* species (Quideau, 2009). Due to a high structural similarity to geraniin and difficulties to purchase reference standards of phyllanthusiin D and elaeocarpusin, both

compounds were quantified by using the calibration curve of geraniin, and their amounts were expressed as an equivalent of geraniin.

Table 4.4. Chemical profile of *P. phillyreifolius* extracts analyzed by UPLC-QTOF-MS

Peak No.	RT	Compound (proposed)	Molecular formula	m/z		
				$[M-H]^-$	$[M-2H]^{2-}$	MS Fragments
1.	0.50	Mucic acid lactone (Sousa et al., 2016)*[b]	$C_6H_8O_7$	191.0195	-	-
2.	0.55	Fructose*[c]	$C_6H_{12}O_6$	179.0559	-	-
3.	1.00	Gallic acid[a]	$C_7H_6O_5$	169.0141	-	-
4.	0.75; 1.25	Unknown	$C_{15}H_{20}O_{10}$	359.0983	-	-
5.	1.45	Catalpol, antirrhinoside*[c]	$C_{15}H_{22}O_{10}$	361.1139	-	-
6.	2.15	Phyllanthurinolactone*[c]	$C_{14}H_{18}O_8$	313.0935	-	-
7.	2.20	Geraniin[a]	$C_{41}H_{28}O_{27}$	951.0740	475.033	-
8.	2.55	Elaeocarpusin*[c]	$C_{47}H_{34}O_{32}$	1109.0943	554.0445	1048.48, 972.56, 300.91 $[EA-H]^-$
9.	2.85	Trigalloyl-HHDP-glucose (Regueiro, et al., 2014)*[b]	$C_{41}H_{28}O_{27}$	951.0742	475.0341	-
10.	3.00	Unknown	$C_{42}H_{32}O_{27}$	965.0889	-	-
11.	3.05	Ellagic acid[a]	$C_{14}H_6O_8$	300.9984	-	-
12.	3.10	Rutin[a]	$C_{27}H_{30}O_{16}$	609.1454	-	-
13.	3.10	Unknown	$C_{20}H_{34}O_{10}$	433.2074	-	-
14.	3.15	Phyllanthusiin D*[c]	$C_{44}H_{32}O_{27}$	991.1062	495.0490	990.61, 300.91 $[EA-H]^-$
15.	3.25	Quercetin-3-glucuronide (Sprenger and Cass, 2013; Kajdžanoska *et al.*, 2010)*[b]	$C_{21}H_{18}O_8$	477.0667	-	477.07, 301.04 $[EA-H]^-$
16.	3.55	Quercitrin[a]	$C_{21}H_{20}O_{11}$	447.3761	-	-

[a]Confirmed by a standard; [b]confirmed by a reference; [c]confirmed by parent ion mass using free chemical databases (*ChemSpider*); *tentatively identified

Phyllanthusiin D concentration among the acetonic extracts varied from 35.6 (ACp) to 178 (ACc) mg/g DWE, its recovery was from 2.08 (ACp) to 18.1 (ACs) mg/g DWP, while the amounts of elaeocarpusin in the extracts ranged from 14.2 (EHp) to 30.3 (ACp) mg/g DWE, and its recovery was from 1.77(ACp) to 6.54 (EHs) mg/g DWP.

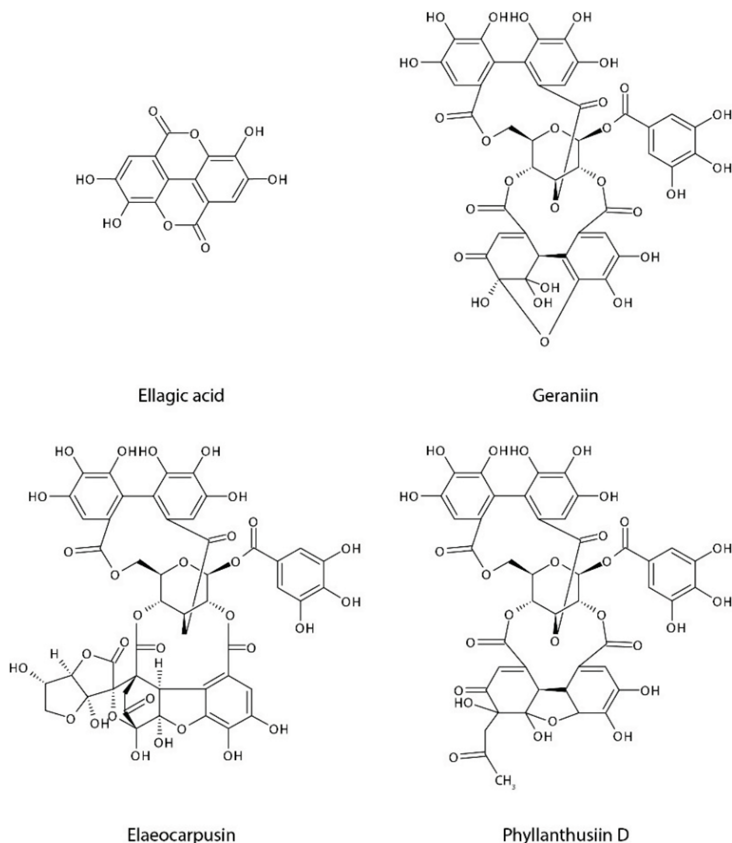


Fig. 4.5. Chemical structures of the main identified compounds of *P. phillyreifolius*

The presence of flavonoids and phenolic acids was also detected in all the extracts of *P. phillyreifolius*. Phenolic acids, such as gallic and ellagic acids, were positively identified according to their chromatographic retention time and the mass spectra in comparison with the commercial standards. The concentration of ellagic acid in different exracts ranged from 31.5 (EHp) to 56.6 (ACc) mg/g DWE, while its recovery was from 2.25 (ACp) to 15.0 (EHc) mg/g DWP. Ellagic acid itself is not thought to be naturally present in plants; it forms when the HHDP group is cleaved from the tannin molecule and spontaneously rearranges; therefore, it is found in many fruits, nut galls and plant extracts in the form of ellagitannins (Bagalkotkar, Sagineedu, Saad and Stanslas, 2006; García-Niño and Zazueta, 2015). The respective values for gallic acid were less than 1 mg/g. Low concentrations of rutin and quercitrin were found in the extracts, and their structures were confirmed by using reference compounds. The compound with m/z =477.0667 corresponding to

the molecular ion of $C_{21}H_{17}O_8$ and the characteristic fragment ion at m/z 301 corresponding to quercetin was tentatively identified as quercetin-3-glucuronide. This is in agreement with other studies on the identification of quercetin-3-glucuronide in the selected *Phyllanthus species* (Sprenger and Cass, 2013; Kajdžanoska, Gjamovski and Stefova, 2010). The identified phenolic acids and flavonoids were previously reported in other plants of the genus *Phyllanthus* (Sprenger and Cass, 2013). The latest studies on another variety of this species *Phyllanthus phillyreifolius* var. *commersonii* Müll., which is an endemic plant of Mauritius (Mahomoodally *et al*., 2018), determined that ellagitannins and gallic acid derivatives are the main compounds of the investigated plant. However, neither geraniin nor its derivatives were identified in that plant species. In both species of *Phyllanthus phillyreifolius*, quercetin-3-glucuronide, gallic and ellagic acids were identified.

It may be observed that the chemical composition of acetonic and hydroethanolic extracts was quite similar. The amounts of phenolics (mg/g DWP) quantified in *P. phillyreifolius* decreased in the following order: geraniin > ellagic acid > phyllanthusiin D > elaeocarpusin > rutin > quercitrin > gallic acid. In general, the extraction solvent and the method had some influence on the recovery of phenolics. The highest amount of quantified constituents was found in the EHs extract followed by EHc, EHp, ACs, ACc and ACp extracts. This confirmed that 70% ethanol extracted more phenolics than acetone. While comparing the extraction techniques, higher amounts of the quantified polyphenols were determined in the extracts obtained by using the conventional techniques; for instance, ACc and EHc recovered 41.0 and 93.6 mg/g DWP, whereas ACp and EHp recovered 19.4 and 87.1 mg/g DWP, respectively. It could be explained by taking into account the fact that high PLE temperatures may increase the risk of decomposition of some thermolabile components (Grunovaite, Pukalskiene, Pukalskas and Venskutonis, 2016). Furthermore, consecutive extraction while applying conventional techniques yielded 135 mg/g DWP of the quantified compounds, and this amount was very close to the value obtained by using 70% ethanol in a single-step process, while the high pressure extraction recovered 107 mg/g DWP of the total quantified phytochemicals. These results are in agreement with the results obtained in the *QUENCHER* assay when the residue after consecutive extraction while using conventional techniques demonstrated a lower antioxidant activity than the residue after PLE.

From the antioxidant activity point of view, the high antioxidant potential of geraniin as a major compound which might be responsible for the strong antioxidative activity observed in the extracts. The high positive correlation was observed between the geraniin content and antioxidant properties (0.911–0.969) (Fig. 4.6). This assumption is supported by the previously reported results (Thitilertdecha, Teerawutgulrag, Kilburn and Rakariyatham, 2010; Thitilertdecha, Teerawutgulrag and Rakariyatham, 2008) when the high antioxidant activity of methanolic extracts of *N. lappaceum* rind was attributed to the presence of geraniin as a major constituent in the rind extract.

Table 4.5. Concentration of phenolic compounds (mg/g) in *P. phillyreifolius* hydrophilic extracts by UPLC-MS

Sample	Gallic acid[a]		Ellagic acid[a] (EA)		Geraniin[a]		*Elaeocarpusin[c]	
	DWE	DWP	DWE	DWP	DWE	DWP	DWE	DWP
Single step extraction								
ACs *(SE)*	0.78±0.06[d]	0.09[b]	51.5±1.15[c]	5.94[c]	288±19.5[b,c]	33.2[b]	27.0±1.15[b,c]	3.11[b]
EHs (*MBS*)	0.52±0.03[a]	0.18[c]	40.1±0.09[b]	13.5[e]	327±7.66[c]	110[d]	19.4±0.93[b]	6.54[e]
Consecutive extraction								
ACc *(SE)*	0.67±0.01[b,c]	0.05[a]	56.6±0.80[d]	4.42[b]	262±7.66[b]	20.5[a,b]	24.9±0.93[b]	1.95[a]
EHc (*MBS*)	0.59±0.01[a,b]	0.18[c]	48.7±0.53[c]	15.0[f]	236±12.3[a]	72.7[c]	15.5±0.57[a]	4.08[c]
Σ		0.23		19.4		93.2		6.03
ACp (*PLE*)	0.59±0.03[a,b,c]	0.04[a]	38.5±0.05[b]	2.25[a]	226±14.6[a]	13.2[a]	30.3±0.97[c]	1.77[a]
EHp (*PLE*)	0.61±0.03[b]	0.17[c]	31.5±1.38[a]	9.06[d]	248±6.66[b]	71.5[c]	14.2±0.77[a]	4.77[d]
Σ		0.21		11.3		84.7		6.54

Table 4.5. (continued)

Sample	*Phyllanthusiin D[c]		Rutin[a]		Quercitrin[a]	
	DWE	DWP	DWE	DWP	DWE	DWP
Single step extraction						
ACs *(SE)*	157±6.86[b]	18.1[c]	1.63±0.06[c]	0.19[c]	0.24±0.01[a]	0.03[a]
EHs (*MBS*)	nd	nd	3.68±0.05[d]	1.24[d]	1.04±0.01[c]	0.35[b]
Consecutive extraction						
ACc *(SE)*	178±6.19[b]	13.9[b]	1.43±0.00[b]	0.11[b]	0.57±0.03[b]	0.04[a]
EHc (*MBS*)	nd	nd	4.19±0.04[e]	1.29[f]	1.22±0.05[d]	0.38[c]
Σ		13.9		1.4		0.42
ACp (*PLE*)	35.6±2.01[a]	2.08[a]	0.19±0.00[a]	0.01[a]	0.54±0.01[b]	0.03[a]
EHp (*PLE*)	nd	nd	4.38±0.03[f]	1.26[e]	1.12±0.01[c,d]	0.32[b]
Σ		2.08		1.27		0.35

[a]Confirmed by a standard; [c]confirmed by parent ion mass using free chemical databases (Chemspider); *expressed as geraniin equivalent. nd – not detected. Values are represented as mean ± standard deviation (n=3), DWE – dry weight extract; DWP – dry weight of the initial plant

Purified geraniin demonstrated high DPPH• scavenging activity and a dose-dependent inhibition of lipid peroxidation. In was determined that 50% inhibition concentration (IC50) of geraniin in the DPPH• assay was 10.3 and 14.5 times lower than that of ascorbic acid and BHT, respectively. Furthermore, the study of Tabata *et al*. (2008) demonstrated that geraniin exhibited a remarkably stronger antioxidative activity than gallic acid, rutin, ellagic acid, quercetin, and chlorogenic acid, and was as active as epigallocatechin gallate (EGCG), a strong antioxidant of green tea. Its strong antioxidative activity might be attributable to the high levels of hydroxyl substitutions.

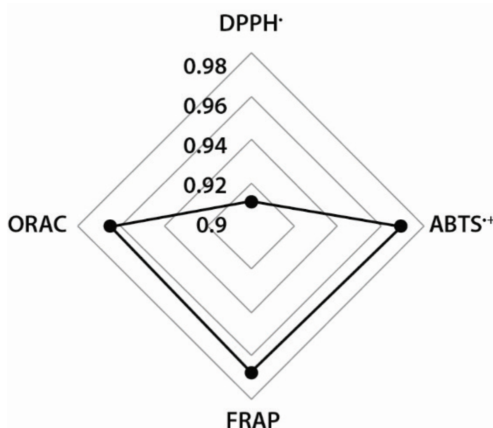


Fig. 4.6. Correlation coefficients between the geraniin content and different antioxidant measurement assays

A relatively high concentration of ellagic acid could also contribute to the high antioxidant activity of extracts. This is supported by Kilic, Yeşiloğlu and Bayrak, (2014) who demonstrated the role of ellagic acid as an effective antioxidant in different *in vitro* assays when it is compared to the well-known reference antioxidants, such as BHA, BHT, tocopherol and ascorbic acid. The high positive correlation was also observed between ellagic acid, gallic acid and rutin content and the antioxidant properties (0.843–0.989). This indicated that these compounds are also responsible for the strong antioxidant properties of *P. phillyreifolius*.

4.2. The effects of phytochemical antioxidants rich *P. phillyreifolius* extracts against hydrogen peroxide induced oxidative stress[2]

4.2.1. Cytotoxic effect of *P. phillyreifolius* extracts in HEK293 cells

Previous studies have demonstrated that plant extracts, due to the presence of different phytochemicals, may exhibit cytotoxic activity against various cell lines (Hu and Kitts, 2004; Silva, Barreira and Oliveira, 2016). With regard to this, the assessment of plant extracts for potential cytoxicity is considered as an important step in evaluating their suitability for further applications (Qader *et al*., 2011). The

[2] This section was prepared with reference to Grauzdytė, Pukalskas, Viranaicken, El Kalamouni and Venskutonis (2018)

cytotoxic effect of AC and EH extracts of *P. phillyreifolius* at a concentration ranging from 12.5 to 1000 µg/mL was investigated by using the *HEK293* cell line by the MTT cell viability assay which relies on the mitochondrial metabolic capacity of viable cells. Fig. 4.7 A shows the cell viability results after 24 h of incubation with *P. phillyreifolius* extracts. It may be observed that the cell viability decreased with the increase of extract concentration. Both extracts were toxic at a higher than 500 µg/mL concentration. However, the cells exposed to lower (< 250 µg/mL) extract concentrations retained a higher than 90% cell viability. The viability of the concentrations of the extracts responsible for a 50% reduction in cells (IC_{50}) was also calculated; the values were 489±31.8 and 387±6.84 µg/mL for the AC and EH extracts, respectively. The obtained results indicated a higher cytotoxic potential of the EH extract than AC.

The cytotoxicity of the extracts was also evaluated by using the Trypan Blue dye exclusion test which is based on the cell selectivity of the blue color trypan blue dye that passes through the membrane: in a viable cell, the trypan blue is excluded, and a cell has a clear cytoplasm, while a dead cell takes up the dye, and thus the cytoplasm is blue-colored (Strober, 1997). These results (Fig. 4.7 B) support the results obtained by using the MTT cell viability assay and indicate that both extracts at concentrations of 250 µg/mL or less did not induce cytotoxicity. Previous studies by Hossen *et al*. (2015) demonstrated that methanolic extract of the same genus plant *P. acidus* was not cytotoxic to RAW264.7, U937 and HEK293 cells at concentrations of up to 300 µg/mL. Based on these results, 250 µg/mL and lower concentrations of AC and EH extracts were chosen for the treatment of cells in the further experiments.

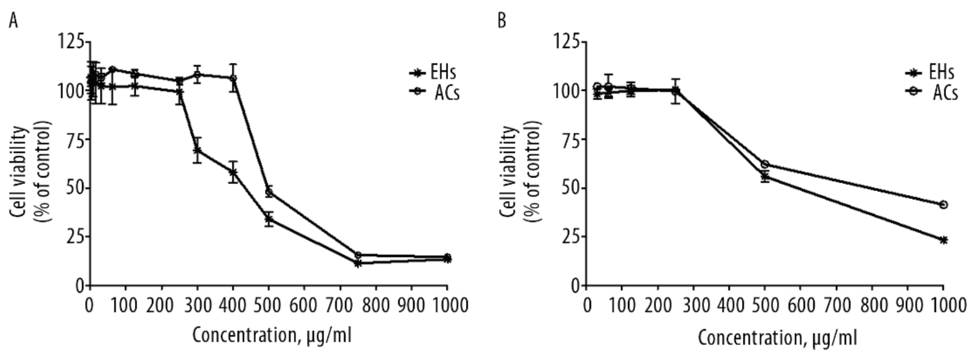


Fig. 4.7. Effect of *P. phillyreifolius* extracts on HEK293 cells viability analyzed by the mitochondrial metabolic activity (MTT) assay (A) and by the Trypan Blue dye exclusion test (B). Cells were treated for 24 h with an increased concentration of hydroethanolic (EH) or acetonic (AC) extracts of *P. phillyreifolius*. The data is represented as a mean ± standard deviation (n=3)

4.2.2. Effect of *P. phillyreifolius* extracts on intracellular ROS level in HEK293 cells

The effect of the preconditioning of HEK293 cells with EH and AC extracts of *P. phillyreifolius* on the intracellular ROS level was determinated by using the cell-based assay, in which, the DCFH-DA fluorescent probe is used as an indicator of the reactive oxygen species (ROS) and oxidative stress. In order to evaluate the protective action of *P. phillyreifolius* extracts against oxidative stress, firstly, the effect of extracts on the intracellular ROS content was determined in HEK293 cells without inducing exogenous oxidative stress. The cells were preconditioned with various concentrations of *P. phillyreifolius* extracts for 24 h. As shown in Fig. 4.8., *P. phillyreifolius* extracts decreased the basal level of ROS in a dose-dependent manner. A similar effect was observed in the previous studies undertaken by Septembre-Malaterre, Stanislas, Douraguia and Gonthier (2016) who reported that the passion fruit, the lychee, and the American mango extracts decreased the basal level of ROS in 3T3-L1 murine preadipose cells.

To model the oxidation stress in the cells, H_2O_2 was chosen as the intracellular oxidizing agent. The optimal concentration of H_2O_2 to induce oxidation was selected as 100 µM since this concentration did not show any significant decrease in the cell viability, and, at this concentration, DCF exhibited good fluorescence intensity in comparison with the control sample (Fig. 4.8).

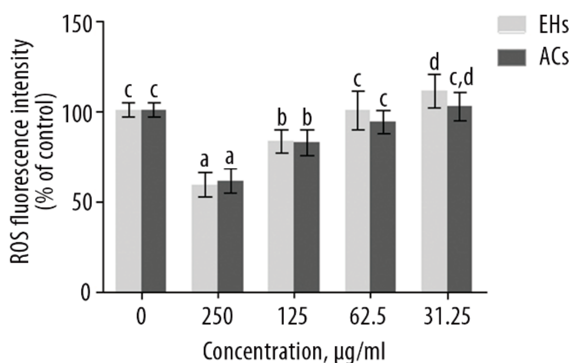


Fig. 4.8. Effect of *P. phillyreifolius* extracts on the basal level of ROS. HEK293 cells were treated with the noted concentrations of acetonic (AC) and hydroethanolic (EH) extracts for 24 h, then they were washed and incubated with DCFH-DA (the fluorescent probe as an indicator of ROS) for 45 min. The data is represented as a mean ± standard deviation (n=3) with one way ANOVA. Columns with different letters (a–d) differ significantly for Tukey's test at $p < 0.05$

Two different preconditioning times with extracts (3 and 24 hours) were chosen to evaluate the antioxidant activity in H_2O_2-stimulated HEK293 cells. As shown in Fig. 4.10, while H_2O_2 increased the ROS production (from 98.7±5.74 to 150.9±7.89% of the control sample), the cells preconditioned with nontoxic concentrations of *P. phillyreifolius* extracts significantly inhibited ROS generation in a dose-dependent manner. Although the cells preconditioned with extracts for

3 hours had sufficient time to inhibit ROS production, however, the cells preconditioned with 250 and 125 µg/mL of both extracts for 24 hours completely counteracted the H_2O_2 induced oxidative stress.

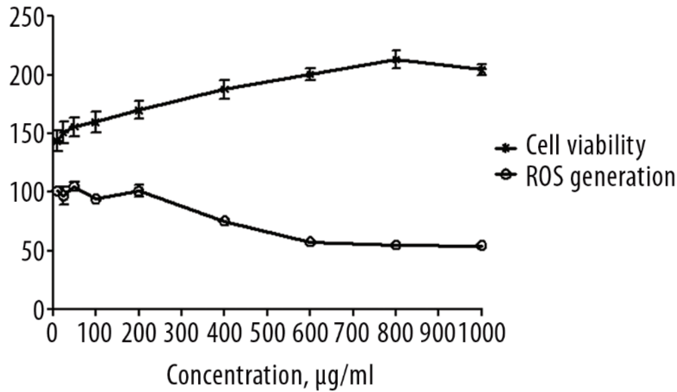


Fig. 4.9. Cytotoxicity of H_2O_2 and its dose response on ROS generation

When comparing the two extracts, the AC extract showed a better intracellular antioxidant activity in comparison with the EH extract. Geraniin as well as other ellagitannins could be responsible for the high ROS decreasing effect of *P. phillireifolius*. This supports the previous research by Wang *et al*., 2015, who found that the dose of gerannin dependently attenuated H_2O_2 induced ROS production in the human hepatocarcinoma cell line (HepG2).

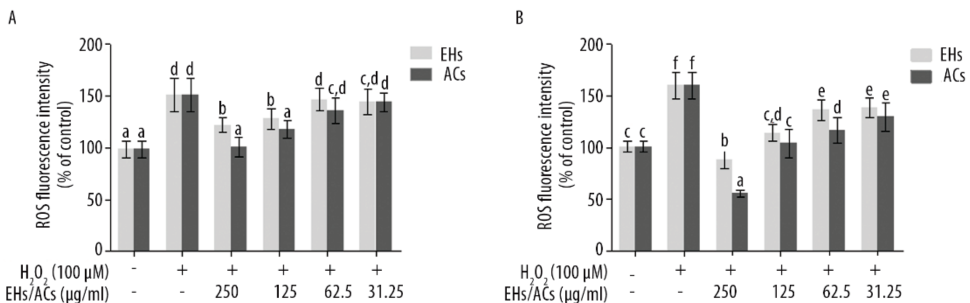


Fig. 4.10. Effect of *P. phillyreifolius* extracts on ROS generation induced by H_2O_2. HEK293 cells were treated with extracts for A–3 h, B–24 h, then, they were washed and incubated with DCFH-DA for 45 min. Then, the supernatant was removed, and the cells were incubated for 1 h with H_2O_2 (100 µmol/L). The data is represented as a mean ± standard deviation (n=3) with one way ANOVA. Columns with different letters (a-e) differ significantly for Tukey's test at $p < 0.05$

The possible mechanism of the ROS generation decreasing effect in cells pretreated with *P. phillyreifolius* extracts might be associated with the direct free radical scavenging activity or indirect protection from oxidative stress (by activating the endogenous defense systems).

4.2.3. Expression of antioxidant enzymes in HEK293 cells

Increasing evidence is being obtained which shows that some antioxidants act as a cellular signaling messenger to regulate the level of antioxidant compounds and enzymes (Wang *et al*., 2010). In order to investigate whether the ROS decreasing effect of plant extracts is mediated by an increase at the antioxidant enzymes level, the preconditioning effect (24 h) of *P. phillyreifolius* extracts (250 μg/mL) on the antioxidant enzymes (SOD1, SOD2, CAT and GPx) gene expression was tested in H_2O_2-stimulated HEK293 cells (Fig.4.11). The gene expression of antioxidant enzymes in preconditioned HEK293 cells with AC and EH extracts was regulated differently. The gene expression of SOD2 and CAT was upregulated, and SOD1 was downregulated by preincubation cells with the EH extract, meanwhile, the gene expression of the SOD2 enzyme increased, but the SOD1, CAT, GPx enzymes decreased in the cells pretreated with the AC extract. Corroborating to our results, Alía *et al*. (2006) reported that some doses of tested quercetin (a well-known antioxidant compound) were able to up- or down-regulate the gene expression of the main antioxidant enzymes, although the acting mechanism was actually unknown. A small but significant decrease in the gene expression of SOD1, CAT and GPx was obtained in H_2O_2-stimulated HEK293 cells. Similar findings were reported in another study conducted by Sowndhararajan *et al*. (2015) who reported that human hepatoma HepG2 cells treated with 200 μM of H_2O_2 significantly reduced the expresions of SOD1, SOD2, CAT and GPx enzymes when compared with untreated control samples. However, cell preincubation with *P. phillyreifolius* extracts prior to exposure to H_2O_2 did not affect the level of antioxidant enzymes, however, the preconditioning with the AC extract markedly increased the gene expression of SOD1 and SOD2 enzymes. Previous studies have shown a different effect of polyphenol-rich extracts on the antioxidant gene expression in H_2O_2 stimulated cells. Marimoutou *et al*. (2015) demonstrated that an increase in the ROS production induced by H_2O_2 in preadipocyte cells was related with the decrease in the SOD gene expression which was counteracted by *A. borbonica* and *D. apetalum* extracts, though *G. mauritania* decreased the ROS generation without any effect on the SOD gene expression. It is of interest that the absence of any effect on the CAT gene expression was also demonstrated in this study. Wang *et al*. (2010) also reported that the antioxidant activities of *Acanthopanax senticosus* Harms aqueous extracts may be related to the upregulation of the gene expression and the activity of CuZnSOD, MnSOD, CAT, and GPx enzymes. From the obtained results, it can be assumed that the intracellular antioxidant activity of *P. phillyreifolius* extracts against H_2O_2 induced ROS production was more related with some other acting mechanism compared to the regulation of the level of antioxidant enzymes.

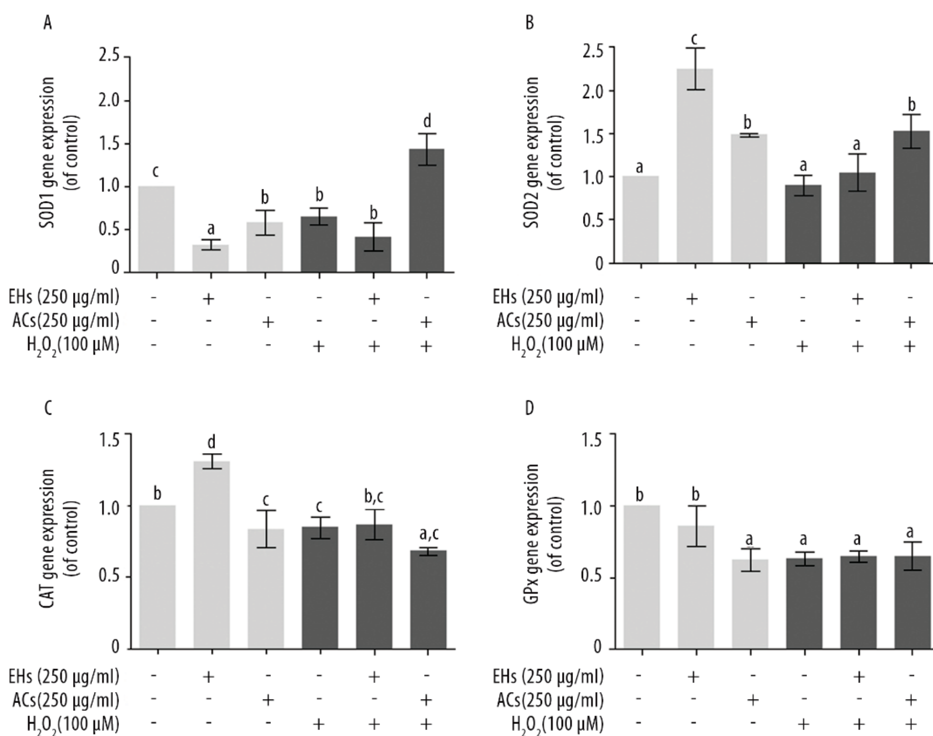


Fig. 4.11. Effect of *P. phillyreifolius* extracts on A – SOD1, B – SOD2, C – CAT, D – GPx enzymes gene expression in unstressed and H_2O_2-stimulated HEK293. Cells were treated with extracts for 24 h, then were washed and incubated for 1 h with H_2O_2 (100 µmol/L). The data is represented as a mean ± standard deviation (n=3) with one way ANOVA. Columns with different letters (a–d) differ significantly for Tukey's test at $p < 0.05$

4.3. Comprehensive evaluation of antioxidant potential and mangiferin content of *A. theiformis* extracts and fractions isolated by applying different methods[3]

4.3.1. Extract yields

Botanicals are very complex biological structures composed of various groups of compounds. Therefore, the proper selection and elaboration of the extraction schemes is an important task for the recovering of products of desirable properties and composition (Kraujalienė, Pukalskas and Venskutonis, 2017). The extractability of phenolic compounds from plant matrices depends on several factors including the solvent, pH, temperature, pressure, the number of steps, and the solvent volume (Nastić et al., 2018). In this study, the single step and consecutive extraction with the increasing polarity solvents was chosen for the isolation of lipophilic and hydrophilic extracts from *A. theiformis* leaves (Table 4.6).

[3] This section was prepared with reference to Grauzdytė, Pukalskas, El Kalamouni and Venskutonis (2020)

Table 4.6. Yields of *A. theiformis* extracts

Sample	Yield, %	
	DWR	DWP
Single step extraction		
HE (*SE*)	2.62±0.04[a]	2.62[a]
ACs (*SE*)	10.97±0.79[e]	11.0[e]
EHs (*MBS*)	34.07±0.66[j]	34.1[g]
Consecutive extraction		
HE (*SE*)	2.62±0.04[a]	2.62[a]
ACc (*SE*)	9.40±0.37[d]	9.15[d]
EHc(*MBS*)	28.79±0.33[i]	25.3[f]
Σ		**37.1**
HEp_{70} (*PLE 70°C*)	2.47±0.23[a]	2.47[a]
ACp_{70} (*PLE 70°C*)	6.65±0.02[c]	6.49[c]
EHp_{70} (*PLE 70°C*)	27.86±0.36[h]	25.4[f]
Wp_{70} (*PLE 70°C*)	13.45±0.29[f]	8.83[d]
Σ		**43.2**
HEp_{140} (*PLE 140°C*)	3.59±0.13[b]	3.59[b]
ACp_{140} (*PLE 140°C*)	6.72±0.01[c]	6.48[c]
EHp_{140} (*PLE 140°C*)	28.80±0.13[i]	25.9[f]
Wp_{140} (*PLE 140°C*)	14.65±0.10[g]	9.38[d]
Σ		**45.4**

Values are represented as a mean ± standard deviation (n=3), columns with different letters differ significantly for Tukey's test at $p < 0.05$; DWR – dry weight extraction residue; DWP – dry weight of the initial plant

Nonpolar and widely used in lipid extraction n-hexane (relative polarity 0.09) gave the lowest extraction yields; however, the yield remarkably increased (by 37% compared to the conventional extraction) when PLE was performed at 140 °C temperature. Higher polarity aprotic solvent acetone (relative polarity 0.355) yielded remarkably higher amounts of extracts both from the initial plant material and from the residues after hexane extraction. It is of interest to note that the yields in PLE with acetone were lower than in the conventional consecutive extraction. The reduced dielectric constant of acetone at a high temperature might be one of the reasons of this finding. The lowest extraction yield was obtained by extraction with hexane, and it increased with the increasing polarity of the solvent. Extraction by maceration with 70% ethanol gave the highest yield of the extract (34.07±0.66%).

The total extract yield in the case of consecutive extraction was fairly similar irrespective of the applied methods and ranged from 36.98% to 40.81%. It was reported by Kraujalienė, Pukalskas and Venskutonis (2017) that PLE yields from *Solidago virgaurea* leaves at 140 °C were almost 2 times higher compared with the values for 70 °C. With regard to this finding, PLE extraction was performed at the same temperatures; however, in the case of *A. theiformis*, the PLE temperature did not have such a significant effect on the extract yield.

4.3.2. Effect of extraction methods on the antioxidant activity of extracts

The evaluation of the antioxidant capacity of natural products cannot be performed accurately by any single method due to the complex nature of phytochemicals and due to the involvement of multiple reaction characteristics and mechanisms (Silva and Sirasa, 2018). Therefore, for evaluating the antioxidant properties of *A. theiformis* extracts, a combination of antioxidant activity assays (hydrogen atom transfer- and electron transfer-based methods) was carried out. Antioxidant activity indicators were measured in different extracts and fractions (DWE/DWF) and recalculated to dry plant material (DWP). Both values are practically important as they show the antioxidant potential of extracts or fractions and evaluate the recovery of antioxidatively active compounds. The antioxidant activity of the hexane extract was left underinvestigated because the main target of this study was hydrophilic fractions.

Overall, the antioxidant activities of the samples were related to the extraction solvents and the employed methods (Table 4.7). The TPC values of extracts obtained by using various solvents and extraction methods ranged from 7.59±0.55 (Wp_{140}) to 362±6.28 (EHs) mg GAE/g DWE. The radical scavenging capacity of plant extracts were from 1395±111 (Wp_{140}) to 3267±81.5 (EHs) µmol TE/g extract in the $DPPH^{\bullet}$ scavenging assay, while the $ABTS^{\bullet+}$ scavenging values ranged from 107±2.89 (Wp_{140}) to 5091±118 (ACc) µmol TE/g DWE. The ability of extracts to reduce ferric Fe (III) ions to their respective lower valency state as determined by the FRAP method varied from 312±54.1 (Wp_{140}) to 4473±129 (EHs) µmol TE/g DWE. Consequently, the antioxidant capacity of the most potential extracts was very strong, i.e., it was equivalent to 0.82–1.27 g of Trolox in 1 g of extract. Hydroethanolic extracts were the strongest antioxidants; acetonic extracts were also strong antioxidants, however, due to their low yield, 70% ethanol was the most suitable solvent for the best recovery of antioxidants from *A. theiformis*. This can be observed from the recalculated values to DWP which were a few times higher for hydroethanolic extracts than for acetonic and water extracts.

When comparing different extraction schemes, it may be observed that the sum of the recovered antioxidants was fairly similar in consecutive extraction and PLE at 70 °C; whereas, PLE at 140 °C gave lower values of the antioxidant recovery. It may be assumed that some sensitive to heat antioxidants degraded at an increased temperature. Antioxidant recovery with 70% ethanol when using single step extraction by stirring was similar or even higher than the recovery of antioxidants obtained when using consecutive extraction; however, the application of consecutive extraction schemes with different polarity solvents enables to achieve preliminary pre-fractionation of botanical bioactive compounds (Kraujalienė, Pukalskas, Kraujalis and Venskutonis, 2016). Other advantages of PLE are the faster process, convenience, and lower volumes of the used solvents.

Table 4.7. Total phenolic content (TPC) and antioxidant capacity of *A. theiformis* extracts obtained by various solvents and extraction methods. TPC expressed in mg GAE/g DWE and mg GAE/g DWP. $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and FRAP expressed as µM

Sample	TPC		$DPPH^{\bullet}$		$ABTS^{\bullet+}$		FRAP	
	DWE	DWP	DWE	DWP	DWE	DWP	DWE	DWP
Single step								
HE (*SE*)	-	-	-	-	-	-	-	-
ACs (*SE*)	247 ± 13.0^{d}	27.1	2451 ± 96.8^{c}	269^{c}	4533 ± 77.6^{g}	497^{c}	3360 ± 176^{d}	369^{d}
EHs (*MBS*)	330 ± 11.9^{f}	112	3267 ± 81.5^{e}	1113^{f}	$4595 \pm 111^{f,g}$	1566^{f}	4473 ± 129^{f}	1524^{f}
Consecutive								
HE (*SE*)	-	-	-	-	-	-	-	-
ACc (*SE*)	275 ± 14.0^{e}	25.1	2902 ± 46.8^{d}	266^{c}	5091 ± 118^{h}	466^{c}	3905 ± 45.1^{e}	367^{d}
EHc(*MBS*)	317 ± 6.76^{f}	80.2	3121 ± 93.8^{e}	791^{e}	$4313 \pm 54.2^{e,f}$	1093^{d}	3388 ± 117^{d}	858^{e}
Σ		**105**		**1057**		**1559**		**1225**
HEp_{70} (*PLE 70°C*)	-	-	-	-	-	-	-	-
ACp_{70} (*PLE 70°C*)	172 ± 5.00^{c}	11.2	1884 ± 51.1^{b}	122^{a}	3025 ± 76.8^{d}	196^{b}	2160 ± 79.5^{b}	144^{b}
EHp_{70} (*PLE 70°C*)	322 ± 23.4^{f}	88.5	2867 ± 34.4^{d}	824^{e}	4598 ± 146^{g}	1166^{e}	$3504 \pm 102^{d,e}$	889^{e}
Wp_{70} (*PLE 70°C*)	138 ± 3.65	12.2	2314 ± 89.9^{c}	204^{b}	2378 ± 30.9^{b}	210^{b}	2317 ± 34.1^{c}	205^{c}
Σ		**112**		**1053**		**1572**		**1238**
HEp_{140} (*PLE 140°C*)	-	-	-	-	-	-	-	-
ACp_{140} (*PLE 140°C*)	154 ± 4.05^{c}	10.0	1748 ± 26.4^{b}	113^{a}	2683 ± 87.4^{c}	174^{b}	2119 ± 28.7^{b}	142^{b}
EHp_{140} (*PLE 140°C*)	291 ± 1.98^{e}	75.4	2837 ± 76.4^{d}	735^{d}	4165 ± 138^{e}	1079^{d}	3362 ± 97.3^{d}	871^{e}
Wp_{140} (*PLE 140°C*)	7.59 ± 0.55^{a}	0.71	1395 ± 111^{a}	131^{a}	107 ± 2.89^{a}	10.1^{a}	290 ± 26.3^{a}	30.7^{a}
Σ		**86**		**979**		**1263**		**1044**

Values are represented as mean ± standard deviation (n=3); columns with different letters differ significantly for Tukey's test at $p < 0.05$; DWE – dry weight extract; DWP – dry weight of initial plant

4.3.3. Fractionation of crude hydroethanolic extract and characterization of the obtained fractions

Crude extracts usually contain a great number of components which may possess different antioxidant, or, in some cases, prooxidant activities, or even be neutral in terms of their effects on the oxidation and/or radical scavenging processes (Pukalskas, Venskutonis, Salido, Waard and Van Beek, 2012). Therefore, the simple fractionation of hydroethanolic extract of *A. theiformis* by liquid–liquid extraction was performed to separate different classes of compounds and find the most active fractions. Mangiferin is prone to precipitation at ambient and lower temperatures (Danthu *et al*., 2010), and the freshly prepared hydroethanolic extract was also fractionated by cooling down to a temperature of 6 ºC.

Among the solvent-partitioned fractions, the n-butanol fraction (48.0%) of *A. theiformis* EHs extract showed the highest yield followed by the water (17.7%), ethyl acetate (17.0%) and hexane (14.2%) fractions. The yield of the fractions obtained by cooling among the fractionation procedures varied markedly due to the difficulties in controling the precipitation process. Nevertheless, it could be observed that 36.3% of the substances of crude EHs extract are prone to precipitation at a low temperature.

The TPC and antioxidant capacity values of the fractions are listed in Table 4.8 and are compared to pure mangiferin. In general, some fractions exhibited remarkably larger antioxidant capacities than the crude EHs extract. A similar tendency was also observed in a previous study by Larrauri, Zunino, Zygadlo, Grosso and Nepote (2016), where the antioxidant potential of some fractions during the purification process increased relatively to that of crude extracts. Among the fractions obtained by liquid–liquid extraction, the n-butanol fraction had the highest antioxidant activity followed by ethyl acetate and water fractions, while the Sto2p fraction showed the highest antioxidant activity among the fractions obtained by precipitation at a low temperature. The most active fractions also had the highest TPC. As indicated in Table 4.8, mangiferin showed a very high antioxidant activity; therefore, it may be considered responsible for the high antioxidant activity of *A. theiformis*. It is of interest to note that the Sto2p fraction featured higher activities towards $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals than mangiferin, which suggests that other constituents – when being present together with mangiferin – may act synergistically in contributing to the high antioxidant capacity of *A. theiformis* extracts.

Table 4.8. The yield, the total phenolic content (TPC), and the antioxidant capacity of the crude hydroethanolic extract (EHs) and its fractions. TPC expressed in mg GAE/g DWE and mg GAE/g DWF. $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and FRAP are expressed as μM TE/g DWE and μM TE/g DWF

Sample	Yield, %	TPC	$DPPH^{\bullet}$	$ABTS^{\bullet+}$	FRAP
Crude hydroethanolic extract (EHs)	34.07 ± 0.66	362 ± 6.28^{e}	3267 ± 81.5^{f}	4595 ± 111^{e}	4473 ± 129^{e}
Fractions obtained by using liquid-liquid extraction					
Hexane (HEf)	14.2±0.3	42.4 ± 0.62^{a}	302 ± 5.98^{a}	511 ± 21.8^{a}	461 ± 17.1^{a}
Ethyl acetate (ECf)	17.0±0.81	208 ± 2.77^{b}	1340 ± 26.3^{b}	2106 ± 46.0^{b}	2030 ± 104^{c}
n-Butanol (n-Bf)	48.0±0.91	423 ± 4.79^{f}	3496 ± 28.4^{g}	4555 ± 26.2^{e}	4818 ± 85.5^{g}
Water (Wf)	17.7±2.41	257 ± 4.78^{c}	1969 ± 92.4^{d}	2589 ± 14.1^{c}	1476 ± 68.8^{b}
Fractions obtained by cooling at 6 ºC					
Sto1g	2.06÷8.23	201 ± 3.68^{b}	1568 ± 51.5^{c}	1946 ± 47.5^{b}	1857 ± 109^{c}
Sto1p	14.14÷29.12	491 ± 16.6^{g}	4333 ± 21.0^{h}	6878 ± 47.8^{f}	4298 ± 129^{e}
Sto2p	2.06÷19.54	599 ± 4.40^{h}	4816 ± 62.3^{i}	7951 ± 360^{g}	5582 ± 87.7^{f}
Tf	63.7	300 ± 3.56^{d}	2359 ± 39.7^{e}	4054 ± 175^{d}	2656 ± 54.6^{d}
Mangiferin	-	668 ± 8.58^{i}	4282 ± 246^{h}	7227 ± 226^{f}	6750 ± 2.13^{h}

Values are represented as a mean ± standard deviation (n=3); columns with different letters differ significantly for Tukey's test at $p < 0.05$

4.3.4. Characterization of phytochemicals by chromatography-mass spectrometry

The analysis of the chemical composition confirmed that *A. theiformis* is rich in mangiferin and some saponins. The chromatograms of the *A. theiformis* EHs extract and its fractions are demonstrated in Fig. 4.12, while the chemical profile is summarized in Table 4.9. As it can be seen from the chromatograms, xanthone (mangiferin) was the dominating constituent of *A. theiformis*. Two other constituents of *A. theiformis* belonging to triterpenoid saponins were also identified as tormentic and hydroxytormentic acids. These compounds were identified by using UPLC-Q-TOF analysis and were confirmed based on the comparison of their accurate mass and chromatographic retention time with the reference standards. The structure of these compounds is provided in Fig. 4.13.

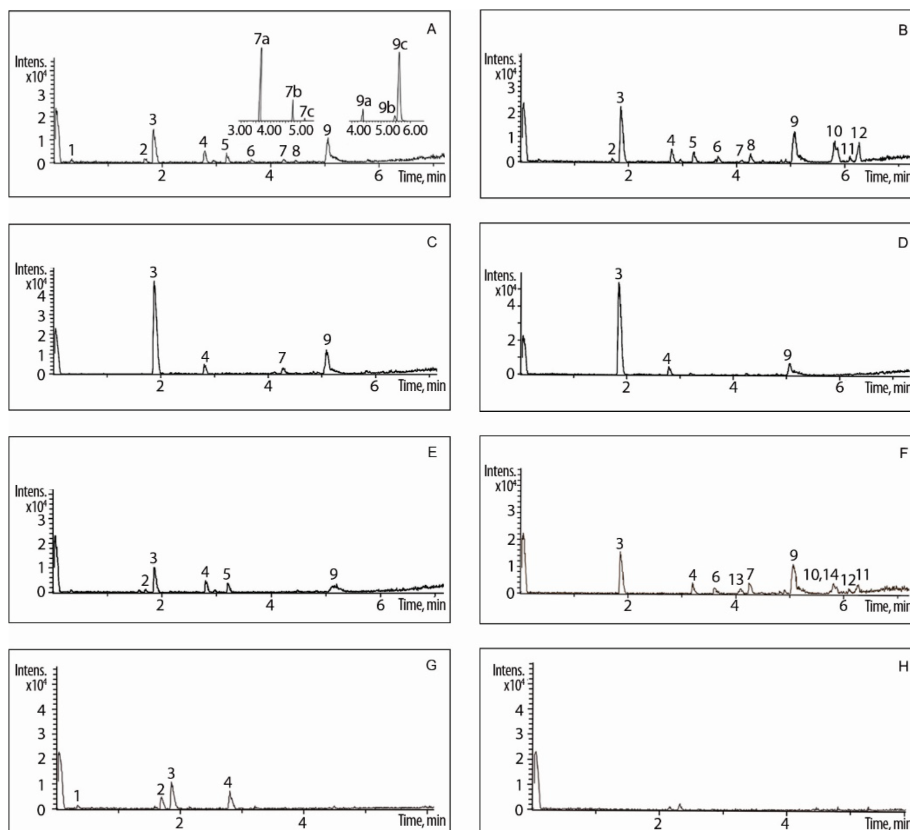


Fig. 4.12. Chromatograms of *A. theiformis* EHs extract (A) and its fractions (B-H) obtained by UPLC-QTOF-MS. Sto1g (B), Sto1p (C), Sto2p (D), Tf (E), n-Bf (F), ECf (G), Wf (H). For peak numbers, see Table 4.9

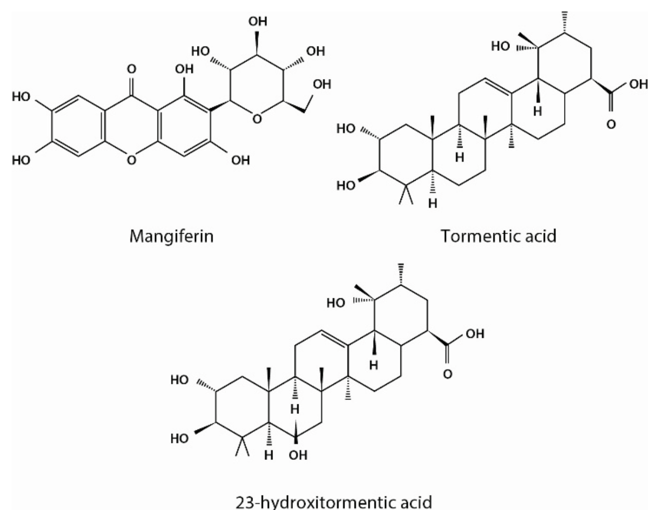


Fig. 4.13. Chemical structures of compounds of *A. theiformis*: 1 – mangiferin, 2 – tormentic acid, 3 – hydroxytormentic acid

Table 4.9. Chemical profile of *A. theiformis* EHs extract and its fractions analysed by UPLC-QTOF-MS

Peak No.	RT	Compound	Molecular formula	m/z	
				$[M-H]^-$	MS Fragments
1.	0.35	Fructose*[b]	$C_6H_{12}O_6$	179.0563	-
2.	1.70	Unknown	$C_{19}H_{20}O_{10}$	407.0984	-
3.	1.85	Mangiferin[a]	$C_{19}H_{18}O_{11}$	421.0777	-
4.	2.75	Unknown saponin	$C_{37}H_{60}O_{14}$	727.3910	-
5.	3.20	Hydroxytormentic acid derivative*	$C_{37}H_{60}O_{13}$	711.3961	503.3371 $[HTA-H]^-$
6.	3.60	Tormentic acid derivative*	$C_{37}H_{60}O_{12}$	695.4012	649.3949, 487.3427 $[TA-H]^-$
7a.	3.61	23-hydroxytormentic acid isomer*	$C_{30}H_{48}O_6$	503.3372	-
7b.	4.73	23-hydroxytormentic acid[a]	$C_{30}H_{48}O_6$	503.3372	-
7c.	5.14	23-hydroxytormentic acid isomer*	$C_{30}H_{48}O_6$	503.3372	-
8.	4.45	Unknown	$C_{16}H_{28}O_6$	315.1813	-
9a.	4.34	Tormentic acid isomer*	$C_{30}H_{48}O_5$	487.3429	-
9b.	5.60	Tormentic acid[a]	$C_{30}H_{48}O_5$	487.3429	-
9c.	5.74	Tormentic acid isomer*	$C_{30}H_{48}O_5$	487.3429	-
10.	5.85	Unknown	$C_{18}H_{30}O_3$	293.2027	-
11.	6.35	Maslinic/corosolic acid*[b]	$C_{30}H_{48}O_4$	471.3480	-
12.	6.40	Unknown	$C_{18}H_{32}O_3$	295.2279	-
13.	4.13	Unknown	$C_{30}H_{48}O_7$	520.7039	-
14.	5.90	Quillaic acid*[b]	$C_{30}H_{46}O_5$	485.3272	-

[a]Confirmed by a standard; [b]confirmed by parent ion mass by using free chemical databases (Chemspider); *tentatively identified

Even though mangiferin, tormentic (TA) and hydroxytormentic (HTA) acids were previously identified in *A. theiformis*, their concentrations were not determined. To fill this gap, the main phytochemicals in *A. theiformis* extracts and fractions were quantified by UPLC-TQ-S by using external standards and the total area under each peak. Their concentrations in different extracts and fractions ranged remarkably and are provided in Tables 4.10 and 4.11. Among the quantified compounds, mangiferin had the highest concentration which ranged from 1.14 to

35.6 mg/g DWP. For comparison, the content of mangiferin found in the mango tree leaf was 1.294 ± 0.002 g mangiferin/100 g DWP (Vo, Nguyen, Nguyen and Ushakova, 2017). Another study determined the content of mangiferin in various fruit tissues (peel, pulp, and seed kernel) of Chinese mango cultivars, and it was in the range from 0.002 to 7.49 mg/g DW; the highest content of mangiferin was found in the fruit peel (Luo *et al*., 2012). It should be taken into account that the amount of mangiferin and other bioconstituents depends on the extraction conditions and the solvents in use. In our case, the highest recovery of mangiferin was obtained by hydroethanolic extraction (22.4÷35.6 mg/g DWP), which confirms that *A. theiformis* is a rich source of mangiferin. The higher temperature (140°C) in the case of PLE had a negative effect on mangiferin extraction in comparison with the lower temperature (70 °C). Among the purified fractions, Sto1p and Sto2p fractions had the highest amounts of mangiferin followed by the n-butanol fraction; therefore, it is not suprising that these fractions demonstrated the highest antioxidant activity.

As presented in Fig. 4.12A, TA and HTA have some isomers with the molecular weights of 487.3430 g/mol (7a, 7c) and 503.3377 g/mol (9a, 9c), respectively. It is worth pointing out that these isomers are reported in *A. theiformis* for the first time. Due to the high structural similarity to TA and HTA acids, these isomers were quantified by using the same calibration curves, and the amounts were expressed as equivalents of TA and HTA acids. Although TA was previously isolated and identified in *A. theiformis*, however, our studies showed that the amount of this compound was rather small (0.02÷0.05 mg/g DWP). Whereas, considerably higher amounts (0.05÷0.40 mg/g DWP) were found for the TA isomer with a retention time of 5.74. This isomer was also dominant in purified fractions. Ethyl acetate and precipitated fractions (Sto1p and Sto2p) had the highest amounts of this isomer, and its amount ranged from 8.30 to 9.98 mg/g DWF. The amount of HTA among the extracts ranged from 0.04 to 5.63 mg/g DWE. As well as in the case of TA, HTA also had two isomers with a retention time of 3.61 and 5.14. The amount of the isomer with a retention time of 3.61 was fairly similar to that of HTA. Although TA isomer with retention time of 4.34 and HTA isomer with RT of 5.14 were also observed in the extracts, however, their amounts were quite low (0.01÷0.32 mg/g DWE). It is worthy of note that TA, HTA and their isomers were more abundant in acetonic extracts than in hydroethanolic and water extracts. The identification of isomers is a fairly complicated process, and it requires a separate study.

In a previous study on the chemical characterization of *A. theiformis*, three triterpene glucosides ($C_{36}H_{58}O_{10}$, $C_{36}H_{58}O_{11}$,$C_{36}H_{58}O_{11}$) were isolated and identified as tormentic acid ester glucoside, 23-hydroxytormentic acid ester glucoside and 6β-hydroxytormentic acid ester glucoside, respectively (Gopalsamy, Vargas, Guého, Ricaud and Hostettmann, 1988). In our study, these compounds were not identified; instead of them, some high molecular mass compounds with a molecular formula of $C_{37}H_{60}O_{14}$, $C_{37}H_{60}O_{13}$ and $C_{37}H_{60}O_{12}$ were determined.

Table 4.10. Quantification of phenolic compounds present in the extracts of *A. theiformis* (mg/g)

Sample	*Mangiferin*		*Tormentic acid* RT=5.60		* *Tormentic acid isomer* RT=4.34		* *Tormentic acid isomer* RT=5.74	
	DWE	DWP	DWE	DWP	DWE	DWP	DWE	DWP
Single step								
ACs (*SE*)	38.10±0.11^{b}	4.18^{b}	0.16±0.01^{b}	0.02^{b}	0.21±0.01^{b}	0.02^{c}	3.70±0.03^{d}	0.40^{d}
EHs (*MBS*)	104.6±3.99^{f}	35.6^{f}	0.15±0.00^{a}	0.05^{c}	0.24±0.00^{c}	0.08^{d}	1.72±0.12^{c}	0.59^{e}
Consecutive								
ACc (*SE*)	43.03±0.05^{b,c}	3.94^{b}	0.18±0.01^{b}	0.02^{b}	0.21±0.00^{b}	0.02^{a}	3.45±0.06^{d}	0.32^{c}
EHc(*MBS*)	101.0±0.01^{f}	25.6^{e}	nd	nd	0.01±0.00^{a}	tr	1.03±0.01^{b}	0.26^{b,c}
Σ		**29.5**		**0.02**		**0.02**		**0.58**
ACp_{70} (*PLE 70°C*)	48.64±0.24^{c}	3.16^{b}	0.24±0.00^{c}	0.02^{b}	0.28±0.00^{d}	0.02^{a}	4.28±0.12^{e}	0.28^{b,c}
EHp_{70} (*PLE 70°C*)	90.46±3.25^{e}	23.0^{d}	nd	nd	0.02±0.00^{a}	tr	0.99±0.00^{b}	0.25^{b}
Wp_{70} (*PLE 70°C*)	87.13±0.02^{e}	7.69^{c}	nd	nd	tr	tr	0.52±0.00^{a}	0.05^{a}
Σ		**33.9**		**0.02**		**0.02**		**0.58**
ACp_{140} (*PLE 140°C*)	72.20±2.64^{d}	4.68^{b}	0.15±0.00^{a}	0.01^{a}	0.24±0.01^{c}	0.02^{b}	4.05±0.11^{e}	0.26^{b,c}
EHp_{140} (*PLE 140°C*)	86.46±0.06^{e}	22.4^{d}	nd	nd	tr	tr	tr	tr
Wp_{140} (*PLE 140°C*)	12.18±0.30^{a}	1.14^{a}	nd	nd	nd	nd	tr	tr
Σ		**28.2**		**0.01**		**0.02**		**0.26**

Table 4.10. (continued)

Sample	*Hydroxytormentic acid* RT=4.73		** Hydroxytormentic acid isomer* RT=3.61		** Hydroxytormentic acid isomer* RT=5.14	
	DWE	DWP	DWE	DWP	DWE	DWP
Single step						
ACs (*SE*)	$2.71±0.02^{e}$	0.30^{c}	$3.19±0.03^{d}$	0.35^{d}	$0.20±0.00^{b}$	0.02^{a}
EHs (*MBS*)	$1.26±0.04^{d}$	0.43^{e}	$1.70±0.21^{c}$	0.58^{e}	$0.01±0.00^{a}$	tr
Consecutive						
ACc (*SE*)	$3.01±0.05^{f}$	0.28^{c}	$3.36±0.06^{d}$	$0.31^{b,c,d}$	$0.22±0.01^{b}$	0.02^{a}
EHc(*MBS*)	$0.34±0.01^{c}$	0.09^{b}	$0.86±0.01^{b}$	$0.22^{b,c}$	nd	nd
Σ		**0.37**		**0.53**		**0.02**
ACp_{70} (*PLE 70°C*)	$3.94±0.02^{g}$	0.26^{c}	$3.72±0.03^{e}$	0.24^{c}	$0.31±0.00^{c}$	0.02^{a}
EHp_{70} (*PLE 70°C*)	$0.31±0.00^{b}$	0.08^{b}	$0.95±0.01^{b}$	0.24^{c}	nd	nd
Wp_{70} (*PLE 70°C*)	$0.04±0.00^{a}$	tr	$0.27±0.00^{a}$	0.02^{a}	nd	nd
Σ		**0.34**		**0.50**		**0.02**
ACp_{140} (*PLE 140°C*)	$5.63±0.08^{h}$	0.36^{d}	$3.25±0.01^{d}$	$0.21^{b,c}$	$0.32±0.01^{c}$	0.02^{b}
EHp_{140} (*PLE 140°C*)	$0.14±0.00^{a}$	0.04^{a}	$0.56±0.01^{a}$	0.14^{b}	nd	nd
Wp_{140} (*PLE 140°C*)	tr	tr	tr	tr	nd	nd
Σ		**0.40**		**0.35**		**0.02**

nd – not detected, tr – trace. *expressed as tormentic or hydroxytormentic acids equivalents. Values are represented as mean ± standard deviation (n=3); columns with different letters differ significantly for Tukey's test at $p < 0.05$

Table 4.11. Quantification of the phenolic compounds present in the fractions of *A. theiformis* mg/g DWF

Sample	*Mangiferin*	*Tormentic acid* RT=5.60	**Tormentic acid isomer* RT=4.34	**Tormentic acid isomer* RT=5.74	*Hydroxytormentic acid* RT=4.73	**Hydroxytormentic acid isomer* RT=3.61	**Hydroxytormentic acid isomer* RT=5.14
Ethyl acetate	191.7 ± 3.21^{c}	tr	tr	8.51 ± 0.01^{b}	4.02 ± 0.22^{b}	5.83 ± 0.06^{d}	tr
n-Butanol	416.3 ± 3.77^{d}	nd	nd	tr	nd	1.30 ± 0.06^{b}	nd
Water	12.57 ± 0.10^{a}	nd	nd	tr	tr	tr	nd
Sto1g	152.5 ± 7.11^{b}	tr	nd	1.00 ± 0.01^{a}	6.31 ± 0.01^{c}	0.59 ± 0.03^{a}	0.05 ± 0.00^{a}
Sto1p	459.7 ± 11.2^{e}	tr	nd	9.98 ± 0.10^{c}	4.59 ± 0.28^{b}	nd	tr
Sto2p	557.0 ± 15.4^{f}	nd	nd	8.30 ± 0.09^{b}	0.10 ± 0.00^{a}	tr	tr
Tf	182.6 ± 5.97^{c}	nd	tr	tr	tr	3.19 ± 0.03^{c}	nd

nd – not detected, tr – trace. *expressed as tormentic or hydroxytormentic acids equivalents. Values are represented as mean ± standard deviation (n=3); columns with different letters differ significantly for Tukey's test at $p < 0.05$

The compound with a molecular formula of $C_{37}H_{60}O_{12}$ after MS/MS fragmentation of the compound gave two fragment ions with *m/z* 649.3949 and 487.3427. The ion with *m/z* 487.3427 fits to the tormentic acid ion formula, thus this compound was preliminarily assigned to the TA derivative. Previous studies also noted that TA derivatives (for example, tormentic acid 3fl-O-a-L-rhamnopyranoside) gave this fragment (Tommasi, Rastrelli, Cumanda, Speranza and Pizza, 1996). Judging from the MS/MS fragment ion with m/z 503.3371, the value fits to the HTA ion formula; the compound with the molecular formula of $C_{37}H_{60}O_{13}$ was preliminarily assigned to the HTA derivative.

Some constituents were left unidentified because of the lack of information about their structures. The exact mass data obtained by UPLC-QTOF-MS was not sufficient for their identification. Some of them were tentatively identified according to mass spectra libraries. However, the identified compounds due to various previously reported biological activities, such as antioxidant, anti-inflammatory, hepatoprotective activities (Jian, Li, Zheng, He and Ren, 2015; Wang *et al*., 2016; Das, Ghosh, Roy and Sil, 2012), could play the crucial role in the bioactivity of *A. theiformis*. In addition, it should be highlighted that, in a previous study by Hsoidrou *et al*. (2014), tannins and flavonoids were also reported in *A. theiformis*.

4.3.5. Correlation between different values

It is known that the specificity of the antioxidant activity assays affects the correlations between the results obtained via different methods (Sieniawska *et al*., 2017). Therefore, it was of interest to assess whether there are any correlations between TPC and different antioxidant activity assays used in this study (Fig. 4.14 A). Based on the calculated correlation coefficients, strong correlations exist between TPC and different antioxidant activity assays of *A. theiformis* extracts (0.936–0.952) and fractions (0.947–0.993). The strongest correlation occurred between the TPC and FRAP values which were measured for extracts, while, in the case of fractions, the strongest correlation was observed between the TPC and $DPPH^{\bullet}$ scavenging values. It is important to note that the antioxidant activity values expressed in DWE were used for calculation

This study indicated mangiferin as the major compound of *A. theiformis*, therefore, it was also of interest to determine if any correlations exist between the mangiferin content and the antioxidant activity of the extracts and fractions (Fig. 4.14 B). A strong correlation (0.892–0.969) was observed between the content of mangiferin and the antioxidant activity of fractions in $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and FRAP assays, while the correlation coefficients of the extracts were fairly weak, specifically, 0.487–0.689. In the case of extracts, the strongest correlation was observed between the content of mangiferin and the $DPPH^{\bullet}$ scavenging assay (of the extracts), and the content of mangiferin and the FRAP assay (of the fractions). These findings reveal that mangiferin is responsible for the high antioxidant activity of fractions, while the absence of the strong correlations of extracts indicated that the presence of other compounds in extracts could also contribute to their antioxidant properties.

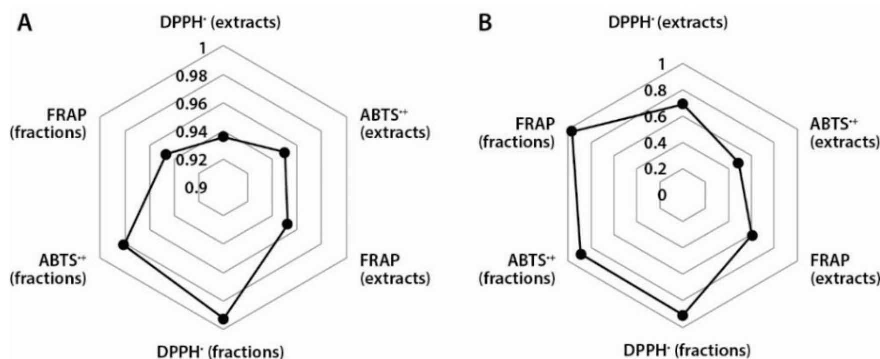


Fig. 4.14. Correlation coefficients between: A) TPC and different antioxidant measurement assays, B) Mangiferin content and different antioxidant measurement assays in extracts and fractions

4.4. Perspective of the investigated plant species as valuable ingredients for nutraceuticals, pharmaceuticals, and cosmetic formulations

It has been identified that oxidative stress is the main cause of the development and progression of several diseases including cancer, cardiovascular and inflammatory diseases. In order to combat the undesirable effects of ROS induced oxidative damage, suplementation of exogenous antioxidants or boosting of endogenous antioxidant defenses of the body is a promising way (Kasote, Katyare, Hegde and Bae, 2015). Plants featuring a high antioxidant activity are suitable sources for this purpose. Therefore, nowadays, many researches are being conducted into the determining new plant sources with various bioactivities.

Both *P. phillyreifolius* both *A. theiformis* demonstrated exclusively high antioxidant activity. This is demonstrated via comparison of the antioxidant activity of these species with worldwide recognized antioxidant active plant species including basil, oregano, *Salvia* spp., *Mangifera indica* and others (Table 4.12). In addition to the high antioxidant activity, such plants also demonstrate other bioactivities including antiinflammatory, antiviral and other aspects; therefore, the investigated species are an interesting subject of further researches in finding other bioactivities and applications.

The high antioxidant activity is inseparable from the presence of bioactive compounds in these plant species. Geraniin was found to be the dominating compound of *P. phillyreifolius*. The amount of this compound in *P. phillyreifolius* reached up to 110 mg/g DWP, while the content of geraniin in *G. thunbergi* was about 8.65 mg/g DWP. In Japan, *G. thunbergi*, due to its richness in geraniin, is certified as an official antidiarrheal drug (Luger *et al*., 1998). This shows that *P. phillyreifolius* is a great source of this compound. The large range of the bioactive properties of geraniin in both the purified form and enriched plant extracts is well reported and discussed in previous sections, therefore, such a compound is a highly suitable candidate for further study in many types of the industry, particularly, the pharmaceutical, nutraceutical and cosmetic industries (Perera, Ton and Palanisamy, 2015). The content of ellagic acid was also high in *P. phillyreifolius* whose amount reaches up to 8.65 mg/g DWP. Various berries are great sources of

ellagic acid, in which, the amount of ellagic acid reaches about 71 mg/100 g fresh weight (Häkkinen, Kärenlampi, Mykkänen and Törrönen, 2000). Some commercial products, such as a lightening cream *White Perfect Laser™* by *L'Oréal*, contain plant-derived ellagic acid which corrects spots.

Table 4.12. Characteristics of the investigated plant species in comparison with other plants

Plant	TPC mg GAE/g DW	ABTS$^{•+}$ μM TE/g DW	FRAP μM TE/g DW	References
P.phillyreifolius	33.5÷153	466÷2316	366÷1907	-
A. theiformis	0.71÷112	10.1÷1566	30.7÷1524	-
Oregano	24.1	245	-	(Brandstetter, Berthold, Isnardy, Solar and Elmadfa, 2009)
M.indica (seed)	86.7	1668	25.32	(Surveswaran, Cai, Corke and Sun, 2007)
Basil	35.6÷126.2	199÷562	-	(Juliani and Simon, 2002)
Salvia spp.	0.5÷43.7	15.6÷514.9	-	(Šulniūtė, Ragažinskienė and Venskutonis, 2016)
B. crassifolia	2.41÷90.5	25.3÷1375	-	(Kraujalienė, Pukalskas, Kraujalis and Venskutonis, 2016)

Mangiferin was the most abundant constituent of *A. theiformis.* This compound exerts protective effects against degenerative diseases, such as atherosclerosis, cancers (breast, colon, neural, skin, and cervical), obesity and diabetes (Imran *et al*., 2017). A mangiferin-rich extract of mango (*Mangifera indica*) bark *Vimang* is commonly used as a traditional medicine in Cuba in the colorectal cancer therapy, and it also shows excellent results in the improvement of the quality of life of terminally ill patients with HIV/systemic lupus, and infertility in women. This compound was extensively studied for various bioactivities, and the research yielded many patents related to the therapeutic or cosmetic application of mangiferin and its derivatives that have been issued in recent years (Telang, Dhulap, Mandhare and Hirwani, 2013). The mangiferin concentration in *A. theiformis* extracts reached up to 35.6 mg/g DWP. Comparing to other mangiferin-rich species, the content of mangiferin in the mango fruit was 2.59 mg/g DWP (Marcela *et al*., 2017), mango tree leaves scored 12.94 mg/g DWP (Vo, Nguyen, Nguyen and Ushakova, 2017), whereas the mango peel had a value of 7.49 mg/DW (Luo *et al*., 2012).

A few successful examples of the application of bioactive compounds which are present in *P. phillyreifolius* and *A. theiformis* for various purposes demonstrated that these plant species may find various uses as functional ingredients for nutraceuticals, pharmaceuticals and cosmetic formulations. These studies are only the first step in demonstrating the importance of these plant species. The practical application of these plants requires further study.

CONCLUSIONS

1. The application of different extract preparation schemes affected the yield of the extracts of *Phyllanthus phillyreifolius* (PP) and *Aphloia theiformis* (AT). The yields obtained with the water/ethanol (3:7) mixture were higher (27.86–34.03%) compared to water (13.45–14.65%), acetone (5.98–11.5%), hexane (2.14–2.62%), and SFE-CO_2 (1.26–2.52%). The conventional extraction methods gave higher yields than the high pressure extraction techniques. In the PP case, consecutive extraction was more effective in the recovery of bioactive extracts than the single step extraction.
2. *In vitro* antioxidant activity (AA) assays indicated that the hydrophilic extracts of the investigated plants were very strong antioxidants. A considerable amount of the antioxidant active material remained after the extraction of PP. Direct maceration by stirring with the water/ethanol (3:7) mixture most efficiently decreased the antioxidant potential of the PP plant material. The fractionation of the hydroethanolic extract of the AT extract gave some fractions with a very high AA, which was comparable to mangiferin AA. A strong correlation between the total content of polyphenols and the AA measured by *in vitro* AA assays was observed in PP and AT extracts, (0.975–0.994) and (0.936–0.994), respectively.
3. Geraniin was the major quantified compound of hydrophilic extracts of PP in concentrations ranging from 13.2 to 110 mg/g DWP. Among the quantified tocopherols, α-tocopherol was most abundant (0.27–0.38 mg/g DWP) in the lipophilic extracts of PP. Mangiferin was the main constituent of AT whose concentration reached 36.5 mg/g DWP. The content of mangiferin in the fractions ranged from 12.57 to 557.0 mg/g DF. Tormentic and hydroxytormentic acids as well their derivatives and isomers were also present in AT extracts and fractions.
4. The preconditioning of HEK293 cells with the acetonic and hydroethanolic extracts of PP extracts strongly inhibited the generation of ROS induced by H_2O_2 thus protecting cells against oxidative stress. The gene expression of antioxidant enzymes was also affected by the pretreatment cells with PP extracts. The cytotoxicity assay indicated that both investigated extracts were nontoxic at 250 μg/ml and lower concentrations.

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LIST OF PUBLICATIONS ON THE THEME OF THIS DISERTATION

Scientific publications published in the WOS List of Scientific Information by *Clarivate Analytics* (formerly *Thomson Reuters*):

1. GRAUZDYTĖ, D., PUKALSKAS, A., VIRANAICKEN, W., EL KALAMOUNI, C. and VENSKUTONIS, P.R. Protective effects of *Phyllanthus phillyreifolius* extracts against hydrogen peroxide induced oxidative stress in HEK293 cells. *PLoS One*. San Francisco, CA: Public library of science. eISSN 1932-6203. 2018, vol. 13, iss. 11, art. No. e0207672, p. 1-15. (IF: 2.766 (2018).
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1. GRAUZDYTĖ, D. VENSKUTONIS, P.R. PUKALSKAS, A. and EL KALAMOUNI, C. Antioxidant and enzyme inhibitory activities of *Aphloia theiformis* extracts/fractions and chemical characterization of major phenolic compounds. In: *FoodBalt – 2018: 12th Baltic conference on food science and technology "Food R&D in the Baltics and beyond", May 17-18, 2018 Kaunas, Lithuania: abstract book*. Kaunas University of Technology. Department of Food Science and Technology. Kaunas: Kauno technologijos universitetas, 2018, p. 29 eISBN 9786090214626.
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7. GRAUZDYTĖ, D., VENSKUTONIS, P.R. and EL KALAMOUNI, C. Antioxidant potential of *Phyllanthus phillyreifolius* - the tropical plant from Reunion Island. In: *Foodbalt - 2015 [e-resource]: 10th Baltic conference on food science and technology "Future food: innovations, science and technology", May 21-22, 2015, Kaunas, Lithuania: abstract book*. Kaunas University of Technology. Department of food science and technology. Kaunas: KTU, 2015, p. 53 eISBN 9786090211380.

Other scientific publications published in the wos list of scientific information by clarivate analytics (formerly thomson reuters):

1. BENDIF, H., MIARA, M.D., KALBOUSSI, Z., GRAUZDYTĖ, D., POVILAITIS, D., VENSKUTONIS, P.R. and MAGGI, F. Supercritical CO_2 extraction of *Rosmarinus eriocalyx* growing in Algeria: chemical composition and antioxidant activity of extracts and their solid plant materials. *Industrial Crops and Products*. Amsterdam: Elsevier. ISSN 0926-6690. eISSN 1872-633X. 2018, vol. 111, p. 768-774. (IF: 4.191 (2018).
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