UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Head office: Università degli Studi di Padova

Department of Pharmaceutical and Pharmacological Sciences

Ph. D. Course in Molecular Sciences

Curriculum: Pharmaceutical Sciences

Series XXXI

Extraction and characterization of chemicals from vegetal matrices and assessment of their properties for nutraceutical and cosmetic applications

Thesis written with the financial contribution of SICIT CHEMITECH S. p. A.

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To my family,

To Alberto

Contents

Preface

This Ph. D. grant was funded by the company SICIT CHEMITECH S. p. A., with the purpose of contributing to the research on raw materials of the company EDYNEA s. r. l.

EDYNEA s.r.l. develops and produces food supplements, functional foods and cosmetics, mainly characterized by the presence of high quality plant extracts. For this purpose, EDYNEA has invested in Paraguay in a natural oasis of 400 hectares dedicated to organic farming, of which 1.5 hectares as a *vivarium* and experimental field, where research and the selection of the best varieties of their plants begins.

Acknowledgements

I wish to thank SICIT CHEMITECH S.p.A. for funding this Ph. D. grant, which gave me an important opportunity for professional and personal growth.

I am grateful to EDYNEA s.r.l., in particular to Massimo Neresini, who considered me part of the research group since the very first beginning and for transmitting me the enthusiasm for the construction of this project.
A deep thank to the R&D group of EDYNEA s.r.l. and in particular to Alberto Rumignani, Elisabetta Rigoni, Alberto Foschini, Elisa Rancan, Chiara Saggiotto, Chiara Pituello and Fabio Grandi: thanks for the work that we made together.

I wish to thank the University of Padua, in particular Prof. Giuseppe Zagotto, for having hosted me in his laboratory.

Thanks to the Universidad Nacional de Asunción, and in particular to Prof. Zully Vera De Molinas, Prof. Nelson Alvarenga, Prof. Laura Mereles, Prof. Nuri Cabral, Prof. Rosa Degen, Prof. Yenny Gonzalez and Prof. Pablo Sotelo, for the collaboration in this Ph. D. project and for the warm welcome during my internship there.

Thanks to my colleagues from Padua University: Giovanni Ribaudo, Enrico Zanforlin and Alberto Ongaro. Even if we worked on very different projects, we shared together lot of unforgettable moments.

I am also grateful to SICIT CHEMITECH quality control laboratory and in particular to Eliana Franco, Michele Cristofani, Sonia Sella, Stefano Zordan, Michela Serraglio and Roberta Bauce for being kind and helpful with me since the very beginning of this experience, that actually started more than three years ago in their laboratory.

I wish to thank my friends, Valentina, Silvia, Laura, Gina and Stefano for having supported and encouraged me in this period of study.

Thanks to Giuseppe and Letizia for supporting me like a daughter.

The greatest thank to my family and to Alberto for having shared completely this experience.

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Abstract

Present days lifestyles that include bad eating habits as well as consistent exposure to sunlight (i.e. UV rays), cigarette smoke and air pollutants increase the number of free radicals in our body and predispose people to oxidative stress, which is responsible for the development of several diseases as well as aging. Therefore, the demand for effective inhibitors of oxidative processes has become essential, also to attenuate the inability of the endogenous antioxidant defence system of the body to cope with the increasing radical production. Plant-derived antioxidant compounds are preferred to animal-derived or synthetic antioxidants because they are safer and they do not face ethical issues.

Hibiscus sabdariffa L. is a plant of the Malvaceae family, native to Sudan. Its calyces represent the most exploited part of the plant and they are very rich in anthocyanins. These compounds are responsible for many of the biological properties reported for the extracts of calyces, like the strong antioxidant activity.

Moringa oleifera Lam. is a plant of the Moringaceae family, with antioxidant properties mainly attributed to its phenolic compounds and, recently, to its isothiocyanates, deriving from the hydrolysis of glucosinolates catalysed by the thioglucosidase myrosinase.

Both these plants are source of seed oils that are interesting because of their composition in antioxidant compounds.

This research work was divided into three project lines:

The first project line focused on *H. sabdariffa* calyces. The future perspective was to obtain an antioxidant extract for the production of nutraceutical products. Therefore, it was performed the selection of a variety of *H. sabdariffa* calyces rich in anthocyanins (among the varieties at disposal of EDYNEA s.r.l. in its Paraguayan plantation), by evaluating the total monomeric anthocyanin content of the extracts. Then, it was optimized an extraction method suitable for the recovery of anthocyanins in high yield but also transferable on large scale. Finally, it were tried different procedures to obtain an extract enriched in anthocyanins using polyvinylpolypyrrolidone (PVPP) as adsorbent matrix. It was concluded that Tenonderà Tempranera variety was the richest in anthocyanins and that the production of extract on large scale could be performed at room temperature, for 15 minutes, with the application of ultrasounds. It was also concluded that further trials were required to obtain an anthocyanins-enriched extract by using PVPP, as the total monomeric anthocyanin value observed for

the most concentrated extract was not satisfactory if compared with the value of an extract not treated with PVPP.

The second project line was devoted to *M. oleifera* tissues. Many studies are reported in literature about leaves and seeds, but as far as we know there is few or no literature about branches that actually represent a waste in the processing of *M. oleifera* and about the patented matrix labelled in this study as "EDYNEA". Therefore, it was interesting to investigate these new tissues in comparison with the traditionally studied ones. Moreover, this study concerns plant material grown in Paraguay and therefore extends the information available on the chemical composition of *M. oleifera* plants cultivated in different agro-climatic regions of the world. As far as we know, this is the first study concerning the characterization of both direct and indirect antioxidant compounds in different tissues of *M. oleifera*.

Hence, in the first part of the study, it was compared the total phenolic content, the direct antioxidant capacity (DPPH and $ABTS^{\bullet+}$ assays) and the glucomoringin content (main glucosinolate of *M. oleifera* and precursor of the isothiocyanate with indirect antioxidant activity) of aqueous extracts of different tissues of *M. oleifera*, grown in Paraguay. Two different drying treatments (freeze-drying and oven drying, 40°C) were performed on the vegetal samples before extraction, with the objective of verify the influence of the drying treatment on the chemical composition of the final extract.

The study was continued with the selection of an extraction condition able to maintain a high extraction yield of total polyphenols and suitable for the conversion of glucomoringin into its bioactive isothiocyanate inside the extracts that were obtained from the tissues selected in the first part of the study because of their richness in glucomoringin or phenolics. A further objective was the quantification of glucomoringin and its isothiocyanate inside the extracts, the determination of total polyphenols and the evaluation of the direct antioxidant capacity. It was observed that, in these extraction conditions, the branches (that have a lower phenolic content and total antioxidant capacity than leaves) could bring to an extract with an equal phenolic content but a higher total antioxidant capacity than the extract of leaves, thus becoming a valid alternative to leaves in the preparation of extracts rich in antioxidant compounds. Even more, the matrix "EDYNEA" showed to be an interesting alternative to leaves for the preparation of extracts richer in glucomoringin-isothiocyanate.

The third project line was dedicated to oilseeds and oily extracts. The future perspective was the production of an oily extract enriched in antioxidant compounds, with a higher total antioxidant capacity than a pure oil, to be used as ingredient in cosmetic or nutri-cosmetic products, against

oxidative stress damages on skin. It was decided to use *M. oleifera* oil and *H. sabdariffa* oils for the preparation of the oleolites together with two commonly used oils in the preparation of cosmetic products and for human consumption (refined olive oil and sunflower oil). The oil of *H. sabdariffa* was selected between the seed oil of different varieties of *H. sabdariffa* available at the Paraguayan plantation of EDYNEA s.r.l. The oleolites were prepared by ultrasound-assisted extraction. The main classes of antioxidant compounds (total phenolics, total carotenoids and α-tocopherol) were characterized and it was assessed the total antioxidant capacity. It was observed that the production of an oily extract higher in antioxidant components has not lead to a better antioxidant capacity compared to the pure oil, at least in the extraction conditions used. Among the oils, the more active was the one extracted from the seeds of the selected variety of *H. sabdariffa*, Kibeleza.

Abbreviations

ABTS$^{\bullet+}$: *2,2'-azino-bis (3-etilbenzothiazoline-6-sulphonic acid) cationic radical*

BHI: *brain heart infusion medium*

CFU: *colony-forming unit*

DAD: *diode array detector*

DMSO: *dimethyl sulfoxide*

DPPH: *1,1-Diphenyl-2-picryl-hydrazyl radical*

EtOH: *ethanol*

GAE: *gallic acid equivalents*

GLUCOMORINGIN (GMG): *4-(α–L-rhamnopyranosyloxy)-benzylglucosinolate*

GLUCOMORINGIN ISOTHIOCYANATE (ITC): *4-(α–L-rhamnopyranosyloxy)-benzyl isothiocyanate*

HO: *H. sabdariffa oil*

HO-ML: *oily extract obtained by extracting M. oleifera leaves with H. sabdariffa Kibeleza oil*

HPLC: *high performance liquid chromatography*

MDA: *malondialdehyde*

MeOD: *deuterated methanol*

MeOH: *methanol*

MO: *M. oleifera oil*

MO-ML: *oily extract obtained by extracting M. oleifera leaves with M. oleifera oil*

NMR: *nuclear magnetic resonance*

NQO1: *NAD(P)H quinone oxidoreductase 1*

OO: olive oil

OO-ML: *oily extract obtained by extracting M. oleifera leaves with olive oil*

OO-HL: *oily extract obtained by extracting H. sabdariffa leaves with olive oil*

OO-EDYNEA03: *oily extract obtained by extracting EDYNEA03 with olive oil*

PVPP: *polyvynilpolypyrrolidone*

SO: *sunflower oil*

SO-ML: *oily extract obtained by extracting M. oleifera leaves with sunflower oil*

SO-HL: *oily extract obtained by extracting H. sabdariffa leaves with sunflower oil*

SPE: *solid phase extraction*

TAC: *total antioxidant capacity*

TCC: *total carotenoid content*

TE: *Trolox equivalents*

TMA: *total monomeric anthocyanins*

TPC: *total phenolic content*

Trolox: *6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid*

1. INTRODUCTION

1.1. Free radicals and oxidative stress

Free radicals are atom or molecules containing one or more unpaired electrons, making them very unstable and reactive[1]. Their reactivity relies on the need to acquire electrons or hydrogen atoms from donor molecules.

Free radicals are constantly produced during normal physiological metabolism in tissue, both from oxygen (reactive oxygen species, ROS) and nitrogen (reactive nitrogen species, RNS)[2].

Intracellular free radicals are generated from the autoxidation and consequent inactivation of small molecules such as reduced flavins and thiols, and from the activity of certain oxidases, cyclooxygenases, lipoxygenases, dehydrogenases, and peroxidases. Oxidases and electron transport systems are prime, continuous sources of intracellular, reactive oxygenated free radicals[3](See Figure 1).

Such molecules can also originate as a consequence of diseases (e.g., inflammations)[2], as a product of activated phagocytes (e.g., HOCl), as components of tobacco smoke and air pollutants (ozone and nitrogen dioxide) and indirectly through the metabolism of certain solvents, drugs, and pesticides as well as through exposure to radiation[3].

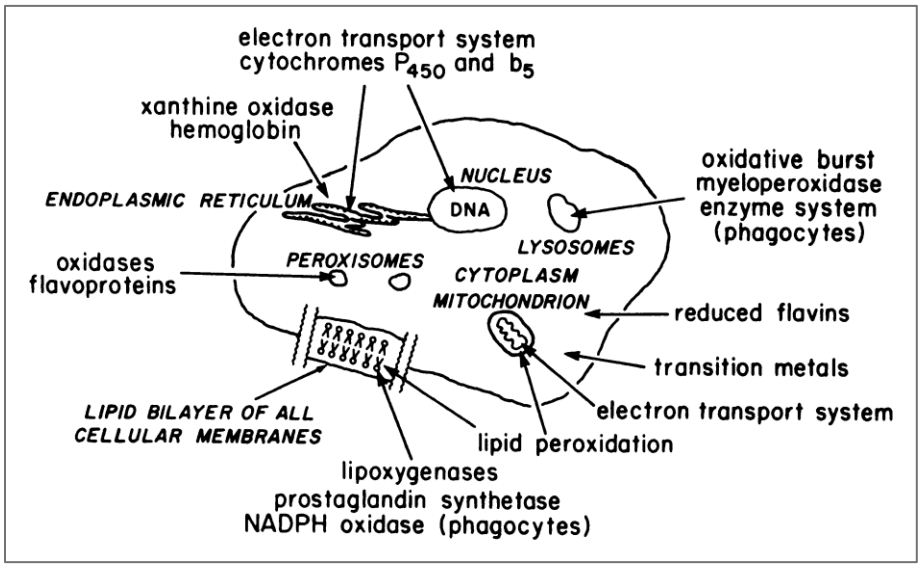


Figure 1: Cellular sources of free radicals (main intracellular oxidase systems and electron transport chain)[3].

The main examples of free radicals playing a major role in the damage of macromolecules are the hydroxyl radical, $OH^{\bullet}$(the most potent oxidant known[4]), the superoxide anion, $O_2^{\bullet -}$, and hydrogen peroxide, H_2O_2[5].

Mitochondria are thought to be the main source of ROS in the body with one in vitro study showing the conversion of 1-2%[6] of all oxygen molecules they consume into $O_2^{\bullet-}$ during the conversion of oxygen to water by the movement of electrons down the mitochondrial electron transport chain[7].
The formation of these three major ROS mainly occurs initially by the reaction of NADPH with oxygen (by NADPH oxidase) to give a superoxide anion radical, which is rapidly converted into hydrogen peroxide by an enzyme, called superoxide dismutase (Scheme 1).

Initiation step:

$$2O_2 + NADPH \xrightarrow{\text{NADPH oxidase}} 2O_2^{\cdot-} + NADP^+ + H^+$$

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{\text{Superoxide dismutase}} H_2O_2 + O_2$$

Scheme 1: Reactions for the production of ROS in the cells.

Once produced, H_2O_2 can pass easily through the semi-permeable cell membrane. Here more ROS can be generated from H_2O_2 because of the Fenton reaction and because the hydroxyl radical (that is produced in the Fenton reaction) reacts with H_2O_2 with the production of superoxide ($O_2^{\bullet-}$). Then superoxide reacts again with H_2O_2, so hydroxyl radical and hydroxyl anion (^-OH) are formed; this part of reaction is known as the "Haber-Weiss Reaction". Superoxide ($O2^{\bullet-}$) reduces Fe^{3+} rather than H_2O_2[8] (Scheme 2).

Propagation step:

$$H_2O_2 + Fe^{2+} \longrightarrow {}^{\cdot}OH + OH^- + Fe^{3+} \qquad \text{Fenton reaction}$$

$${}^{\cdot}OH + H_2O_2 \longrightarrow O_2^{\cdot-} + H^+ + H_2O$$

$$O_2^{\cdot-} + H_2O_2 \xrightarrow{(Fe^{3+}/\ Fe^{2+})} {}^{\cdot}OH + OH^- + O_2 \qquad \text{Haber-Weiss reaction}$$

Scheme 2: Fenton and Haber-Weiss reactions for the production of ROS.

H_2O_2 can also react with enzymes, ions (iron, copper and aluminium) and other oxidants to form more damaging oxidants such as HOCl, which can add chloride to DNA bases[7].

When free radicals react with non-radical molecules, a chain reaction occurs causing the formation of new free radicals. Free radicals such as peroxyl radicals, the superoxide anion, and the hydroxyl radical are responsible for many of the damaging reactions[3].

If not quenched by antioxidants, these highly reactive compounds will react non-enzymatically with several cellular or extracellular components, such as cell membranes, lipoproteins, proteins, carbohydrates, RNA, and DNA. In this way, the structure and the function of those macromolecules get altered[2].

The unsaturated bonds in membrane lipids represent an important target of free radicals. Consequent peroxidation results in a loss in membrane fluidity and receptor alignment and potentially in cellular lysis[3]. Free radical damage to sulfur-containing enzymes and other proteins culminates in inactivation, cross-linking, and denaturation. The attack to nucleic acids causes damages that result in mutations that may be carcinogenic[3]. Oxidative damage to carbohydrates can alter any of the cellular receptor functions including those associated with hormonal and neurotransmitter responses[3]. In addition, certain aldehydes such as malondialdehyde (MDA) and hydroxynonenal, arising from the free radical degradation of polyunsaturated fatty acids, can cause cross-linkings in lipids, proteins, and nucleic acids[3]. Lipid peroxidation can be observed by the concentration of MDA in the cell, as it is a byproduct of the reaction.

Nevertheless, the extent of tissue damage is the result of the balance between the free radicals generated and the antioxidant defense systems of the body[3].

When the critical balance between generation of free radicals and other ROS or RNS and the antioxidant defense is unfavourable, oxidative damage can accumulate. Oxidative stress is defined as "a condition that is characterized by accumulation of non-enzymatic oxidative damage to molecules that threaten the normal function of the cell or the organism"[2]. Compelling evidence has emerged in the past two decades demonstrating that oxidative stress is intimately involved in the pathophysiology of many seemingly unrelated types of disease. Thus, oxidative stress is now thought to make a significant contribution to all inflammatory diseases (arthritis, vasculitis, glomerulonephritis, lupus erythematosus, adult respiratory distress syndrome), ischemic diseases (heart disease, stroke, intestinal ischemia), cancer, hemochromatosis, emphysema, organ transplantation, gastric ulcers, hypertension, neurologic diseases (multiple sclerosis, Alzheimer's

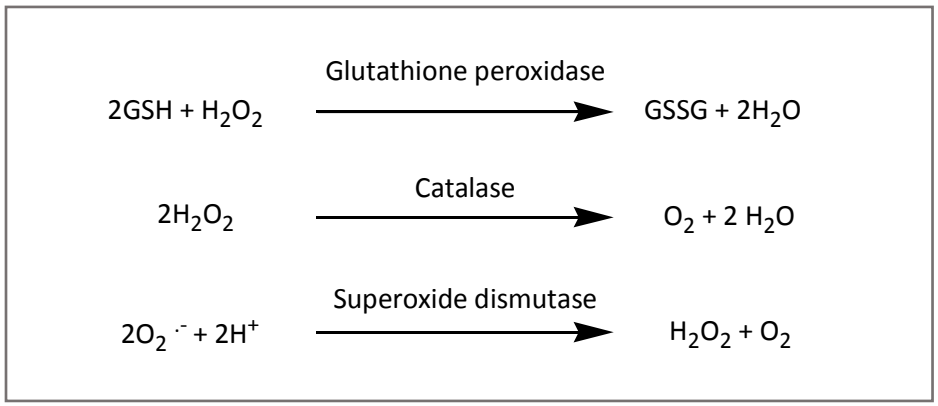
disease, Parkinson disease, amyotrophic lateral sclerosis, muscular dystrophy), alcoholism, and smoking-related diseases[2].

1.2. Antioxidant defence systems of the body

An antioxidant is defined as "a redox active compound that limits oxidative stress by reacting non-enzymatically with a reactive oxidant," while an antioxidant enzyme is "a protein that limits oxidative stress by catalysing a redox reaction with a reactive oxidant"[9].

1.2.1. Endogenous antioxidant defence systems

All aerobic cells developed a very efficient and complex defense system to counteract oxidative damage and oxidative stress. The endogenous antioxidant defense has both enzymatic and non-enzymatic components that act by preventing radical formation, removing radicals before damage can occur, repairing oxidative damage, and eliminating damaged molecules[2]. The endogenous antioxidant defense, which is produced by cells themselves, consists of components such as glutathione, thioredoxin, and various antioxidant enzymes[2], especially metallo-enzymes such as glutathione peroxidase (selenium dependent), catalase (iron dependent) and superoxide dismutase (copper, zinc and manganese dependent)[3].



Scheme 3: Removal of ROS by endogenous antioxidant enzymes.

Glutathione peroxidases, that are present in cytosol and mitochondria, facilitate the removal of H_2O_2 by oxidizing glutathione (GSH). This enzyme converts the hydrogen peroxide to water, and therefore, prevents the continuation of free radical production[4].

Catalases that are present in peroxisomes in many tissues remove hydrogen peroxide when present in high concentrations[4].

Superoxide dismutases (containing copper and zinc at the native site) in cytosol and in mitochondria

(containing manganese) catalyse the dismutation of superoxide to hydrogen peroxide and oxygen: The product of this reaction, hydrogen peroxide, is a weak oxidant and is relatively stable. However, unlike superoxide, hydrogen peroxide can rapidly diffuse across cell membranes, and in the presence of transition metal ions it can be converted to hydroxyl radicals via Fenton chemistry, as previously described[4].

The mitochondrial cytochrome oxidase greatly diminishes the intracellular free radical production, by preventing the release of ROS in the electron transport chain[4].

Mutations in genes coding for these peptides or proteins often lead to increased incidence of oxidative stress-related diseases[2].

N-acetylcysteine is the precursor of glutathione (GSH), which is the most abundant non-enzymatic endogenous antioxidant in the body. Glutathione plays the role of intracellular protective substance and functions as an effective scavenger of oxygen radicals. A decrease in GSH cell synthesis increases the oxidative state of the cell, leading it to a condition of oxidative stress[10].

1.2.2. Exogenous antioxidant defence systems

In addition to the endogenous antioxidant defense, the body also exploits dietary components introduced with diet or with nutritional supplementation that can contribute to the antioxidant defense and that are referred to as exogenous antioxidants[11].

Exogenous antioxidants can be classified in several categories according to their mechanism of free radical scavenging, to their pathway of synthesis, to their molecular structure.

Therefore, considering the mechanisms of action, it is possible to distinguish direct and indirect antioxidants: direct antioxidants are redox active, short-lived, small molecules that directly scavenge reactive oxygen and/or nitrogen species. Indirect antioxidants induce a variety of phase II xenobiotic metabolizing enzymes (e.g. NAD(P)H quinone reductase 1), resulting in a higher antioxidant capacity and a long-lasting protective effect compared to direct antioxidants. As a matter of fact, both mechanisms work together to protect the body from free radical damages[12]. It is believed that if plant compounds induce the phase II enzymes, cells are more readily able to neutralize toxic agents such as free radicals and other toxic electrophiles when they appear[2].

Even more, considering direct antioxidants, it is possible to do a further classification based on the mechanism with which those compounds interfere with the autoxidation process[13]. Autoxidation is a

radical-chain reaction that begins with the generation of free radicals that subsequently react with oxidizable substrates (such as unsaturated lipids) both by abstracting hydrogen atoms or by addition to C=C π systems, thus generating carbon-centered radicals. The latters generate peroxyl radicals in presence of oxygen that are able to react with another molecule in the organism, thus producing another radical molecule and propagating the chain reaction[13]. Termination reaction occurs if two peroxyl-radical molecules react or by cross-reaction of two radicals, but this is a very unlikely event in the absence of antioxidants[13]. Therefore, following any initiation event in absence of antioxidants, several propagation cycles will occur before termination takes place, determining the so-called chain length and the number substrate molecules transformed into the primary oxidation products, that is a) hydroperoxides (ROOH) if propagation occurs by hydrogen atom abstraction b) polyperoxides if it proceeds by C=C addition[13].

Therefore, direct antioxidants can be classified into three categories:

1. *preventive* antioxidants, that interfere with the initiation process.
2. *chain-breaking* antioxidants, that are able to react with peroxyl radicals to form products that do not propagate the radical chain[13]. Chain-breaking antioxidant can scavenge free radicals by several mechanism such as HAT[14](hydrogen atom transfer), ET[13] (electron transfer) or by addition to C=C π systems[13].
3. *termination enhancing* antioxidants, that co-oxidize with the substrate and form peroxyl radicals that do propagate the chain but have much higher rate of chain termination, thereby decreasing the chain length and saving the oxidizable substrate[13].

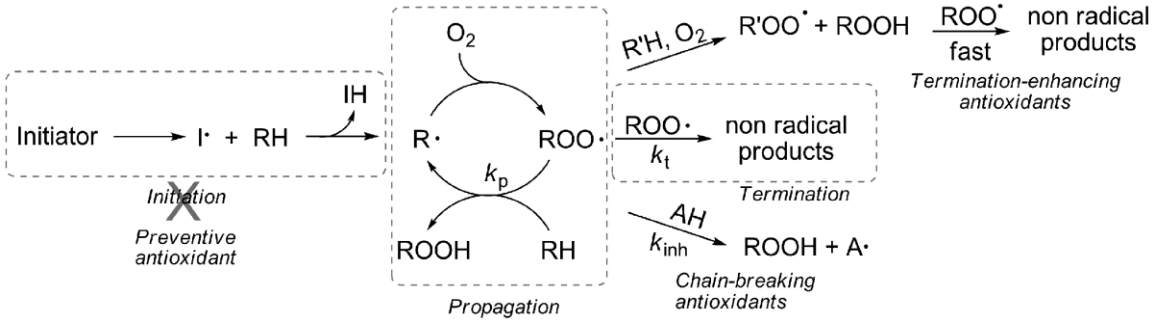


Figure 2: Autoxidation of an organic substrate and mechanism of interference by direct antioxidants[13].

Based on the pathway followed for the production of antioxidant compounds, these molecules can be classified into natural and synthetic antioxidant compounds[15]. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are compounds with phenolic structures of various degrees of alkyl substitution, whereas natural antioxidants can be phenolic compounds (tocopherols, flavonoids, and phenolic acids), nitrogen containing compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), or carotenoids as well as ascorbic acid[15]. Nevertheless, synthetic antioxidants might exert toxic side effects at higher concentrations than their permitted limits and, on the other side, animal-derived antioxidants face ethical issues[16]. Consequently, plants actually represent the most promising reservoir of health-protective antioxidants and they have already been showed to be an important source of compounds able to counteract free radicals, because of their antioxidant activity[17].

Therefore, considering plant-derived antioxidants, a classification can be done based on the differences in the chemical structures of these molecules. The main classes of antioxidant phytochemicals are reported below:

1.2.2.1. Phenolic compounds: Flavonoids and phenolic acids belong to the group of phenolic compounds.

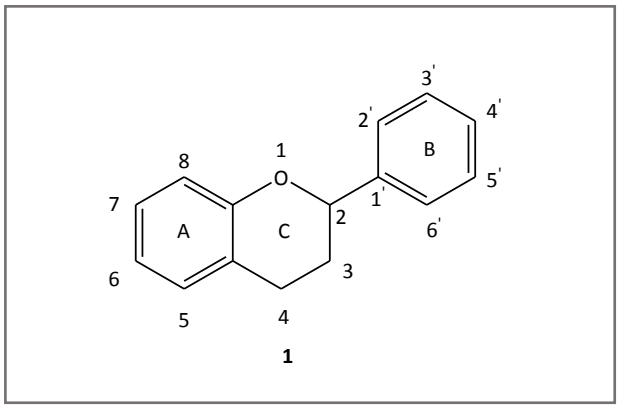


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Figure 3: The C_6-C_3-C_6 skeleton and numbering system for most flavonoids[18].

From a chemical point of view, flavonoids are molecules based on a C_6-C_3-C_6 configuration in the flavan nucleous. The flavan structure (**1**) has three rings, named A, B and C. Various substituting groups are

numbered according to their position on the rings. To the class of flavonoids belong flavonols (**2**, **3**, **4**), anthocyanidins (**5**, **6**), catechins (**7**, **8**) and procyanidin[18].

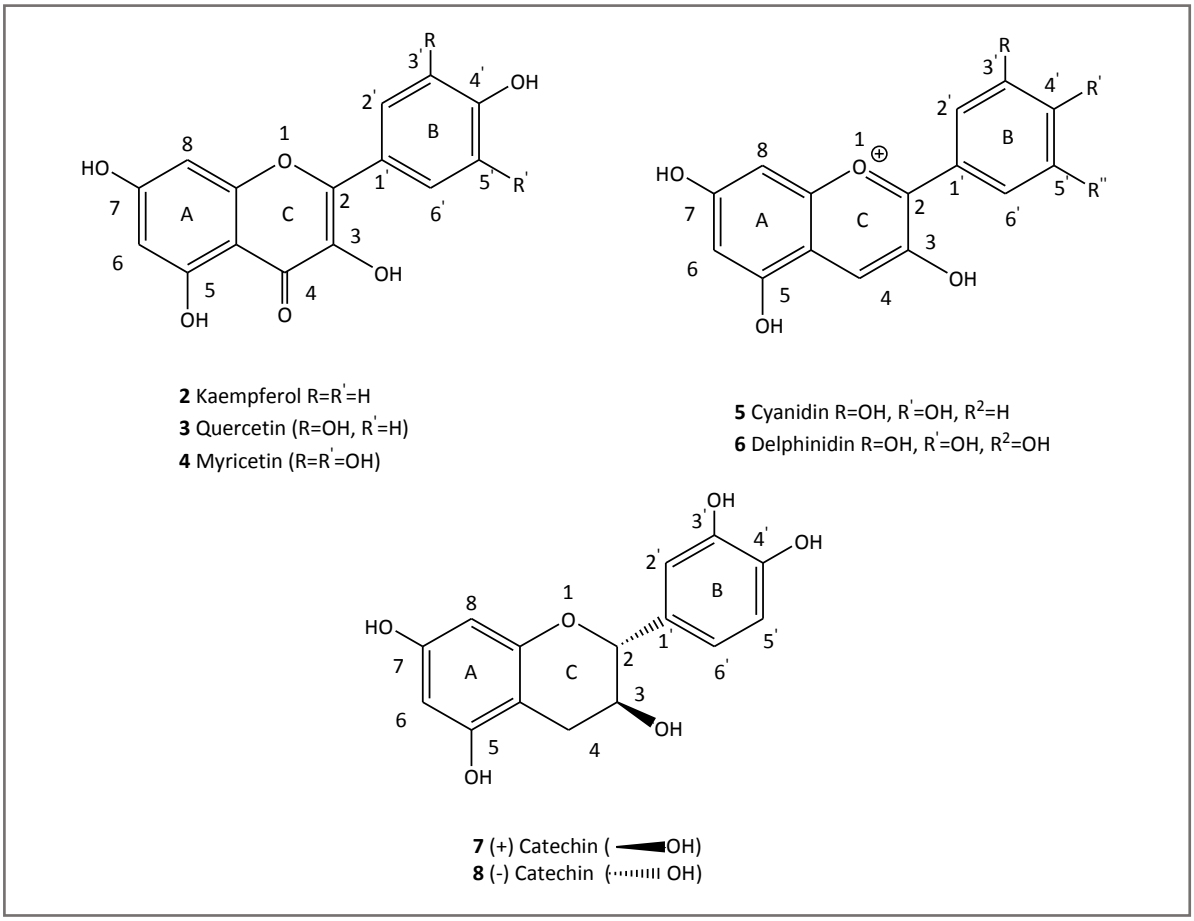
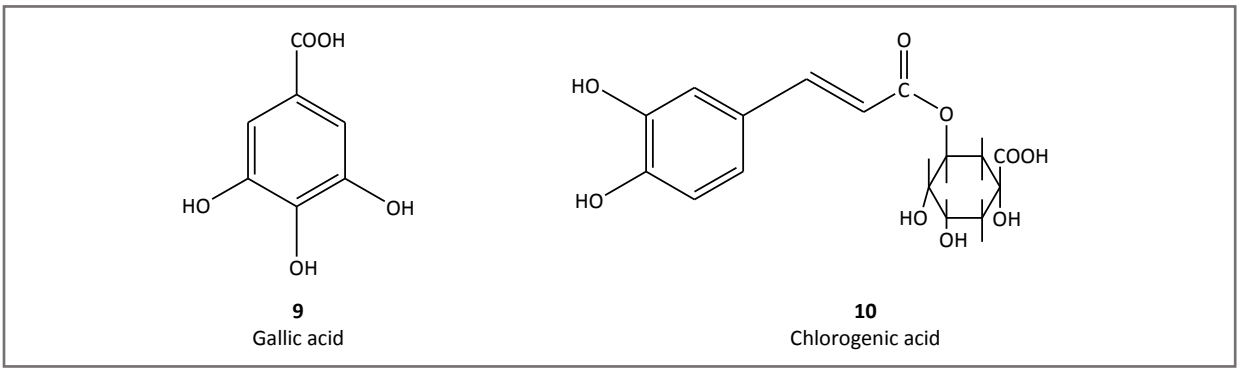


Figure 4: Chemical structures of common flavonoids found in nature[18].

Most flavonols and anthocyanidins occur as glycosides in plants that means that they are linked to one or more monosaccharides. The sugar moiety may have one or more aliphatic or aromatic acids attached in an acyl linkage[18].

An important function of flavonoids is to colour plants (from red/bluish due to anthocyanins to white or pale yellow). Other functions of flavonoids in plants are the protection from UV radiations of the sun, the protection against the attack of parasites, to function as signal substances and to provide flavour to various plant products for human consumption[18].

Phenolic acids are characterized by the presence of one or more carboxylic functions in their molecules and they also contribute to the flavour of the vegetal product. Examples of these compounds include caffeic acid, gallic acid (**9**), and chlorogenic acid (**10**).



9
Gallic acid

10
Chlorogenic acid

Figure 5: Chemical structures of gallic acid and chlorogenic acid[18].

The ability of some phenolic compounds to act as antioxidant has been demonstrated in literature[15]. Flavonoids can react readily with radicals thus forming flavonoid radicals that are stabilized by electron delocalization within the molecule[18]. The radical scavenging properties of flavonoids are strictly related to their chemical structure[18]. Briefly, Shi et al.[18] reported three main criteria that facilitate the donation of a hydrogen atom by flavonoids and thus their protective activity against free radicals. These are the *o*-dihydroxy structure in the B ring, the 2, 3-double bond in conjugation with a 4-oxo-function in C ring and finally the additional presence of the 3- and 5-OH groups in rings A and C[18]. The antioxidant activity of individual flavonoids depends also on the number and arrangements of the hydroxyl groups on the flavonoid structure, and depends on the type and extent of alkylation and glycosylation of the hydroxyl groups, too[18]. It is reported in literature an increase in the antioxidant activity with the increasing number of hydroxyl groups and a decrease antioxidant activity with glycosylation[18].

Flavonoids also showed metal-chelating abilities that are important to prevent the formation of free radicals, catalysed by metallic ions[18].

There is an increasing evidence that in addition to traditional dietary antioxidants such as vitamins, also other plant-derived compounds, such as flavonoids, have important antioxidant function in the human body[18]. Therefore, phenolic compounds may contribute to the delay or reduction of the damages caused by free radicals and oxidative stress.

In summary, flavonoids are a class of natural compounds extensively present in foods of vegetal origin showing a good potential in terms of usefulness for human health, as antioxidant molecules but also

because of some ancillary yet pharmacologically interesting properties. The action mechanism of polyphenols is pleiotropic, however, it mostly relates to their antioxidant activity. Natural polyphenols decrease the level of reactive oxygen species protecting biomolecules against oxidative damage[19]. Nonetheless, they need to be managed carefully, and their supplementation into the diet (as diet enrichment or as nutraceuticals) have to take in account also some potential drawback concerning human health and wellness[20]. Under conditions like high concentrations, high pH, and the presence of redox-active metals, phenolic compounds can acquire a pro-oxidant behaviour, mainly based on the generation of a radical species or a labile complex with a metal cation exerting redox activity[20]. However, maximum levels of polyphenols in dietary supplements are indicated by law.

1.2.2.2. Tocopherols: Tocopherols together with tocotrienols constitute a set of compounds referring to Vitamin E, as each of them exhibits the biological activity of α-tocopherol[18].

From a chemical point of view, they consist of a chromanol ring and a hydrophobic side chain linked to the ring at carbon 2. The tocopherols family includes α-tocopherol (**11**), β-tocopherol (**12**), γ-tocopherol (**13**), δ-tocopherol (**14**) that differ from each other by the number and position of methyl groups on the phenolic part of the chromanol ring[18]. Tocopherols have phytyl side chains with three chiral centers. They differ from tocotrienols in the structure of the side chain as tocotrienols have an isoprenyl side chain with only one chiral center and three double bonds[18].

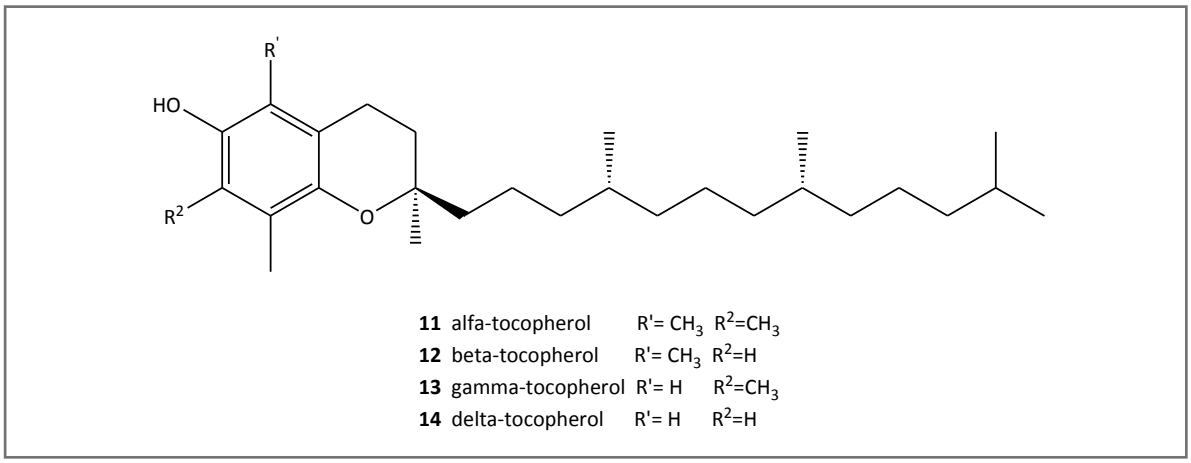
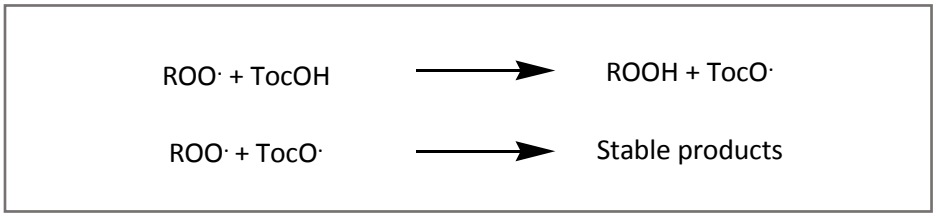


Figure 6: General structure of tocopherols.

Tocopherols and tocotrienols are present in all photosynthetic organisms and they are always located in the membranes of the cells. The most important sources of vitamin E in the diet are the plant seed oils (50-200 mg α-tocopherol equivalents per 100 g). Most other plant tissues contain less than 0.5 mg per 100 g[21]. In general, total amount of tocopherols in plants are greater in mature leaves and other light-exposed tissues and are smaller in roots and other tissues grown under less intense light[18]. Their concentration in plant tissues depends therefore on the intensity of sunlight, as well as soil state and other growing conditions[18].

The role of vitamin E as a whole is to prevent a range of oxidation reactions of polyunsaturated lipids in vivo and to function as an antioxidant.

The main reaction responsible for tocopherol antioxidant activity in foods and biological systems is HAT, with the formation of a tocopheroxyl radical. Tocopherols together with tocotrienols scavenge free radicals and thus delay the oxidation of lipids. As showed in Scheme 4, first a tocopherol or tocotrienols (TocOH) molecule reacts with a peroxyl radical ($ROO^{\bullet}$) to form a tocopheroxyl o tocotrienoxyl radical ($TocO^{\bullet}$) and a lipid hydroperoxide (ROOH), which is relatively stable product[18]. The $TocO^{\bullet}$ then scavenges another $ROO^{\bullet}$ by radical-radical coupling thus forming a variety of stable secondary oxidation products[18]. The slowness of reaction between α-$TocO^{\bullet}$ and oxygen is one of the main reason why α-tocopherol seems to have been selected as nature's major lipid-soluble chain-breaking antioxidant. In biological systems, α-tocopherol works together with ascorbic acid and GSH in an antioxidant network, effective in counteracting lipid oxidation[18].



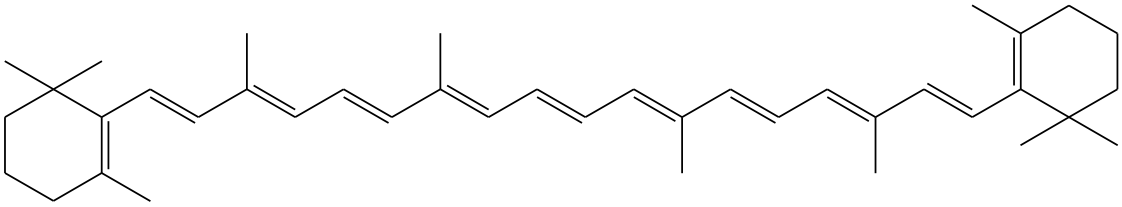
Scheme 4: Radical scavenging activity of tocopherols[18].

All tocopherols and tocotrienols are bioactive in vivo, but α-tocopherol is the most bioactive of all and it has also the highest bioavailability[18]. Tocotrienols have lower activity than the respective tocopherols.

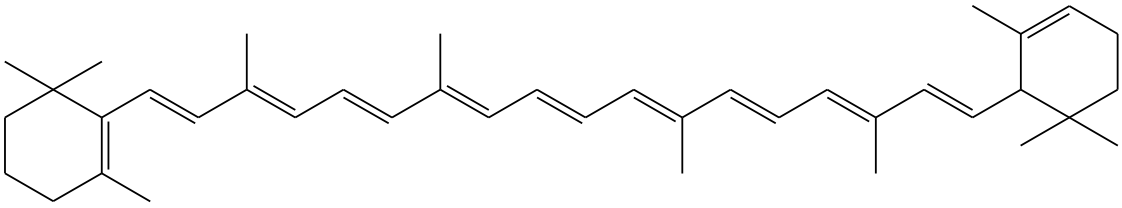
It is reported in literature[20]that α-and γ-tocopherol forms of vitamin E exert a differential set of biological effects, which cannot be always regarded as positive to human health; this is something that needs to be taken in account when considering to enrich the content of vitamin E into a diet with

antioxidant purposes. For example, γ-tocopherol administered orally showed a potent pro-inflammatory function during allergic inflammation[20]. Even more, a research found a correlation between the prevalence of asthma and the average plasma tocopherol in several countries, based on nutritional consumption of foods and oils rich in tocopherol. Briefly, countries with an average plasma γ-tocopherol concentration of 2–7 µmol/L had the highest asthma prevalence compared to those with a concentration of 1-2 µmol/L, independently from α-tocopherol plasma levels[20]. Finally, in mice prone to allergic disease, supplementing allergic mothers (at the time of mating) with α-tocopherol was enough to inhibit the pup allergic responses[22], while γ-tocopherol supplementation amplified pup responses to allergens[20].

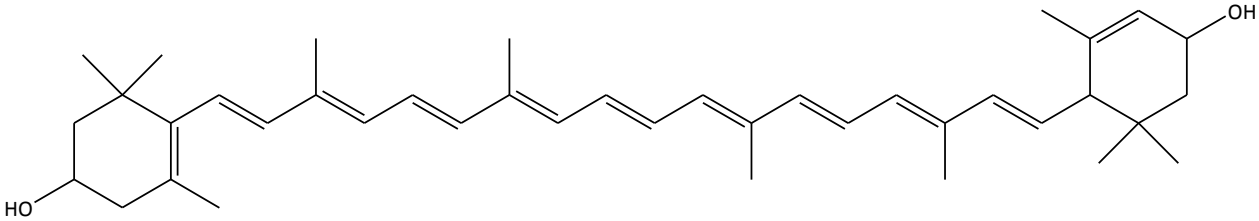
1.2.2.3. Carotenoids: Carotenoids are a wide family of natural pigments, produced by photosynthetic plants and bacteria[23]. Hundreds of different structures have been described in literature, of which almost 50 became constituents of products used by humans[23]. The most important are β-carotene (**15**), α-carotene (**16**), lutein (**17**), zeaxanthin (**18**), lycopene (**19**), just to name a few.



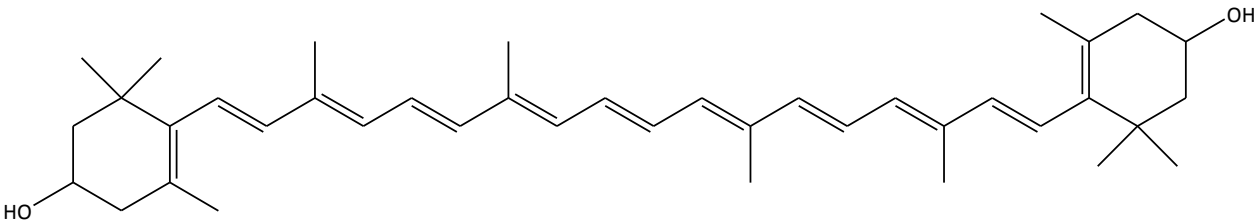
15
beta carotene



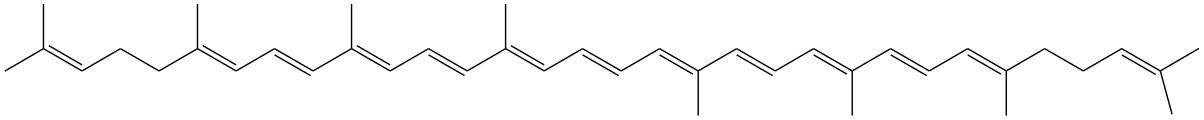
16
alfa carotene



17
lutein



18
zeaxanthin



19
lycopene

Figure 7: Chemical structures of carotenoids

From a chemical point of view, most carotenoids exhibit a characteristic, symmetrical tetraterpene skeleton formed by the tail-to-tail linkage of the two C_{20} moieties. The linear C_{40} hydrocarbon backbone is susceptible to diverse structural modifications (e.g. hydrogenation level, cis-trans isomerization, cyclization at one or both ends or the addition of side groups with their subsequent glycosylation/acetylation)[23].

The typical feature of carotenoids is their brilliant coloration, which is a consequence of light absorption deriving from the presence of an extensive system of conjugated double bonds. The ability to absorb light is in accordance with the role that such a class of compounds have in plants and some bacteria (photosynthesis) and in all-living organisms (photo-protection)[23].

Carotenoids are known to be very efficient physical and chemical quenchers of singlet oxygen ($^1O_2$) as well as potent scavengers of ROS. This is of special significance, because the uncontrolled generation and concomitant increase of ROS level in the body results in “oxidative stress”, an essential contributor to the pathogenic processes of many diseases, as previously described.

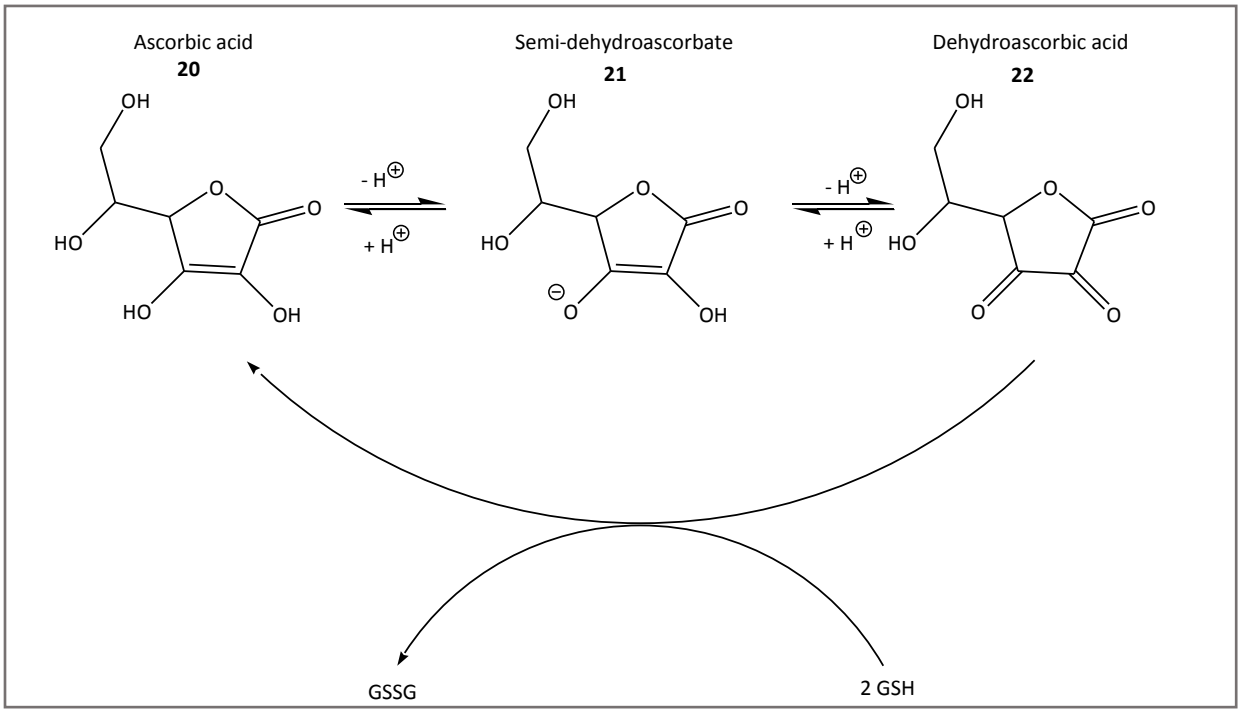
Carotenoids are highly non polar compounds that are localized inside cell membranes. In particular, strict hydrocarbons, like β-carotene and lycopene, are placed in the inner part of cell membrane, while those carotenoids, that contain also oxygen atoms in their molecule, are oriented perpendicular to the membrane surface and they expose their hydrophilic moieties toward the aqueous compartments[23].

The presence of carotenoids in the cellular membranes can modify the properties of the membranes and confer resistance toward the attack of ROS.

The β-carotene (**15**), the lycopene (**18**) and lutein (**17**) each have a protection at low concentrations when incorporated into the membranes. However, with the increase in carotenoid concentrations, pro-oxidative effects have been noted[24].

1.2.2.4. Ascorbic acid: Ascorbic acid (**20**) is a natural hydro-soluble antioxidant. The ascorbate reacts with ROS, eliminating them and promoting the conversion into semi-dehydroascorbate radical (**21**), which is a less reactive species that converts subsequently to dehydroascorbate (**22**) (Scheme 5).

Even more, ascorbic acid protects tocopherols, especially α-tocopherol from oxidation, by restoring the reduced form of the molecule, which oxidizes because of the reaction with free radicals[20].



Scheme 5: Vitamin C in the three different forms in which occurs in the living organisms, from the reduced form to the completely oxidized one. Regeneration takes place thanks to glutathione (GSH) which provides the two electrons needed to convert dehydroascorbate back to ascorbic acid.

1.2.2.5. Glucosinolates and Isothiocyanates: The main class of compounds believed to be able to support the antioxidant defense through an indirect mechanism of action is represented by glucosinolates (**23**) and isothiocyanates (**24**)[2].

Glucosinolates are widespread plant constituents, and it is believed that glucosinolate breakdown products with an isothiocyanate group (such as the isothiocyanate sulforaphane from Broccoli) induce phase II enzymes and are therefore responsible for the protective effects shown by Brassica vegetables. The latter are known to be a rich source of glucosinolates. Glucosinolates can be converted into isothiocyanates by an S-glycosidase called myrosinase that is present inside vegetable cells apart from glucosinolates. Nevertheless, a damage or a rupture of the tissue allows myrosinase to get in contact with glucosinolates, thus producing the bioactive product.

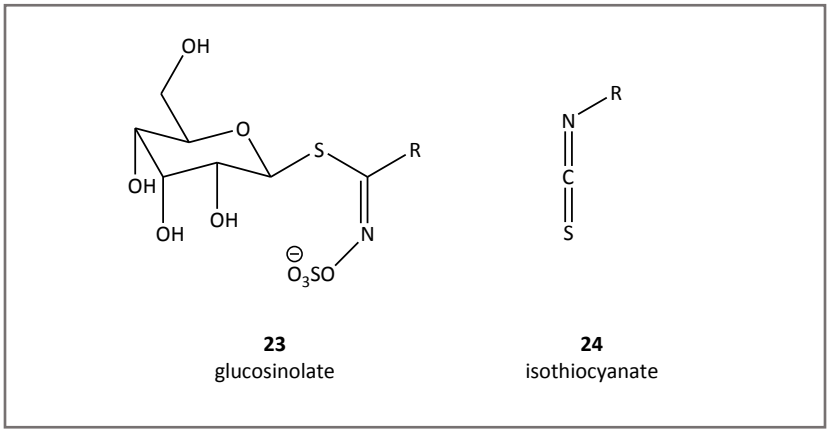


Figure 8: General structures of a glucosinolate and an isothiocyanate molecule. R indicates a variable group, synthesized from different amino acids, so it can be either aliphatic or aromatic.

The evidence for phase II enzyme inductions at ordinary intake levels of plant foods in humans is, however, limited, and the importance of this defense mechanism in the overall protection against oxidative damage is still not completely known[2].

Several studies have shown that after consuming cooked cruciferous vegetables with no myrosinase activity, isothiocyanates can be detected in urine but only between 10% and 30% of that which would have occurred with functional plant myrosinase, probably due to the conversion of glucosinolates to isothiocyanates by the colonic microflora[25].

Blanching cruciferous vegetables prior to freezing also denatures myrosinase, so that intact glucosinolates are consumed and any breakdown products entering the systemic circulation is dependent upon bacterial myrosinases in the colon. There is no evidence that intact glucosinolates are absorbed[25].

Increased phase II enzyme activity has also been documented *in vivo*, in animal models but more importantly in humans. When healthy male volunteers consumed 300 g of cooked Brussels sprouts per day there was a significant increase in the plasma levels of phase II enzymes compared to volunteers that consumed a glucosinolate-free diet, according to a study reported in literature[25].

More recently, in skin biopsies of healthy subjects, a specific phase II enzyme (NQO1) increased by 1.5-fold after a single topical application of broccoli sprout extract and 4.5-fold after three applications compared to skin biopsies that received placebo controls[25]. These studies highlighted the ability of isothiocyanates to induce antioxidant enzymes both after oral and topical applications in humans.

Despite clear evidence that isothiocyanates stimulate cellular antioxidant proteins that protect against oxidative stress and carcinogens there is some evidence that isothiocyanates induce themselves cellular stress. Following passive diffusion of isothiocyanates into the cell, conjugation with intracellular GSH occurs immediately, leading to a rapid depletion of glutathione levels in the cells as early as 30 minutes following exposure. This initial depletion of GSH is likely to be perceived by the cell as critical stress, which is dealt with by increasing the production of GSH[25].

As reported in literature, the pro-oxidative activity of isothiocyanates depends on their concentration, as it is effective only at high dosages of a specific isothiocyanate. For this reason, it is recommended the use of isothiocyanates inside plants or inside plant extracts, so that their concentration will be lower and the presence of other phytoconstituents with antioxidant activity can integrate their activity.

1.2.3. Final remarks

In conclusion, present days lifestyles that include consistent exposure to sunlight, radiation, cigarette smoke as well as pollutants in air and the bad eating habits dramatically increase the number of free radicals in our body and predispose people to oxidative stress. Therefore, the demand for effective inhibitors of oxidative processes has become essential[5].

Some clinical researches showed that the antioxidant supplementation attenuates the endogenous antioxidant depletion thus alleviating the associated oxidative damage[26].

The enzymatic endogenous antioxidants act primarily at an intracellular level. Therefore, extracellular free radicals, either endogenously produced or from the environment, need to be inactivated by the circulating antioxidants such as the one introduced in the body with the diet or supplementation[3].
The level of dietary intake of all the antioxidant micronutrients directly affects the circulating level of these nutrients. Low intakes of one or more of these antioxidant nutrients could reduce the body's defense against free radical damage and increase susceptibility to health problems associated with free radical damage[3]. On the other hand, it is important not to exceed with the dosages, as some phytoantioxidants showed pro-oxidant activity at high concentration.

1.3. Phytoantioxidants for cosmetic applications

Skin is the largest organ of the body that functions as the necessary interface between the internal and the external environment. It continuously protects the body from noxious stimuli like microorganisms, ultraviolet (UV) irradiation, allergens, and irritants. Its unique role and function is a direct result of its structure and in particular of the most superficial part, the epidermis[27]. Under the epidermis there is the dermis, which is a connective tissue containing collagen and elastin fibers. Finally, under the dermis there is the hypodermis, an adipose tissue.

The skin has a high metabolic activity and therefore a high risk of undergoing oxidative stress, because of the production of ROS inside its cells. Moreover, it is also interested by the production of free radicals induced by UV rays because of solar exposition. UV rays damages are responsible for photoaging[28]. UV radiation is part of the electromagnetic spectrum with wavelengths that range from 200 nm to 400 nm. It is divided into three main categories: long wave UVA (320-400nm); medium wave UVB (280-320nm) and shortwave UVC (200-280 nm)[29]. Since UVC are completely filtered from the atmosphere, they are not dangerous for the skin as are instead UVA and UVB. UVA rays penetrates deeply into the epidermal junction and the dermis to damage collagen and elastic tissue. The UVA directly accelerates the appearance of extrinsic aging signs by overregulating the expression of matrix metalloproteases that degrade collagen and elastin, thus causing wrinkles and skin laxity[29]. Moreover, UVA rays can indirectly produce structural damage to the DNA, generate free radicals, damage the immune defense and lead to cancer like malignant melanoma[30]. On the other hand, UVB causes direct damage to DNA[28].

Photoaging by chronic exposure to UV radiation is characterized by dyspigmentation (mostly lentigo and freckling), solar elastosis (that is due to collagen degradation by ROS and its accumulation in the dermis), actinic keratosis, and seborrheic keratosis[27].

It is reported in literature that the exposure to solar rays causes serious losses in antioxidant compounds such as tocopherols, carotenoids and polyphenols that constitute the antioxidant defence system of the skin together with enzymatic endogenous antioxidants[28].

Therefore, it seems evident that a balanced diet rich in antioxidant agents can act favorably to combat, both generally and locally, degenerative processes related to both aging and solar radiation. This balanced diet should be followed for the entire life span, preferably[28]. In the particular case of aging of the skin and free radical damages to the skin, it is reported in literature that topical application of antioxidants is characterized by a higher bioavailability of these compounds, giving a better localized effect on skin[27]. Another study showed that the topical application of α-tocopherol clearly reduces the damages of skin, and a positive effect was observed even in the case of an oral intake of this antioxidant on the aspect of skin, even if to a lower degree[28]. Carotenoids are reported to be protective skin factors, too[28].

Concerning the action of the phenolic compounds on the skin, it is reported in literature their ability to inhibit the activity of proteases that catalyse the degradation of collagen and elastin. Furthermore, the pre-treatment of keratinocytes with polyphenols or polyphenol-rich extracts leads to a reduction of the intracellular ROS formation induced by UVA or hydrogen peroxide. Not only the pre-treatment but also the post treatment of human epidermal keratinocytes is effective in suppressing the overproduction of peroxides and mediators of inflammation. It seems that polyphenolic extracts can be considered useful for both sunscreens and products to be applied after exposition to sun[31].

A review reported in literature stated that polyphenol enriched extracts can be effective for the prevention and therapy of premature skin aging, provoked by oxidative stress. This review comprises the beneficial properties of polyphenols, which are mostly relevant by the topical application, because of their antioxidant activity, protective action against UV damages, inhibition of dermal proteinases anti-microbial activity and anti-carcinogen action, which were determined *in vitro* using cell line assays[31].

Some controlled clinical studies demonstrated the photoprotective or anti-aging effects of topically applied or orally supplemented polyphenols, in natural extracts[31].

In conclusion, an optimal supply of antioxidant micronutrients in the skin increases basal dermal defense against UV rays, supports longer-term protection, and contributes to maintenance of skin health and appearance[31].

1.4. *Hibiscus sabdariffa* L.

Hibiscus Sabdariffa L. (syn. *Hibiscus cruentus* Bertol. and *Sabdariffa rubra* Kostel[32], just to name a few) is a plant native to the regions of western Sudan. Subsequently, it spread to other areas of the world over the years and nowadays it is widely grown in the tropical and subtropical areas of both hemispheres like Sudan, China, Thailand, Egypt, India, and Mexico[33].

Figure 9: Plantation of *H. sabdariffa* L., in Paraguay. (Courtesy of EDYNEA S.R.L.)

1.4.1. Taxonomic classification and botanical description

There are numerous common names with which this plant is known: in the English-speaking countries it is called "Roselle" or "Jamaican sorrel"; "Rohzelu" in Japanese. From the Eritrean dialects comes the name “karkadeh”, which means "health drink". The term “karkadeh” is also used in Switzerland and in North Africa[33].

Kingdom	Viridiplantae
Sub Kingdom	Tracheophyta
Super Division	Spermatophyta
Division	Magnoliophyta
Class	Eudicotyledons
Subclass	Rosids
Order	Malvales
Family	Malvaceae
Genus	Hibiscus

Table 1: Taxonomic classification of *H. sabdariffa* L., according to the NCBI's taxonomy database

There are two main varieties of *H. sabdariffa*: var. *altissima* that is cultivated mainly for its jute-like fiber and var. *sabdariffa*. The latter includes shorter bushy forms classified into four races (*bhagalpuriensi*, *intermedius*, *albus*, *and ruber*) that differ from each other for the colour of their calyces[32].

H. sabdariffa var. *sabdariffa ruber* has red calyces and it is an annual, erect, bushy, herbaceous subshrub that can grow up to 2.4 m tall, with smooth, cylindrical, typically red stems[32]. The leaves are green with reddish veins and long or short petioles. The margins of the leaves are toothed[32]. Flowers, borne singly in the leaf axils are initially yellow but turn pink as they wither at the end of the day[32]. At this time, the typically red calyx, consisting of 5 large sepals with an epicalyx, begins to enlarge and becomes fleshy, crisp and juicy[32]. At this point, the calyx fully encloses the capsule that is green when immature, turning brown when mature. Inside the capsule are found kidney-shaped seeds[32].

Figure 10: Fleshy fresh calyces of *H. sabdariffa*, var. *sabdariffa ruber*. (Courtesy of EDYNEA S.R.L.)

Figure 11: Flower of *H. sabdariffa*, var. *sabdariffa ruber*. (Courtesy of EDYNEA S.R.L.)

1.4.2. Phytochemistry

The main classes of phytochemicals responsible for the nutraceutical, cosmetic and pharmacological properties of *H. sabdariffa* are anthocyanins, flavonoids, organic acids and polysaccharides.

1.4.2.1. Anthocyanins

The anthocyanins are a group of flavonoids derivatives and natural pigments that are highly concentrated in the calyces of *H. sabdariffa*, but that were identified in the leaves of the plant, too[32]. Anthocyanins (from the Greek "antos" flower, and "kyanos" blue), are water-soluble pigments present especially in the epidermal cells of the plant, at the level of the vacuole. These substances are classified according to the number and position of the hydroxyl and methoxyl groups present on the flavylium cation that is the core of their structure.

As well as other polyphenolic substances, anthocyanins occur naturally in the form of glycosides, in which the aglycone is called anthocyanidin. There are six different anthocyanidins in nature: pelargonidine, cyanidine, delphinidin, peonidine, petunidin and malvidin[18], but the variety increases considerably based on the types of glycosylation[21].

In the calices of *H. sabdariffa* the most expressed forms are cyanidin (**25**) e delphinidin (**26**)[34]. The most common sugars present in these glycosides are glucose, galactose, xylose, rhamnose and arabinose; these sugars also occur in the form of disaccharides linked to the central structure and are named rutinoside (glucose + rhamnose), sambubioside (glucose + xylose), etc. More often, glycosylations occur at position 3 in the case of monoglycosides, in positions 3 and 5 in the case of diglucosides[18].

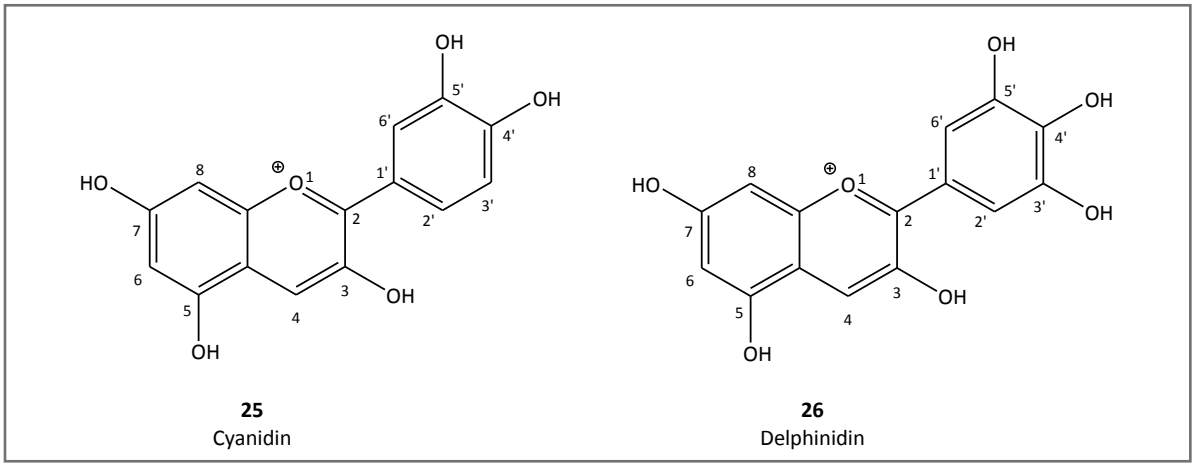
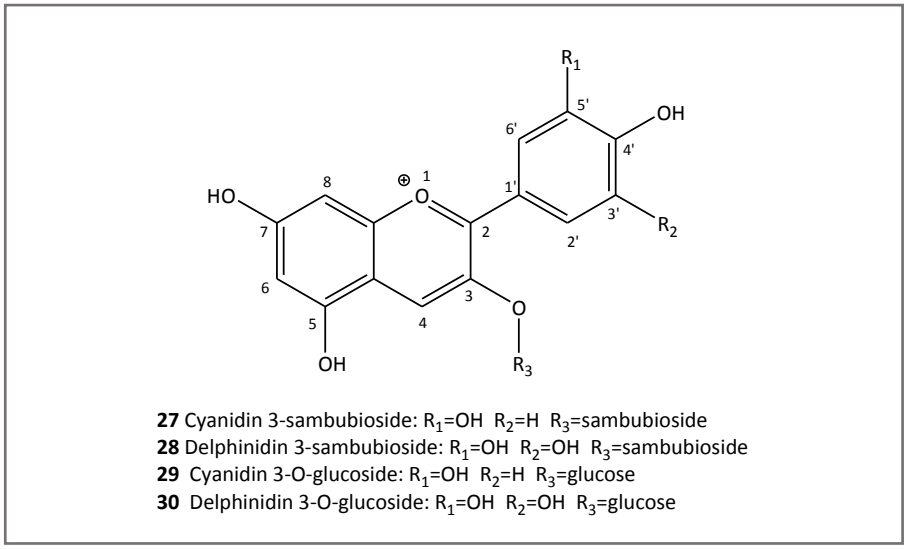


Figure 12: Main anthocyanidins of *H. sabdariffa*.

Glycosylation gives a higher stability to anthocyanins, thus they are less sensitive to variations of pH and less susceptible to degradation by ascorbic acid and light. Even more, there is also an increase in water solubility with respect to anthocyanidins.

Often the glycosidic portion of the anthocyanins is acylated in one or more positions with derivatives of cinnamic acid, but also with aliphatic acids (succinic, malonic, etc.): this also improves stability as well as increase its variability[18]. Anthocyanins are characterized by a strong absorption of visible light; in fact they show a maximum absorption between 465 and 550 nm, according to data reported in literature[35].



27 Cyanidin 3-sambubioside: R_1=OH R_2=H R_3=sambubioside
28 Delphinidin 3-sambubioside: R_1=OH R_2=OH R_3=sambubioside
29 Cyanidin 3-O-glucoside: R_1=OH R_2=H R_3=glucose
30 Delphinidin 3-O-glucoside: R_1=OH R_2=OH R_3=glucose

Figure 13: Major (cyanidin 3-sambubioside and delphinidin 3-sambubioside) and minor (cyanidin 3-O-glucoside and delphinidin 3-O-glucoside) anthocyanins of *H. sabdariffa* [32].

1.4.2.1.1. Stability of anthocyanins

The stability of anthocyanins is a very important factor, especially if these are constituents of supplements, nutraceuticals and in general of products for humans. So much information on the stability of such substances turns out to be useful in the production of products intended for humans[18].

Effects of pH:

The pH value of an aqueous solution of anthocyanins has an effect both on the colour of those molecules (that depends on the structure that the molecule assume at that specific pH) and on the stability of the molecules[18].

In fact, it has been studied that in an acidic or neutral environment, four different structural forms of anthocyanins exist in equilibrium (see Figure 14): the flavylium cation (**31**), (red), the quinoidal base (**33**), (blue), the pseudo-base carbinol (**32**), (colourless) and the structure of the chalcone (**34**)[36].

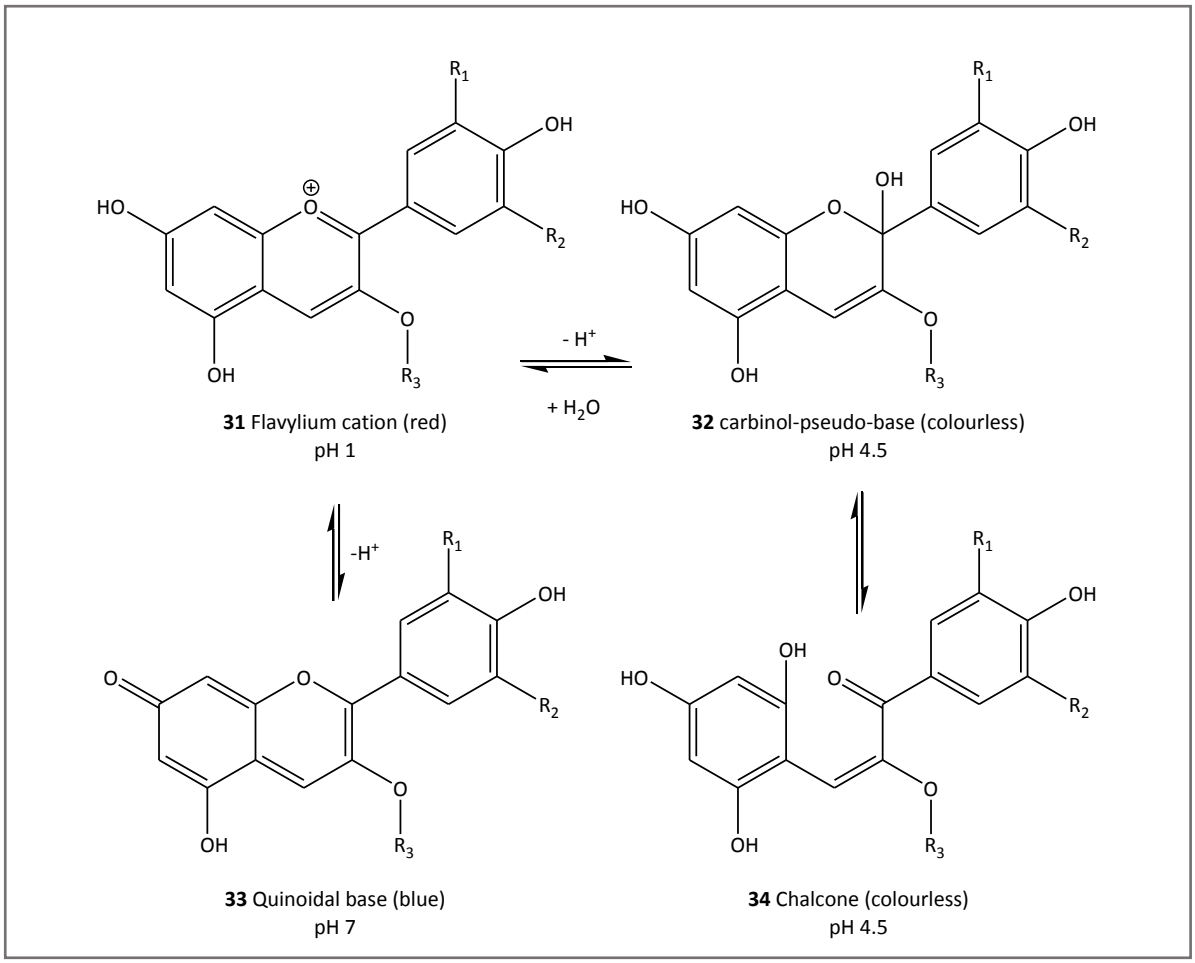
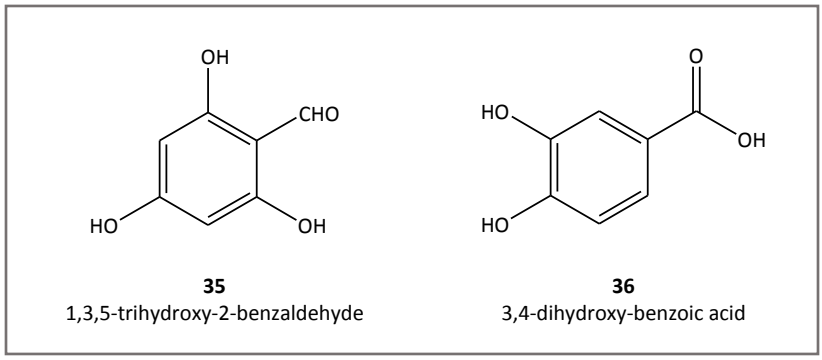


Figure 14: Changes in anthocyanins structures based on the pH of the medium[36].

Effects of temperature:

There is a logarithmic relationship between the temperature and the degradation of anthocyanins[35]. It was suggested a mechanism that at high temperatures favours the transition of cation flavylium to chalcone structure. The opening of the ring subsequently leads to the loss of sugar moieties of the chalcone and the formation of degradation compounds, which causes browning of products. It is thought that in the case of cyanidin the degradation products are 1,3,5-trihydroxy-2-benzaldehyde (**35**) and 3,4-dihydroxy-benzoic acid (**36**)[37].



35
1,3,5-trihydroxy-2-benzaldehyde

36
3,4-dihydroxy-benzoic acid

Figure 15: Anthocyanins degradation products.

Effects of light:

UV-VIS light is another factor that damages anthocyanins and favours their degradation; this effect seems to be dependent on the wavelength of the radiation: the short ones are the most harmful. The exclusion of UV radiation could therefore improve the stability of anthocyanin extracts. Glycosylation and acylation improve the stability of these substances on exposure to light[18].

Effects of water and sugars:

Several studies have showed that the stability of anthocyanins increases by decreasing the water content of an extract, as it decreases its activity (this is the quantity of water free from bonds with other components and therefore available for chemical). The dry powders of anthocyanins are stable for several years if stored in containers hermetically sealed. It was also studied that a high sugar content results to be protective for these substances, but this effect seems to be due to the resulting reduction of water activity: a low amount of sugars and their degradation compounds accelerate the deterioration of these pigments, especially in the presence of oxygen[18].

Effects of oxygen, hydrogen peroxide and ascorbic acid:

Oxygen has deleterious effects on anthocyanins; it is well known that these substances, if stored under vacuum or in a nitrogen atmosphere, are more stable than oxygen exposure[18]. Oxygen is able to alter the anthocyanins either by acting directly or indirectly through the oxidation of compounds that in turn favour the decomposition of these substances. Among the secondary oxidation compounds, we find metal ions and ascorbic acid. For the latter two mechanisms of action have been proposed. The first involves the oxidation of ascorbic acid by the oxygen with the generation of hydrogen peroxide, which would oxidize the anthocyanins[18]. The second mechanism provides instead the direct reaction of ascorbic acid on these substances in the absence of oxygen: the acid would condense with the anthocyanins and form unstable products that would determine colourless compounds. The condensation of the anthocyanins with the flavonols, however, prevents the formation of this complex and therefore promotes their stability[18].

In addition, other factors like the activity of some glycosidases may affect the stability of anthocyanins, but generally enzymes that degrade anthocyanins have an optimum pH greater than 4[38], that is not the case of an aqueous extract of *H. sabdariffa* calyces.

1.4.2.2. Flavonoids

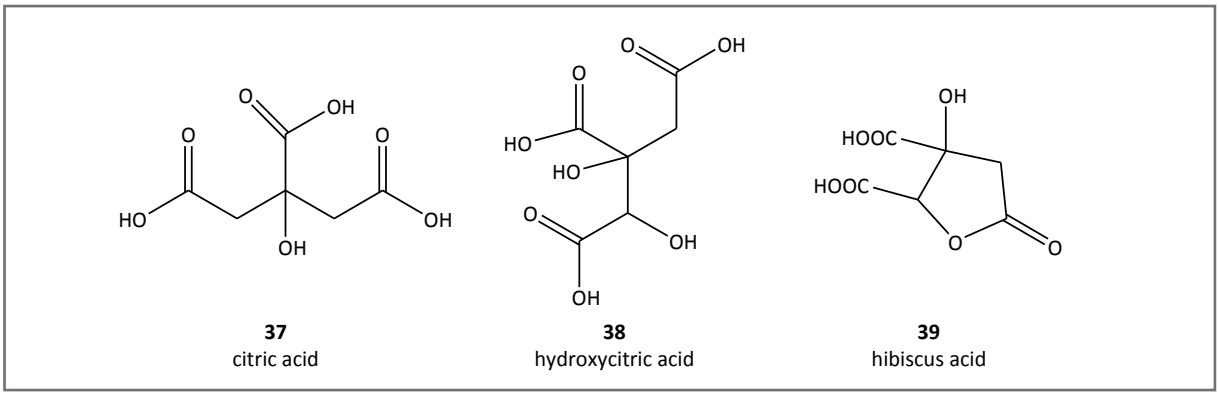
Studies reported in literature highlighted the presence of quercetin, rutin and kaempferol in *H. sabdariffa* calyces, while catechin and ellagic acid were reported to be present in the leaves[18]. Concerning phenolic acids, caffeic acid and chlorogenic were identified in both the leaves and calyces of *H. sabdariffa*[32].

Figure 16: Leaves of *H. sabdariffa*, var. *sabdariffa ruber*. (Courtesy of EDYNEA S.R.L.)

1.4.2.3. Organic acids

H. sabdariffa extracts contain a high percentage of organic acids, including citric (**37**), hydroxycitric (**38**), hibiscus acid (**39**), malic, tartaric as major compounds and oxalic and ascorbic acids as minor compounds[32].

Hydroxycitric acid (**38**), hibiscus acid (**39**) and its derivatives were identified as the major organic acids in the leaves and calyces of *H. sabdariffa*[32].



37
citric acid

38
hydroxycitric acid

39
hibiscus acid

Figure 17: Main organic acids in *H. sabdariffa* calyces and leaves[32].

1.4.2.4. Polysaccharides

Polysaccharides are present in large quantities in *H. sabdariffa* calyces. They are mainly constituted of sugars like arabinose, galactose, glucose, rhamnose[32]. The content in mucilage depends on the variety of the calyces and reaches 24-28% in the varieties from Central America and Egypt, but only 15% in the varieties from India[32]. The content in pectin, on the other hand, results to be quite low, 2-4%, while the free sugars reach maximum values of 3-5%[32].

1.4.3. Antioxidant activity

Several studies about the antioxidant activity of *H. sabdariffa* extracts have been reported in literature[32]. The antioxidant activity observed in both *in vitro* and *in vivo* studies is due to different mechanisms like strong scavenging effect on ROS and free radicals or to the inhibition of xanthine oxidase activity or the protective action against the *tert*-butyl hydroperoxide-induced oxidative damage or to the inhibition of the formation of the malondialdehyde (100-300 mg/Kg). The effects were observed for both aqueous and ethanolic extracts of different tissues of *H. sabdariffa* (calyces, seeds, leaves)[32].

For example, the group of Tseng at the Chung Shan Institute of Biochemistry in Taiwan conducted a study on oxidative stress in a mouse hepatocyte and identified in the anthocyanins of *H. sabdariffa* the fraction with a greater antioxidant effect[39]. The results showed that the anthocyanins of *H. sabdariffa at* concentrations of 100 and 200mg / kg taken orally for 5 days before a *tert*-butyl hydroperoxide dose of 0.2mmol / kg drastically reduce the serum level of liver enzymes (ALT and AST)[39]; these extracts therefore also have a hepatoprotective activity due to their strong antioxidant activity.

It is also reported in literature one clinical study that was conducted with eight healthy volunteers that allowed to observe that one single dose (0.05g/ml) of an aqueous extract of *H. sabdariffa* calyces increased the systemic antioxidant potential in plasma and urines. In fact, following the administration of the extract, it was observed an increase in the hippuric acid excretion (that is a product of the metabolism of anthocyanins and flavonoids) and a decrease in malondialdehyde in urines (that is a biomarker for oxidative stress)[32,40], compared to volunteers that drank water in the place of the extract.

According to the review by Da Costa-Rocha et al.[32], anthocyanins are key classes of compounds linked to the antioxidant activity that is also the basis for other activities reported for the extracts of *H. sabdariffa* (e.g. hepatoprotective and nephroprotective).

The preparations of *H. sabdariffa*, especially aqueous extracts, have a long-standing traditional use both in food and in medicine, and in general are considered to be safe[32]. However, for the future, are recommended more well designed clinical trials, based on the use of phytochemically characterized extracts.

1.5. *Moringa oleifera* Lam.

Moringa oleifera Lam. (syn. *M. pterygosperma* Gaertn.[41]) is an evergreen tree native to the Sub Himalayan tracts of India[42]. It grows at elevation from sea level to 1400 m. It is also cultivated in north-eastern Pakistan, north-eastern Bangladesh, Sri Lanka, West Asia, the Arabian Peninsula, East and West Africa, throughout the West Indies, in Central and South America from Mexico to Perù, as well as in Brazil and Paraguay[42]. *M. oleifera* is also cultivated in southern and southwestern China, with a cultivation area of 3000 ha in the year 2014[43].

Figure 18: *M. oleifera* tree in Paraguay. (Courtesy of EDYNEA S.R.L.)

1.5.1. Taxonomic classification and botanical description

The plant is known with different synonyms: Latin name is "*Moringa oleifera*"; in Hindi it is called "*Saguna*" or "*Sainjna*"; in Spanish it is called "*Àngela*", "*Ben*" or "*Moringa*" and in Portuguese it is called "*Moringueiro*". The English name is "*Drumstick tree*", "*Horseradish tree*" or "*Ben tree*"[42].

Kingdom	Viridiplantae
Sub Kingdom	Tracheophyta
Super Division	Spermatophyta
Division	Magnoliophyta
Class	Eudicotyledons
Subclass	Rosids
Order	Brassicales
Family	Moringaceae
Genus	Moringa

Table 2: Taxonomic classification of *Moringa oleifera* Lam., according to the NCBI's taxonomy database

M. oleifera is a small, fast-growing, evergreen tree that usually grows up to 10 o 12m in height, with a soft and white wood and with corky and gummy bark. Leaves have long main axis and jointed branch. The leaflets are finely hairy, green and almost hairless on the upper surface, paler and hairless beneath, with entire (not thooted) margins and are rounded or blunt-pointed at the apex and short-pointed at the base[42]. The twigs are finely hairy and green. Flowers are white, scented, pods are pendulous, ribbed, and seeds are 3-angled[42].

Figure 19: *M. oleifera* plantation in Paraguay. (Courtesy of EDYNEA S.R.L.)

1.5.2. Phytochemistry

This plant has important nutritional and medicinal properties, as it is rich in nutrients and phytochemical compounds.

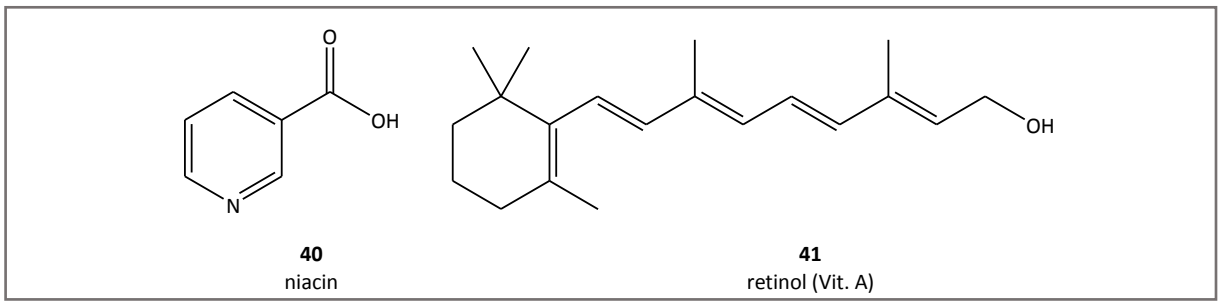
The main classes of phytochemicals are discussed below.

1.5.2.1. Macro and micro nutrients

M. oleifera contains more than 90 kinds of nutrients, with synergic effects and high bioavailability[44]

Leaves and flowers of *M. oleifera* have a good mineral profile that is expressed as ash content[45].

Fresh leaves of *M. oleifera* have a high content of vitamin A (**41**), carotenoids, vitamin C, vitamin E and some vitamins of the group B[46]. Vitamin A is involved in many physiological processes such as vision, reproduction, embryonic growth and development and brain function[46]. Its deficiency causes child and maternal mortality[46]. Carotenoids are mainly represented by β-carotene, with pro-vitamin A activity and lutein[46]. Vitamin C acts as antioxidant, protecting the body from free radicals, facilitating the conversion of cholesterol into bile acids and increasing the absorption of iron in the gut by reducing ferric to ferrous state[46]. Because of its sensitivity to heat and oxygen, vitamin C rapidly oxidizes, and this is the reason why dry leaves have a lower vitamin content compared to fresh leaves[46]. Thus, a good method for the preservation of vitamin C is freeze-drying rather than the traditional drying methods. Concerning tocopherols, all parts of the plant also contain high levels of γ-tocopherol[47]. Concerning the vitamins of the group B, literature reports studies about thiamine (B1), riboflavin (B2) and niacin (B3) (**40**) in *M. oleifera* leaves. These vitamins act as coenzymes of many enzymes that participate to the metabolism of nutrients and to the production of energy in the human body[46]. The review of Godinez-Oviedo *et al.*[44]indicates also the presence of vitamins B7 and B12 in fresh leaves and fruits of *M. oleifera*, and the content of B12 in the dried leaves[44]. More studies are required concerning other vitamins of the group B and, more in general, about vitamin D and K in the leaves of *M. oleifera*[46]. Further studies are necessary on the other tissues of the plant.



40
niacin

41
retinol (Vit. A)

Figure 20: chemical structures of some vitamins in *M. oleifera* leaves.

M. oleifera indeed is a good source of nutritional compounds and elements, even better than the majority of known and consumed vegetables. Nevertheless, Leone *et al.*[46] reported that it also has a high content of oxalates and phytates in the leaves. Even more, Ferreira *et al.*[47] indicated the presence of phytates in the seed kernels, too. It is widely known that oxalates and phytates are anti-nutritional compounds that are able to reduce the intestinal absorption of minerals, especially calcium and iron through the formation of insoluble salts or insoluble metal complexes. Thus, it is also important to consider the presence of these compounds when *M. oleifera* is considered as a supplement food of minerals[46].

Figure 21: *M. oleifera* leaves. (Courtesy of EDYNEA S.R.L.)

Figure 22: *M. oleifera* seeds. (Courtesy of EDYNEA S.R.L.)

1.5.2.2. Polyphenols

Polyphenols are the most investigated class of phytochemicals in *M. oleifera*[44].

As previously discussed, flavonoids have beneficial effects on human body, and it is documented that they can protect the body also from bacterial and viral infections and degenerative diseases like cancer or cardio-vascular and age-related diseases[46]. Phenolic acids also have documented healthy effects on human beings beyond the antioxidant activity, such as anti-inflammatory, antimutagenic and anticancer effects[46].

Polyphenols are distributed in different amounts in the tissues of *M. oleifera.* The concentration of these phytochemicals in a specific tissue depends on the environmental conditions in the various origin countries, the harvesting season, the genotype of the plant, the drying method, the maturity stage of the tissue and the extracting method used[46].

Characteristic	Total phenols (mg GAE/g extract)	Total flavonoids (mg QE/ g extract)
Ethanol extract of dried powder flowers	19.3	ND
Aqueous extract of dried powder fruit	72.8	8.8
Aqueous extract of dried powder leaf	105	31.3
Aqueous extract of fresh leaf	36.0-45.8	15.0-27.0
Aqueous extract of fresh leaf	120	40.5
Aqueous extract of freeze-dried powder leaf	53-74	33-108*
Dried powder leaf	2.5-53.5	2-25.1
Ethanol extract of freeze-dried powder leaf	81-100	59-101
Ethanol extract of dried powder leaf	23-132.3***	9-62**
Freeze-dried leaf	29-43	21-44.3*
Methanol extract of freeze-dried powder leaf	89-123	59-140*
Methanolic extract of dried leaf	83-135	69.3-125
Aqueous extract of dried powder seeds	45.8	9.9
Methanol extract of seed flour	41.73	2.3
Seed oil	40.2	18.2*

Table 3: total phenolic and flavonoids in *M. oleifera*[44]. GAE: gallic acid equivalent; QE: quercetin equivalent; *RE: rutin equivalent; **IQE: isoquercetin equivalent; ***CAE: chlorogenic acid equivalent; ND: not determined

According to data reported by Godinez-Oviedo *et al.*[44] and considering the study by Vyas *et al.*[16], the leaves of *M. oleifera* are the tissue with the highest concentration of polyphenols. A possible explanation of the high polyphenol content and, the high antioxidant activity of leaves or leaves extracts is that leaf has a longer life span compared to other parts of the plant. Moreover, it is the site of energy production. So, it may be exposed to a great oxidative damage[16].

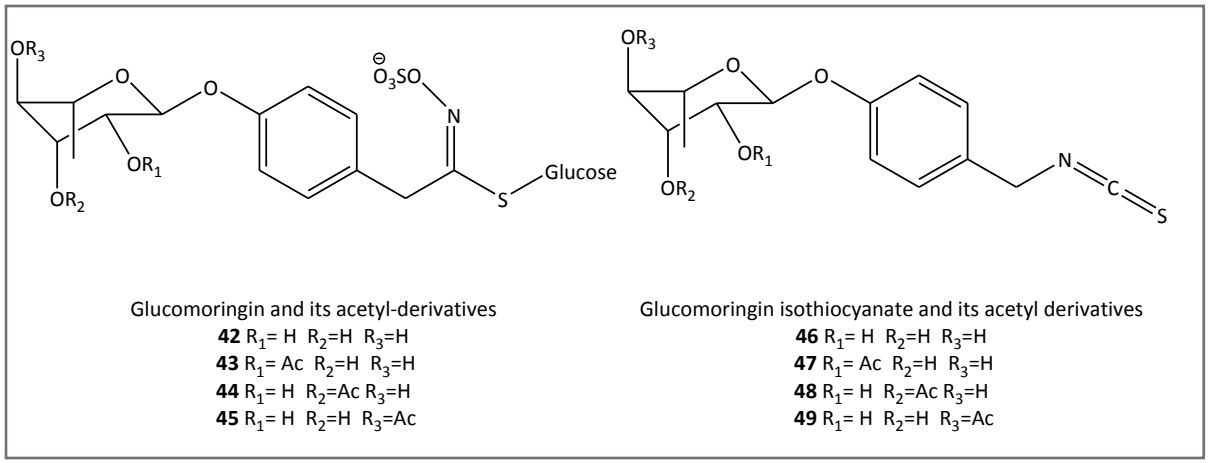
Being that leaves are the most used part of *M.oleifera* and the richest in total phenols, there are several studies available on their specific flavonoids and phenolic acids[47] that were identified with kaempferol (**2**), quercetin (**3**), myricetin (**4**) and gallic acid (**9**).

1.5.2.3. Glucosinolates and isothiocyanates

Glucosinolates are compounds typical of those plants that belong to the order Brassicales. These compounds have a backbone of glucose that is attached to a sulfonated aldoxime, with a variable side chain.

Inside plant cells, glucosinolates are stored separately from myrosinase, the thioglucosidase that catalyzes the hydrolysis of the glucose moiety, resulting in the formation of the isothiocyanates (ITCs). In intact plant tissues of *M. oleifera*, the concentration of ITCs is very low or undetectable, but upon mechanical rupture or damage of the fresh tissues, myrosinase gets in contact with glucosinolates thus producing the isothiocyanates. Even more, it has been reported that the intake of glucosinolates with diet can results in the conversion to ITCs, operated by the gut microbiota[12]. In contrast with the glucosinolates and isothiocyanates of other crucifers, the glucosinolates and the isothiocyanates of *M. oleifera* are solid at room temperature and stable analogues, due to the presence of a sugar moiety of rhamnose in the aglycone portion of the molecule[12].

The principal glucosinolate of *M. oleifera* is 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate (glucomoringin) (**42**). Glucomoringin is present in all parts of the plant in different concentrations[48]. Even more, in some parts of the plant, three acetyl isomers of glucomoringin **43, 44, 45** and benzyl glucosinolate were detected as well[48].



Glucomoringin and its acetyl-derivatives
42 R_1= H R_2=H R_3=H
43 R_1= Ac R_2=H R_3=H
44 R_1= H R_2=Ac R_3=H
45 R_1= H R_2=H R_3=Ac

Glucomoringin isothiocyanate and its acetyl derivatives
46 R_1= H R_2=H R_3=H
47 R_1= Ac R_2=H R_3=H
48 R_1= H R_2=Ac R_3=H
49 R_1= H R_2=H R_3=Ac

Figure 23: Chemical structures of glucomoringin (**42**) and its acetyl derivatives (**43**, **44**, **45**). Chemical structures of glucomoringin isothiocyanate (**46**) and its acetyl derivatives (**47**, **48**, **49**).

According to studies by Bennett et al.[48], seeds only contain 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate at high concentrations. Roots have high concentrations of both glucomoringin and benzyl glucosinolate. Leaves contain 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate and three acetyl isomers. Bark only contains 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate[48]. Table 4 reports the content of different glucosinolates in different tissues of *M. oleifera*.

	Seeds	***Old leaves***	***Young leaves***	***Stem***	***Whole root***
4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate (8)	202±15	33.9±0.9	59.4±1.4	16.3±0.09	20.4±0.8
4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate monoacetyl isomer I (9)	0	2.9±0.06	5.0±0.1	1.6±0.01	0
4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate monoacetyl isomer II (10)	0	1.2±0.02	1.5±0.08	0	0
4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate monoacetyl isomer III (11)	0	17.4±0.04	50.2±1.7	3.2±0.2	0
benzyl glucosinolate	0	0	0	0	22.7±0.7

Table 4: Glucosinolates content in different tissues of *M. oleifera*. Glucosinolate content is expressed as mean±σn-1 mg compound/g dry weight of tissue[48].

Seeds represent the tissue with the highest concentration of glucomoringin, and that green parts of the plant contain also the acetyl isomers of glucomoringin.

It is noteworthy that Amaglo et al.[49] reported the presence of sinalbin (the glucosinolates of the seeds of white mustard) in *M. oleifera* leaves, grown in Ghana, with a concentration ranging between ND and 2.36 mg/g DW.

The isothiocyanates of *M. oleifera* are new and interesting compounds because of their reported anti-oxidant[12], anti-inflammatory[50,51,52], antibacterial[53,54], anticancer[55,56,57]properties.

As the leaves of *M. oleifera* are the most used part of the plant[46], there are available many studies related to the mineral, vitamin, polyphenolic and other compounds derived from amino acids (i.e. glucosinolates and isothiocyanates) profile of this tissue. On the contrary, fewer researches have been performed on the other parts of the plant like branches. This is the reason why there are less available data and further research is recommended to identify new and hopefully better sources of those compounds.

1.5.3. Antioxidant properties

This plant is known for its nutritional properties, mainly attributed to the leaves, and also, for its wide range of claimed medicinal properties such as antimicrobial, antiasthmatic, hepatoprotective, cancer protective, spasmolytic, hypotensive, cholesterol lowering and hypoglycemic[16], but it is also known for its antioxidant properties[16].

The antioxidant properties of *M. oleifera* have long been attributed to the phenolic compounds present in the plant, especially in the leaves. Polyphenols, like flavonoids and phenolic acids directly eliminates free radicals in the body, by breaking the propagation chain reactions of free radicals, as it is widely known. Nevertheless, the indirect antioxidant activity of the isothiocyanates (ITCs) from *M. oleifera* has recently been demonstrated[12].

Both the ITCs generated from glucomoringin and the ITC generated from the isomer of glucomoringin acetylated in the position 4' of the rhamnose moiety proved to have an indirect antioxidant capacity comparable to that of sulphorafane. Sulphorafane is the characteristic ITC of Broccoli that is able to induce phase II metabolizing enzymes, thus contributing to the scavenging of free radicals, through an indirect mechanism of action[12].

1.5.4. *M. oleifera* and *Propionibacterium acnes*

In this thesis a brief investigation was dedicated to the assessment of the inhibitory activity of glucomoringin-isothiocyanate and isothiocyanate-containing *M. oleifera* extracts on the growth of *Propionibacterium acnes*.

Acne vulgaris is a disease that affects the pilosebaceous units in the skin and manifests when hair follicles get clogged with oil and dead skin cells, because of an increased production of sebum. Clogged pores are a good environment for the anaerobic bacterium *P. acnes*, which is normally found on the surface of the skin. The hyper-proliferation of *P. acnes* provokes an inflammatory response in the skin[58]. Even if the trials for the treatment of acne aim to hit the different mechanisms that contribute to its development, antimicrobials remain the mainstay of acne therapy[58].

Nevertheless, the increasing resistance to antibiotics by *P. acnes* has become a problem that makes it necessary to identify new and suitable agents that are active against *P. acnes*[58]. Nowadays, the use of vegetal extracts, also in association with conventional therapy is becoming an option.

It is reported in literature a study in which an aqueous extract of *M. oleifera* was effective in inhibiting the growth of *P. acnes*[59]. Nevertheless, that extract was not deeply characterized in order to understand which components may be responsible for the antibacterial activity observed.

On the other side, the antibacterial properties of glucomoringin isothiocyanate from *M. oleifera* toward both gram-positive and gram-negative bacteria are reported in literature[53,54]. Thus, it was thought to verify the activity of the isothiocyanate of glucomoringin from *M. oleifera* as well as of isothiocyanate-containing extracts against the growth of *P. acnes*.

1.6. Seed oils

1.6.1. *H. sabdariffa* oil

H. sabdariffa seeds are an unexploited source of oil that could be use for cosmetic applications as well as for nutraceutical applications, after a proper analysis of its fatty acids composition.

According to data reported in literature, *H. sabdariffa* oil (HO) contains 27% and 73% saturated and unsaturated fatty acids respectively[60]. The major saturated fatty acids in HO are palmitic (20.84%) and stearic (5.88%) and the main unsaturated fatty acids are linoleic (39.31%) and oleic (32.06%).

Nevertheless, the seed oils of plants belonging to Malvaceae family contain some unusual fatty acids known as cyclopropenoid fatty acids (CPFA) and epoxy fatty acids (EFA)[60]. HO is also known to contain these abnormal fatty acids that are dihydrosterculic acid (DHSA) and vernolic acid (VA)[61]. According to Wang et al.[61], when *H. sabdariffa* seeds or oil is consumed by animals or human, DHSA in HO can have detrimental health effects such as toxicity and physiological disorders[61]. Therefore, refined HO may be considered as edible after proper processing such as refining, bleaching, deodorization and heating. Partial substitution or blending with other vegetable oils for bringing down abnormal fatty acid level has also been advised to make it suitable for consumption[60].

It may be considered to refine or dilute HO even before using it for cosmetic applications, even if no references were found in literature concerning the toxicity of these two fatty acids following topical applications of the oil.

Moreover, HO showed interesting antioxidant activity[62] and phenolic content. A study reported in literature showed that vanillic acid was the predominant acid, followed by caffeic acid, among the phenolic acids. As reported in literature, the objectionable flavour of some oilseeds is due to the presence of phenolics that possess sour, bitter, astringent and/or phenolic-like flavour characteristics[62]. Phenolics have been shown to play important roles as antioxidants and influence the flavour of oils. Different oils may contain different types and concentration of phenolic compounds. High levels of vitamin E, detected in the oils, may contribute to the greater stability toward oxidation.

H. sabdariffa seeds are often discarded as by-products of production process and they have high prospective for the exploitation of the oil for the development of natural products as a source of antioxidant for cosmetic application. In Malaysia, the oil is already used to produce scrubs and soaps[32] and it is possible to find on the market HO of African origin[63], indicated as an ointment for the prevention of damages due to free radicals.

1.6.2. *M. oleifera* oil

M. oleifera seeds are rich source of oil, that is present in high percentages (35-40%) depending also on the geographical area of cultivation and on the agro-climatic conditions[64]

M. oleifera oil (MO) has light yellow colour with mild nutty flavour and a fatty acids composition that makes it highly suitable for both edible and non-edible applications.

Due to its pleasant flavour and odour and due to very low levels of peroxides, MO is normally used without any pre-processing (refining, bleaching, and deodorization), which is mandatory for most of the commercial vegetable oils that are used in cosmetic or other applications[64].

The fatty acid composition of MO is almost similar to that of olive oil, with a high percentage of oleic acid (75-77%). Studies reported in literature have proved that diets rich in oleic acid have the potential to lower the serum cholesterol and minimize the risk of cardiovascular diseases, thus highlighting the nutraceutical potential of MO. Moreover, it contains also 5-6% of behenic acid that confers to MO good cosmetic properties, as it is a crystallizing agent[64].

It is reported in literature about the antioxidant potential of MO: this oil is a rich source of kaempferol and other phenolics. Even more, it is a rich source of vitamin A and E. The high content of antioxidant compounds is proved by the great stability of MO toward autoxidation. Therefore, it could be also blend with other vegetable oils to increase their stability toward oxidation[64].

Considering the cosmetic applications of MO, it is reported in literature the use of the oil for cosmetic purposes by Egyptians, Greeks and Roman communities. Egyptians used MO in the treatment of skin disorders, as smoothing, moisturizing and oiling agent for the treatment of dry skin and therapeutic massages. It has the capability of absorbing and retaining the flavouring compounds[64].

Currently MO is widely used in the formulations of body creams, lotions, balms, scrubs and anti-hair fall formulations. Even more, it can be easily blended with essential oils, so that it can be considered an excellent massage oil. It can be used in the preparation of different types of soaps, cosmetic cream and lip balm[64].

In cosmetics, it is preferred over other oils as it does not leave greasy after feel and the cost of production is low as compared to other sources of edible oils[64].

1.6.3. *H. annuus* oil (sunflower oil)

Helianthus annuus L. (Asteraceae family) is one of the most important oilseed crop grown in the world[65]. The seeds of the plant in particular are exploited as a source of protein and oil[65].

Sunflower oil (SO) represent almost the 50% of the weight of sunflower seeds and it is interesting for its fatty acids composition as well as for its tocopherols content[65].

The main fatty acids in SO are oleic and linoleic, with higher linoleic acid concentration compared to olive oil. The presence of linoleic acid makes SO a suitable ingredients for skin products due to the positive benefits of linoleic acid[31]. In fact, linoleic acid has a direct role in maintaining the integrity of the barrier of the skin. It is also reported in literature that SO preserves the integrity of the stratum corneum and improves adult skin hydration without causing erythema[27].

SO is a vehicle of high amounts of tocopherols, with α-tocopherol representing the 90 % of total tocopherol content.

Nevertheless, SO is not so stable toward autoxidation in spite of its high content of tocopherols and this is a point of discussion for the scientific community[65].

1.6.4. *O. europaea* oil (olive oil)

Olive oil (OO) is mainly constituted by glycerol esters with different fatty acids. The predominant fatty acid present in olive oil triacylglycerols is monounsaturated oleic acid (up to 83% w/w). Other components are palmitic acid, linoleic acid, stearic acid and palmitoleic acid[19].

As it is widely known, olive oil found a large use in the preparation of cosmetics and for human nutrition, especially in the Mediterranean cousine[19]. The beneficial activity of OO in human health is mainly correlated to its polyphenolic content that ranges from 50 to 1000 mg/kg. However, it depends on the agronomic factors, the ripeness of olives, as well as extraction technology, along with storage or packaging process. In general, the highest value of total phenolics are measured in extra-virgin OO and the lowest values in refined OO[19].

Oleuropein, hydroxytyrosol, and their derivatives are polyphenolic compounds that are highly concentrated in OO and they are powerful antioxidants[19].

It is reported in literature that the presence of polyphenols in OO determines its antioxidant activity that is also the mechanism laying at the basis of other beneficial properties of OO on human health. Therefore, the antioxidant activity of OO is documented in literature as it is stated that the most important protection mechanism given by OO is the alleviation of oxidative stress phenomena[19].

Polyphenols of OO demonstrated also a direct antioxidant action on the skin, in particular oleuropein, which acts as a scavenger of free radicals in the skin[28]. Concerning the use of OO in cosmetic and dermo-protective creams, it is reported in literature the similarity of virgin OO's composition to sebum. This similarity is given by its high content of squalene, β-sitosterol content, optimum fatty acids content (the presence of oleic acid, which acts as a skin softener). Moreover, antioxidant substances present direct protective properties on the skin. When applied to the skin after sun exposure, OO has an inhibitory effect on sun-induced cancer development (especially the virgin one that has a higher content of phenolics and then a higher potential of scavenging free radicals in the skin)[28].

High content of antioxidant polyphenols (hydroxytyrosol, oleuropein) makes virgin olive oil relatively stable and resistant to oxidation upon storage.

In contrast, oleic acid is detrimental to skin barrier function[66]. Oleic acid causes barrier disruption and eventually induces dermatitis under continuous topical application[27].

2. AIM OF THE WORK

2.1. First project line: *Hibiscus sabdariffa* L. calyces

It is known that the ability of a plant to synthesize its metabolites depends both on the plant variety and on the geographical area i.e. the local growing conditions, where the plant is cultivated[32]. Therefore, it is not completely correct to rely on literature data, when looking for information concerning the amount of a metabolite in a plant, in order to select the best source of a specific metabolite.

In the first part of the study, three different varieties of *H. sabdariffa* calyces were studied. All of them were cultivated in the southern part of Paraguay, but the seeds came from three different geographical areas of the earth (Brazil, Paraguay and the equatorial area of Africa).

Therefore, it was important to perform a comparative study among these varieties of calyces in order to assess their anthocyanin content.

In the second part of the study, the first objective was to perform a new selection of a variety of calyces, with the highest content of anthocyanins, among three different varieties, all native to Paraguay, since the variety that had been selected in the first part of the study had subsequently showed some agronomic problems.

The second objective was the optimization of an extraction method to recover anthocyanins in high yield but that could be suitable for the production of extract on large scale, too.

The third objective was the selection of an enrichment method to obtain an anthocyanin-rich aqueous extract, to be exploited in the preparation of nutraceutical products with a higher concentration of the active compounds of *H. sabdariffa*.

2.2. Second project line: *Moringa oleifera* Lam. tissues

In the first part of the study, the aim of the work was to prepare and characterize aqueous extracts of different tissues of *M. oleifera*, in order to identify the best sources of glucomoringin (as a precursor of the bioactive isothiocyanate, with reported indirect antioxidant properties and antibacterial), and polyphenols (known for their direct antioxidant activity). Actually, few tissues of the plant, especially leaves, have been widely studied and characterized, but there is little or no literature concerning branches and the patented matrix here called "EDYNEA". Branches in particular are a waste product of the processing of *M. oleifera*, thus in depth research is suggested to investigate their content in compounds of interest.

Even more, the total amount of each vegetal sample, representing a specific part of the plant, was divided into two aliquots, that were subjected to two different drying treatments (oven-drying at 40°C and freeze-drying), in order to verify how the drying treatment could influence the chemical composition of the final extracts.

In the second part of the study, the aim of the work was the selection of an extraction condition suitable for the conversion of glucomoringin into the bioactive isothiocyanate inside the selected extracts, preserving the total phenolic content. The preparation of *M. oleifera* extracts already containing active isothiocyanates, offers the advantage of dosing a characterized quantity of these compounds, thus providing a better alternative to the administration of the precursor, which could be bio-activated by the intestinal microflora, but in an uncertain measure. A further objective was the characterization of glucomoringin-isothiocyanate inside the extracts, the determination of TPC and the evaluation of the direct antioxidant capacity (DPPH and $ABTS^{\bullet+}$ assays).

This part of the study was continued with those tissues that had been selected in the first part of the study. In fact, they were considered good sources of both glucomoringin as precursor of the bioactive isothiocyanate and polyphenols.

A final objective was the assessment of the inhibitory activity of glucomoringin-isothiocyanate and isothiocyanate-containing extracts toward the growth of *Propionibacterium acnes*.

2.3. Third project line: oilseeds and oily extracts

In this case, the final objective was the production of oily extracts enriched in antioxidant compounds for cosmetic applications.

In the first part of the study, the aim of the work was the selection of a variety of *H. sabdariffa* seeds with a high oil percentage and α-tocopherol content, in comparison with *M. oleifera* seeds. The seeds were obtained from plants cultivated in Paraguay.

This study was continued by performing a new selection of a variety of *H. sabdariffa* seeds with a high oil percentage and α-tocopherol content. In fact, the variety that had been selected in the first part of the study revealed some agronomic problems that required its substitution with another variety.

Subsequently, it was performed the extraction of the oil from the seeds of the newly selected *H. sabdariffa* variety and from *M. oleifera* seeds. These two oils together with two frequently used oils in nutrition and in cosmetic field (olive and sunflower oils) were used for the preparation of oily extracts enriched in compounds with known radical-scavenging activity (carotenoids, polyphenols and α-tocopherol), with the technique of ultrasounds. The oleolites and the respective pure oils were characterized and the radical-scavenging activity was evaluated.

3. MATERIALS AND METHODS

Materials:

Reagents

The reagents were purchased from *Sigma- Aldrich*.

Propionibacterium acnes (ATCC®6919™) was purchase from *LGC standards*.

Solvents

Solvents were purchased from *Carlo Erba* and *Sigma-Aldrich*.

Deuterated solvents

Deuterated solvents of *Sigma-Aldrich* Company were used.

Chromatographic columns

The following columns were used:

Zorbax SB-C18 (150 x 4,6 mm; 3.5 µm) column, *Agilent*

Zorbax 300SB-C18 (4.6 x 250 mm; 5µm) column, *Agilent*

Instrumentation:

Balances:

Balance *OHAUS adventurer*

Balance *Radwag*, AS220.R2

Drying and Lyophilisation

Freeze-dryer *Edwards* (collector temperature -40°C, vacuum 50 mBar)

Freeze-dryer PY *LabConco*, Freezone 4.5 (collector temperature -45°C, vacuum 0,050 mBar)

Oven PY *Matsui*, Matsui FFG. Co. Ltd, Japan, PO-200

Extractions

Ultrasonic bath *Soltec ultrasonic cleaner*, Sonica 3300 MHS3 (500W, 50/60Hz)

Soxhlet *BÜCHI*, BÜCHI extraction system B-811

Centrifugation

Centrifuge *Heal Force*, Neofuge 15R

Centrifuge *SIGMA*, 3-16KL

Absorption spectroscopy

Spectrophotometer *Agilent*, Cary 60 UV/Vis

Liquid Chromatography

HPLC *Agilent* 1260

HPLC *Shimadzu* Prominence

HPLC *Varian* ProStar 325-Uv

Nuclear magnetic resonance spectroscopy

NMR Bruker AMX 300

NMR Bruker AVANCE III 400

NMR Bruker minispec mq20

Mass spectrometry (HRMS)

Mariner Applied Biosystems, ESI-TOF

Others

pH meter *Mettler Toledo*

Mill *Ika*, A11 basic (160W)

Vortex *VELP Scientifica*, ZX3

3.1. First project line: *Hibiscus sabdariffa* L. calyces

3.1.1. Plant material and drying

Calyces of *H. sabdariffa* were provided by the company MANTIS AGROPY S.A., located in Guairà Department, Paraguay. The samples were harvested, collected and dried at 59°C for 17 hours in MANTIS AGROPY S.A., Py.

The dried material was then pulverized with a mill and stored in a dark, dry and fresh place.

The following varieties were used in the first part of the study:

1. Fortìm
2. Tenonderà
3. Koape guaré

To perform the second part of the study, the varieties showed below were used:

4. Kibeleza
5. Che la Reina
6. Tenonderà Tempranera

and Koape guaré variety was used as a reference in this case.

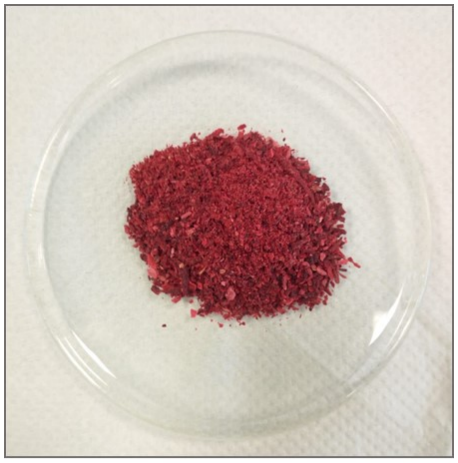


Figure 24: *H. sabdariffa* calyces powder

With the exception of Fortìm (native to Brazil) and Koape guarè (native to the equatorial region of Africa), all other varieties were native to Paraguay. The calyces used in the first part of the study were collected in 2015 while the calyces used in the second part of the study were collected in 2017.

3.1.2. First part of the study: preliminary selection of a variety of calyces rich in anthocyanins.

The aim of the work was the selection of the variety of calyces richest in anthocyanins.

3.1.2.1. Extractions

The extracts were prepared according to the method reported by Sindi et al[67]. This method is based on the extraction of calyces in acidic water: 0.5 g of grounded calyces were added to 50 ml of boiling water with 1% formic acid. After 10 minutes, the extract was filtered by Buchner. It was then dried in a rotary evaporator at 35°C.

3.1.2.2. Characterizations

3.1.2.2.1. SPE-mediated enrichment of the extracts

The raw extracts were then purified by SPE, using reverse phase SPE cartridges (Sep-Pak 1g) according to the method of Rodriguez-Saona and Wrolstad[68] with some modifications.

The raw extracts dried in rotary evaporator were solubilized in 25mL of deionized water. Two solutions were prepared, an aqueous solution (0.01% hydrochloric acid) and a methanolic one (0.01% hydrochloric acid).

Before introducing the extract solution into the SPE cartridge, the column was conditioned by first passing 18 mL of methanol and immediately after 27 mL of acidic water (0.01% of hydrochloric acid). Thereafter, 400 µL of extract solution was introduced into the SPE cartridge and a first elution was carried out with 18 mL of acid water and then with 18 mL of acidic methanol; this last elution was collected in a flask and concentrated by rotary evaporator.

To determine the elution volumes and the solvent volume in which the raw extract is solubilized, the data reported in the reference method were used, making the appropriate proportions. In fact starting from a more concentrated raw extract than that reported in the article, it was loaded almost half the volume of the quantity indicated in the article into the SPE cartridge. This volume and the elution volumes were then determined also on the basis of the load capacity of the SPE cartridge at our disposal (1g versus 300mg reported in the article).

Finally, the purification cycles were carried out several times, in order to collect a quantity of plant material enriched in anthocyanins sufficient for subsequent analysis.

3.1.2.2.2. Total monomeric anthocyanin (TMA)

The spectrophotometric pH differential method was used[36]. Approximately 6 mg of the extract were weighed and were brought to volume in a 2 mL graduated flask; 1 ml was collected and brought to 5 ml of volume with HCl-KCl buffer (pH = 1), the other mL instead was brought to 5 mL with acetic acid-sodium acetate buffer (pH = 4.5). Both solutions after 15 minutes were analyzed at the UV-Vis spectrophotometer at two wavelengths, 520 nm and 700 nm[36]. Some of these samples were subjected to further dilutions so that the measured absorbance value remained within the linearity range. Each analysis was performed in triplicate; before each reading the pH of the two solutions was assessed by pH-meter.

For each sample the absorbance value was measured using the following formula:

$$Abs = (Abs_{520} - Abs_{700})_{pH\ 1,0} - (Abs_{520} - Abs_{700})_{pH\ 4,5}$$

Total monomeric anthocyanins were calculated by the following formula and expressed as mg delphinidin 3-sambubioside/L of sample.

$$TMA(mg.L^{-1}) = \frac{A \times MW \times DF \times 1000}{\varepsilon \times l}$$

MW represents the molecular weight (597.15 g.mol^{-1}, for delphinidin 3-sambubioside), ε is the molar absorptivity coefficient (26000 L.mol^{-1} cm for delphinidin 3-sambubioside), DF is dilution factor and *l* is the light pathway along the quartz cell (1 cm).

3.1.3. Second part of the study

The aim of the work was the selection of the best Paraguayan variety of calyces, the optimization of an extraction method and the selection of an enrichment method to obtain an anthocyanin-rich aqueous extract.

3.1.3.1. Extraction conditions

1. 200 mg of powdered *H. sabdariffa* calyces were extracted for 10 minutes in 20 mL of distilled H_2O (1:100 g/mL) at 100°C, under stirring, according to a method reported in literature[67]. Solid was removed by filtration with filter paper. The liquid was then lyophilized.
2. 200 mg of powdered *H. sabdariffa* calyces were extracted for 60 minutes in 20 mL of distilled H_2O (1:100 g/mL) at 60°C, under stirring. Solid was removed by filtration with filter paper. The liquid was then lyophilized.
3. 200 mg of powdered *H. sabdariffa* calyces were extracted for 15 minutes in 20 mL of distilled H_2O (1:100 g/mL) at 60°C, in an ultrasonic bath. Solid was removed by filtration with filter paper. The liquid was then lyophilized.
4. 200 mg of powdered *H. sabdariffa* calyces were extracted for 15 minutes in 20 mL of distilled H_2O (1:100 g/mL) at 25°C, in an ultrasonic bath. Solid was removed by filtration with filter paper. The liquid was then lyophilized.

All extracts were prepared in triplicate.

Lyophilisation was performed by freezing the samples with dry ice (solid carbon dioxide), before putting them into the freeze-dryer. Samples were protected from light with aluminium foils.

Extraction yield was calculated with the following formula:

$$\frac{grams\ of\ lyophilized\ extract}{grams\ of\ powdered\ H.sabdariffa\ calyces} * 100$$

Results were expressed as mean and standard deviation (n=3).

3.1.3.2. Enrichment conditions

In this study, we also try to optimize a condition to be applied on an aqueous extract of *H. sabdariffa* calyces, to obtain an anthocyanins enriched extract, with a TMA value higher than the value normally found in an extract that was subjected to no enrichment procedures.

Thus, extracts were prepared by following extraction condition number 1. For the standard, with no purification method, the liquid was then lyophilized. Four different enrichment procedures were applied on the other extracts, as indicated below:

Conditions 1, 2 and 3

The pH of the extraction mixture was checked to ensure a pH below 4. Then, 0.600g of PVPP (Polyclar Super R, particle size 110µm) (30 mg/mL) was added to approximately 20 ml of extract. The mixture was left under magnetic stirring for 15 minutes at room temperature. Then it was filtered by Büchner filtration. The PVPP was conserved. Extraction by PVPP was repeated two more times on the filtrate with fresh PVPP.

Combined PVPP was placed in a round bottom flask and 40mL acetone/distilled water (70:30 v/v) (Condition 1) were added. This was extracted by sonication for 15 minutes, followed by 15 minutes under magnetic stirring. The mixture was then filtered by Büchner filtration and the PVPP re-extracted two times more.

All extracts were combined, and solvent removed by rotary evaporation at temperatures not exceeding 35°C. The samples were then lyophilised.

For conditions 2 and 3, the same method was used, but the acetone/water solution was replaced with EtOH: H_2O (50:50 v/v) and EtOH: H_2O (50:50) plus 1% acetic acid, respectively (Table 5). These samples were not lyophilised as dryness was achieved solely through use of a rotary evaporator. The addition of more ethanol during rotary evaporation was required for condition 3 to ensure all acetic acid was fully evaporated.

Products were all dark pink solids and soluble in water.

Condition 4

The pH of the extraction mixture was checked and adjusted if necessary to ensure a pH below pH 4. 0.667g of PVPP (30 mg/mL) was added to approximately 20 ml of extract and left under magnetic stirring for 15 minutes at room temperature. After this time, the mixture was filtered by Büchner

filtration. The PVPP was conserved. Extraction by PVPP was repeated two more times on the filtrate with fresh PVPP.

PVPP was placed in a 250mL round bottom flask and washed with 100 mL of deionised water under magnetic stirring for 10 minutes. The mixture was filtered by Büchner filtration. This was repeated two more times. The aqueous solutions produced by washing were discarded.

Combined PVPP was placed in a round bottom flask and 40mL EtOH: H_2O (50:50 v/v) plus 1% acetic acid was added. This was extracted by sonication for 15 minutes, followed by 15 minutes under magnetic stirring. The mixture was then filtered by Büchner filtration and the PVPP re-extracted a further two times.

All extracts were combined, and solvent removed by rotary evaporation at 35°C, until drying. The extracts were a dark blue colour and insoluble in water.

All extracts were covered with foil and stored at 4°C to reduce degradation of anthocyanins due to heat and light.

Sample	***Elution Solvent Mixture***
Standard	No Purification Method
Condition 1	Acetone: H_2O (70:30 v/v)
Condition 2	EtOH: H_2O (50:50 v/v)
Condition 3	EtOH: H_2O (50:50 v/v) plus 1% Acetic Acid
Condition 4	Initial washing with H_2O, followed by elution with EtOH: H_2O (50:50 v/v) plus 1% Acetic Acid

Table 5: Solutions used for the elution of anthocyanins from PVPP-Anthocyanin complex in each condition

Each enrichment procedure was tried three times. Yields are expressed as mean and standard deviation (n=3).

3.1.3.3. Characterizations

3.1.3.3.1. Total monomeric anthocyanins (TMA)

Total anthocyanins content was evaluated following the pH differential spectrophotometric method described by Ochoa-Velasco et al.[69] with modifications. A pH 1 buffer solution was produced from 50mL 0.1M KCl and 97mL 0.1M HCl, adjusted to final volume of 200mL with deionised water. A pH 4.5 buffer solution was produced from 16.4g CH_3COONa and 20.73mL CH_3COOH made up to 200mL with deionised water (values calculated using Henderson-Hasselbalch equation). The pH of the obtained buffers was checked by a pHmeter. *H. sabdariffa* extracts (2-6 mg) were diluted 1mL of H_2O (except in the case of condition 4, in which 2mg of extract were diluted with 3mL of 49.5:49.5, EtOH:H_2O with 1% acetic acid). 400 µL of this solution were then added to 3.5mL of each buffer solution, in separate test tubes and left for 15 minutes, in the dark. A spectrophotometer was then used to measure absorption at 520 and 700nm. Distilled water was used as blank.

These absorbance values were used in the following equation to calculate total monomeric anthocyanins:

$$A = (A_{520} - A_{700})_{pH=1.0} - (A_{520} - A_{700})_{pH=4.5}$$

$$TMA(mg.L^{-1}) = \frac{A \times MW \times DF \times 1000}{\varepsilon \times l}$$

MW represents the molecular weight (449.2 $g.mol^{-1}$, for cyanidin-3-O-glycoside), ε is the molar absorptivity coefficient (26,900 $L.mol^{-1}$ cm for cyanidin-3-O-glycoside), DF is dilution factor (total volume/ volume of extract, in this case 9.75) and *l* is the light pathway along the quartz cell (1cm). The equations and values were reported in the work of Ochoa-Velasco[69].

Results were then converted to mg of cyanidin-3-O-glycoside per gram of extract by multiplying by the volume of solvent required to dissolve the extract (expressed in litres) and then multiplying by mass of extract used (in grams). Cyanidin-3-O-glycoside is reported, in literature, as standard to use for this formula[69, 70].

Values were expressed as mean and standard deviation (n=3).

3.1.3.3.2. HPLC-DAD analysis of anthocyanins in an aqueous extract of *H. sabdariffa* calyces

The characterization of the single anthocyanins in the aqueous extract obtained by using the best variety of calyces and the most suitable extraction condition was performed in an Agilent 1260 HPLC system, with a DAD detector, according to a method that has been recently reported in literature[71]. It was used a Zorbax SB-C18 (150x4,6 mm; 3.5 µm) column. It was performed a gradient elution with phase A: orthophosphoric acid in milli-Q water (3%V/V) and phase B: orthophosphoric acid (10% V/V), methanol (36.5%V/V) and milli-Q water (53.5%V/V), during an 85 minutes run. Flow rate was 1 ml/min. Temperature of the column was set at 25°C. The sample was dissolved in phase A, filtered with 0,45 µm filter and 20 µl were injected into the column. Detection was at 520 nm on a photodiode array detector. Compounds were identified from retention time and were quantified against external standards. Values were expressed as mean and standard deviation (n=3)

Time (minutes)	***% phase A***	***% phase B***
0	77	23
21	74	26
58	40	60
85	40	60

Table 6: Timetable of solvents gradient for the elution of *H. sabdariffa* anthocyanins.

Standard	***Linearity range (M)***	***R^2***
Delphinidin 3-sambubioside chloride	$2.21x10^{-6}$-$2.21x10^{-5}$	0.9982
Delphinidin 3-O-glucoside chloride	$5.99x10^{-6}$-$5.99x10^{-5}$	0.9961
Cyanidin 3-sambubioside chloride	$1.62x10^{-6}$-$1.62x10^{-5}$	0.9917
Cyanidin 3-O-glucoside chloride	$2.68x10^{-6}$-$2.68x10^{-5}$	0.9622

Table 7: Linearity ranges and R^2 values of anthocyanins standards that were used for the external calibration.

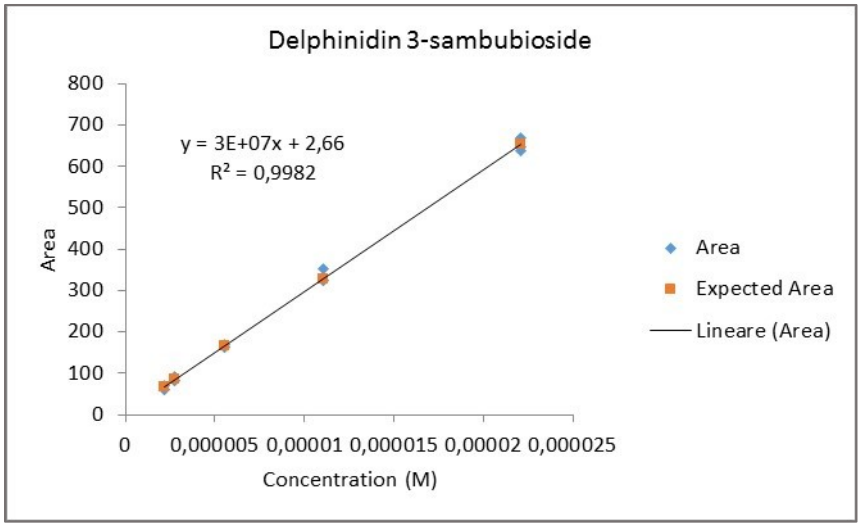


Figure 25: Calibration curve of delphinidin 3-sambubioside on an *Agilent 1260 Infinity* HPLC system.

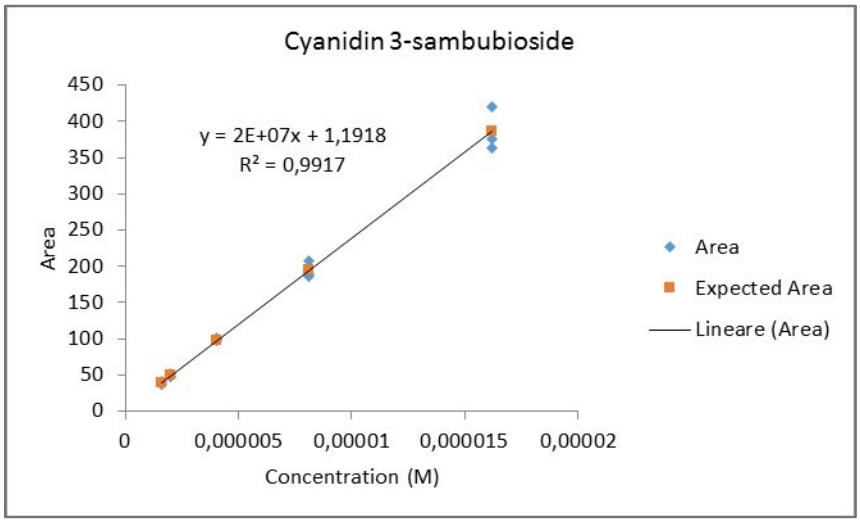


Figure 26: Calibration curve of cyanidin 3-sambubioside on an *Agilent 1260 Infinity* HPLC system.

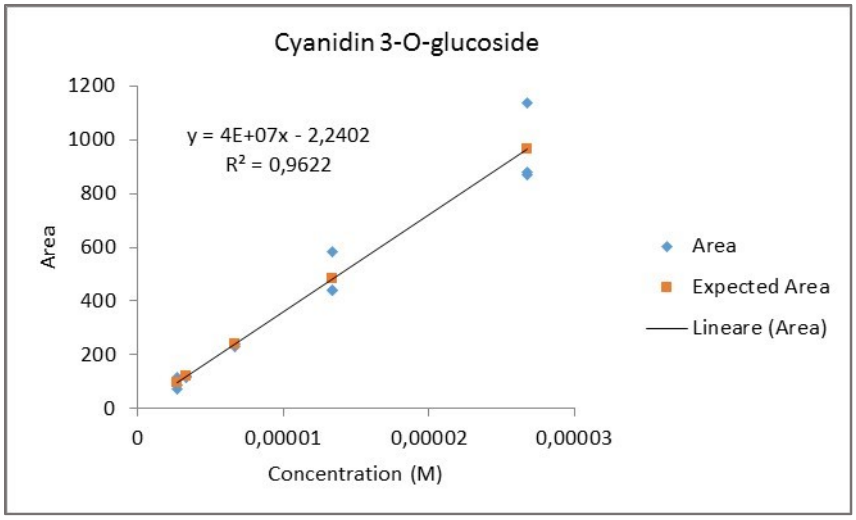


Figure 27: Calibration curve of cyanidin 3-O-glucoside on an *Agilent 1260 Infinity* HPLC system.

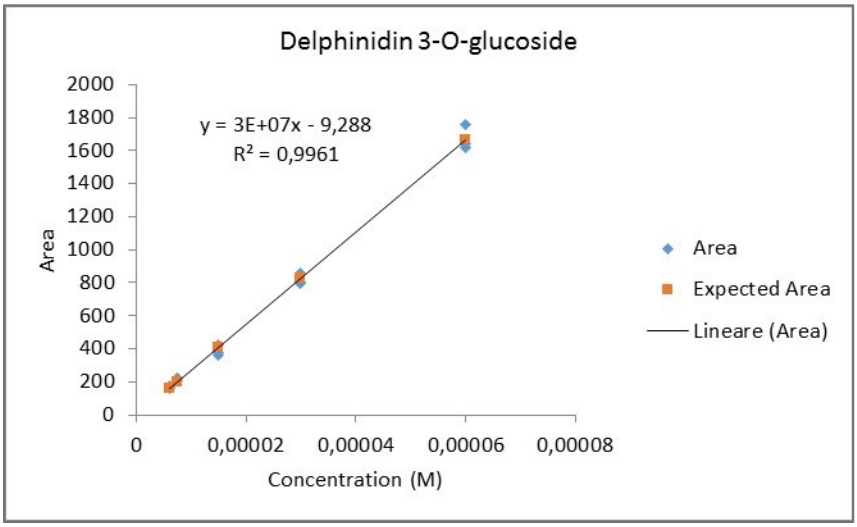


Figure 28: Calibration curve of delphinidin 3-O-glucoside on an *Agilent 1260 Infinity* HPLC system.

3.1.3.4. Assessment of the properties

3.1.3.4.1. Total antioxidant capacity (TAC)

The antioxidant capacity of the extracts was measured by the following *in vitro* assays.

3.1.3.4.1.1. DPPH assay

According to the method reported by Povolo et al.[72], 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) was dissolved in methanol at a final concentration of 56 μg/ml. After that, it was prepared a stock solution of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) in methanol at a concentration of 0.1 mg/ml. Eight different dilutions of the stock solution were prepared in triplicate (1:2; 1:4; 1:6; 1:8; 1:10; 1:15; 1:20; 1:25). For the preparation of the sample, 8 mg (weighed with the precision of 0.1mg) were dissolved in 4 ml of distilled water and they were diluted 1:100 in methanol. Trolox solutions and sample solution (150 μl) were added to DPPH methanolic solution (750μl) and to pure methanol (750 μl).

According to the method of Szydłowska-Czerniak et al.[73], a control sample was prepared using methanol instead of the extracts.

Absorbance of those solutions was read in a spectrophotometer at 515nm against a blank of methanol, after 5 minutes of reaction.

The scavenging of DPPH was calculated as follows[73]:

$$\%\ DPPH\ scavenging = \frac{Abs\ control - Abs\ sample}{Abs\ control} * 100$$

Where *Abs control* is the absorbance of the control sample and *Abs sample* is the absorbance of DPPH solution+ extract or Trolox solution. However, the final results were obtained thanks to the following linear relationship:

$$f\left(concentration\ of\ Trolox\ standard\ solutions\ in\ \mu\frac{mol}{ml}\right) = \%\ DPPH\ scavenging$$

So the standard solutions of Trolox were used to build a calibration curve that was subsequently used to calculate the antioxidant capacity of the samples.

Therefore, the DPPH values were expressed as μmol Trolox equivalents (TE) per gram of extract and they were reported as mean and standard deviation (n=9).

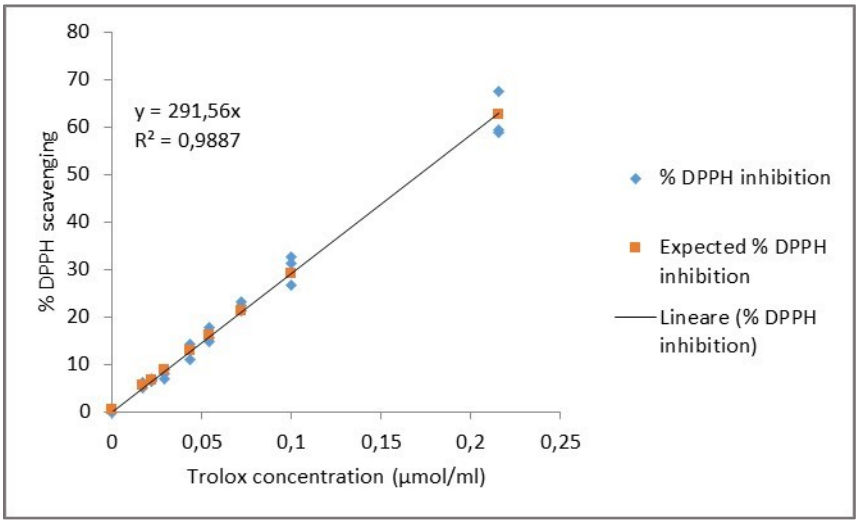


Figure 29: Linear relationship between Trolox concentration and DPPH scavenging (%).

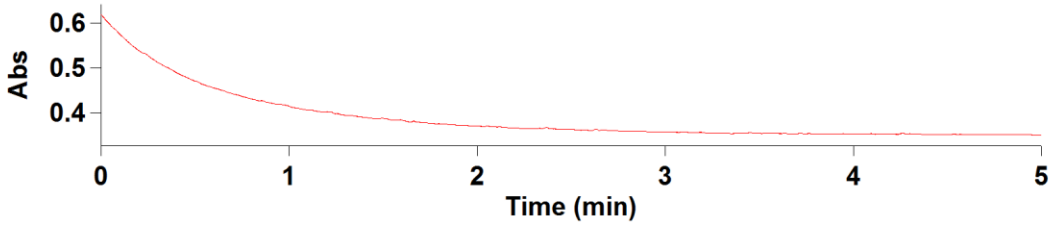


Figure 30: Kinetic of DPPH scavenging by Trolox 0.05 mg/ml in methanol, 515 nm.

3.1.3.4.1.2. ABTS assay

The total antioxidant activity of each extract was measured through the assay of the decoloration of the cationic radical 2,2'-azino-bis (3-etilbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$)[74] with some modifications.

The cationic radical $ABTS^{\bullet+}$was produced by adding 10 ml of a solution of potassium persulfate ($K_2S_2O_8$)2,45mM to 10 ml of a solution of ABTS 7mM in distilled water. This mixture was kept in the dark, at room temperature for almost 20 hours, before use. The mixture was then diluted with ethanol until a final absorbance of 0,700 at 730 nm. To quantify the total antioxidant activity, a calibration curve with Trolox was built: 49.9 mg of Trolox were weighed and dissolved in 5 ml distilled water+5 ml methanol+90 ml ethanol in a 100 ml volumetric flask.

Four different dilutions of the stock solution were prepared five times (1:50; 1:100; 1:500; 1:1000). 200µl of each solution were added to 2000 µl of the radical solution. After 10 minutes at room temperature, the absorbance was read at 730 nm against a blank of ethanol. A control sample was prepared using ethanol in the place of the standard solution.

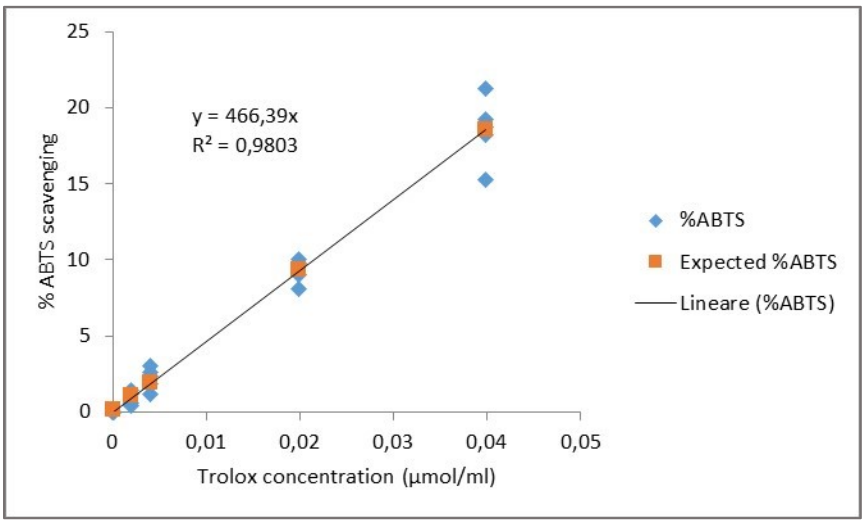


Figure 31: Linear relationship between Trolox concentration and $ABTS^{\bullet+}$ scavenging.

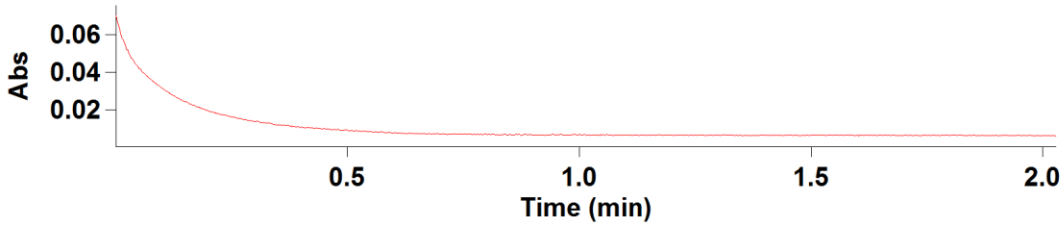


Figure 32: Kinetic of $ABTS^{\bullet+}$ scavenging by Trolox 0.05 mg/ml in ethanol, 730 nm

The sample was prepared by dissolving 2 mg of *H. sabdariffa* calyces extract (weighed with the precision of 0.1mg) in 1 ml of distilled water. The sample was diluted 1:200 with distilled water. 200 µl of sample were added to 2000 µl of the ABTS solution in ethanol. After 10 minutes, the absorbance was read at 730 nm against a blank of 9 % water in ethanol. A control sample was prepared using water in the place of the extract.

The scavenging of $ABTS^{\bullet+}$ was calculated as follows[73]:

$$\%\ ABTSradical\ scavenging = \frac{Abs\ control - Abs\ sample}{Abs\ control} * 100$$

Where *Abs control* is the absorbance of control sample and *Abs sample* is the absorbance of $ABTS^{\bullet+}$ solution+ extract or Trolox solution. However, the final results were obtained thanks to the following linear relationship:

$$f\left(concentration\ of\ Trolox\ standard\ solutions\ in\ \mu\frac{mol}{ml}\right) = \%\ ABTSradical\ scavenging$$

Results were expressed as µmol Trolox equivalents/gram of extract.

Mean and standard deviation (n=3) were calculated.

3.2. Second project line: *Moringa oleifera* Lam. tissues

3.2.1. Plant material and drying

Leaves, seeds, bark and branches of fresh *M. oleifera*, "purple" variety, were collected in Moringa guaranì S.A. (YGUA), located in Compañía Colonia Piraretá, Piribebuy District, Cordillera Department, Paraguay. Samples coded EDYNEA01 and EDYNEA02 were provided by Veterinary Department, Universidad Nacional de Asunción, Asunción, Paraguay. EDYNEA01 and EDYNEA02 represent two different varieties of the same raw material, respectively "green" variety and "purple" variety. All samples were collected carefully, without breaking the vegetal tissues, in February 2017.

A specimen of *M. oleifera* "purple" variety was deposited at the Herbarium FCQ at Botany Department of the Faculty of Chemical Sciences of the National University of Asunción, Campus Universitario, 1144 San Lorenzo, Paraguay (Herbarium codes: Y. González and C. Povolo 139, 140).

Each plant sample was divided in two aliquots: one was oven-dried at 40 °C (Matsui, PO-200, Matsui FFG. Co. Ltd, Japan.), while the other was freeze-dried (Freezone 4.5 LabConco Corp. USA, collector temperature -45°C, vacuum 0,050 mBar). Samples were dried until complete removal of water, then were pulverized with a mixer and stored at -18°C.

Seeds of *M. oleifera*, "green" variety, air-dried in the dark, were provided by Moringa Guaraní S.A. (YGUA), located in Compañía Colonia Piraretá, Piribebuy District, Cordillera Department, Paraguay. They were pulverized with a mixer and stored at room temperature.

Figure 33: A picture of the specimen of *M. oleifera*, deposited at the Herbarium FCQ (on the left). A picture of the pulverized dried tissues of *M. oleifera* (on the right).

3.2.2. First part of the study

The aim of the work was to prepare and characterize aqueous extracts of different part of *M. oleifera*, in terms of 4-(α–L-rhamnopyranosyloxy)-benzylglucosinolate (glucomoringin) content, total phenolic content and total antioxidant activity, in order to find out the more suitable to be used in the preparation of nutraceutical and cosmetic products. Even more, the total amount of each vegetal sample, representing a specific part of the plant, was divided into two aliquots, that were subjected to two different drying treatments (oven-drying at 40°C and freeze-drying), in order to verify how the drying treatment can influence the composition of the aqueous extracts.

3.2.2.1. Extractions

Aqueous extracts of different tissues of *M. oleifera* were prepared according to a method reported in literature[75], with modifications. Briefly, the powders were extracted with deionized and distilled water, at 80°C, for 10 minutes, with the ratio drug/solvent: 1/12. After that, the extracts were filtered through quantitative filter paper (MN 640, Macherey-Nagal, Ø 125 mm), and the residue was extracted again twice with deionized and distilled water, at 80°C, for 5 minutes, with the ratio drug/solvent: 1/4. After each extraction step, the extracts were filtered and at the end of the whole process, the supernatants were unified and centrifuged at 12000g, at 25°C, for 10 minutes, with a Heal Force Neofuge 15R centrifuge.

Potassium sorbate was then added as a preservative and they were stored at -20°C.

3.2.2.2. Characterizations

3.2.2.2.1. Isolation and characterization of glucomoringin

To isolate glucomoringin from *M. oleifera* seeds, in order to use it as external standard for HPLC analysis, an intact glucosinolate extraction method, based on Förster et al.[75] was used, with modifications.

20 g powdered *M. oleifera* seeds ("green" variety) were heated to 80°C in 240 ml 70% MeOH for 10 minutes. After centrifugation at 3000 rpm for 10 min, the pellet was re-extracted twice with 80 ml 70% MeOH for 5 min. Combined supernatants were dried in a rotary evaporator at 45°C up to a final volume of 15 ml.

After that, the solution was transferred to a DEAE Sephadex A-25 column that was equilibrated with MilliQ, and the column washed with 4 volumes of 70% MeOH, followed by 2 volumes of MilliQ, according to the procedure of De Graaf et al[76]. The elution was performed according to a procedure reported by Förster et al.[75], with modifications. Briefly, glucomoringin was slowly eluted with 2 volumes of 0.5 M potassium sulphate and collected into an equal volume of absolute ethanol (99.9%). After centrifugation at 3000 rpm for 10 min, the supernatants were dried in a rotary evaporator at 45°C. Dried residues were re-dissolved in three successive volumes of MeOH, that were equals to 1/25 volume of absolute ethanol added in the previous step. Solutions were centrifuged for 10 min at 3000 rpm. Absolute ethanol (99.9%) was added to the supernatants and solvents were evaporated to dryness on a rotary-evaporator at 45°C.

The identity of glucomoringin was confirmed by mass spectrometry (Mariner ESI-TOF, negative mode) and NMR spectroscopy (Bruker 400 MHz NMR). The purity was confirmed by inspection of the ^{1}H NMR for traces of other glucosinolates. An additional purity estimate (100% of total HPLC area at 223 nm) was obtained from HPLC of intact glucosinolates under the conditions of Förster et al[75].

3.2.2.2.2. Enzymatic conversion of glucomoringin into its isothiocyanate

The isothiocyanate of glucomoringin was obtained according to the procedure reported by De Graaf et al.[76], with modifications. Briefly, about 50 mg of the intact glucosinolate were dissolved in PBS (pH 7.2, $NaH_2PO_4 \cdot H_2O/Na_2HPO_4 \cdot 2H_2O$). To this solution, 4U of Thioglucosidase from *Sinapis Alba* seeds (25u, T4528, Sigma-Aldrich) were added. The mixture was put in a water bath at 37°C and the reaction was monitored through HPLC, under the conditions of Förster et al[75]. The isothiocyanate was then recovered from the reaction mixture by washing the solution with diethyl ether for five times. The organic phase was then dried with sodium sulphate and dried in a rotary evaporator at 40°C.

The identity of the isothiocyanate was confirmed by mass spectrometry (Mariner ESI-TOF, negative mode) and NMR spectroscopy (Bruker 400 MHz NMR). An additional purity estimate (92.3% of total HPLC area at 221 nm) was obtained from HPLC analysis under the conditions of Förster et al[75].

3.2.2.2.3. HPLC-DAD analysis of glucomoringin in aqueous extracts of *M. oleifera*

Glucomoringin determination in aqueous extracts was performed according to the method reported by Förster et al.[75], with modifications. 400 µl sample were diluted to a volume of 800 µl with deionized and distilled water. 200µl of zinc acetate 0.4M were added and the mixture was incubated for 30 min at room temperature. After that, they were centrifuged at 16000g at 25 °C for 10 min (Heal Force Neofuge 15R centrifuge). Supernatants were analysed on a Shimadzu prominence HPLC system. 10 µl sample were injected (SIL-20A auto sampler) on a 4.6 x 250 mm 300SB-C18 column (Zorbax, 5µm, Agilent) and separated using the following gradient program: 0-2 min: 0-1%B, 2-20 min: 1-50%B, 20-24 min: 50-100%B, 24-26 min: 100%B, 26-33 min: 100-0%B, 33-38 min: 0%B. Buffered eluents were used with solvent A: 100% 0.1M ammonium acetate, B: 40% acetonitrile/ 0.1M ammonium acetate. Detection was at 223 nm on a photodiode array detector (SPD-M20A). Temperature of the column was set at 25°C (Column oven CTO-10A). Compounds were identified from retention time and UV spectra and glucomoringin was quantified against external standard (See: *Isolation and characterization of glucomoringin).*

In the same samples, the presence of the glucomoringin-isothiocyanate was checked qualitatively, by using the product of the enzymatic reaction as a reference.

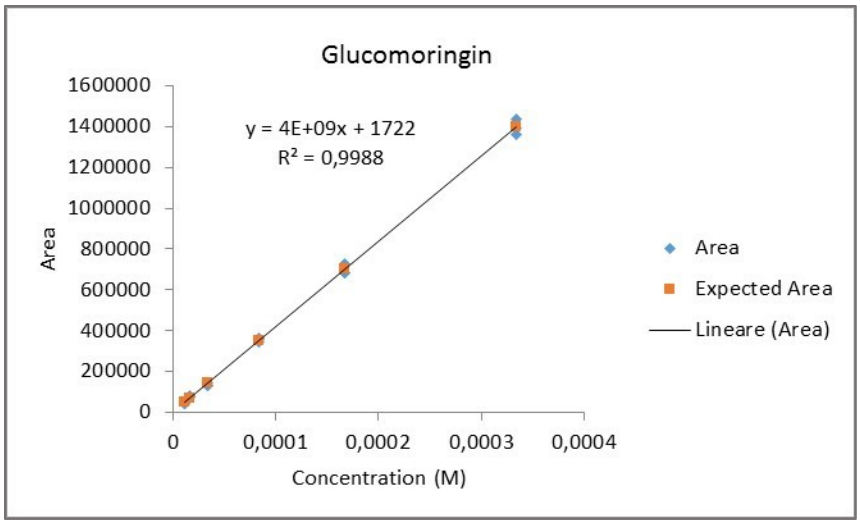


Figure 34: Calibration curve of glucomoringin on a *Shimadzu prominence* HPLC system.

3.2.2.2.4. Determination of total phenolic content (TPC)

The determination of total phenols in the extracts was performed according to the method of Folin-Ciocalteu[77], with modifications.

In this method, the reactant of Folin-Ciocalteu (a mixture of phosphomolybdate and phosphotungstate) reacts at a basic pH with phenolic compounds, producing tungsten (W_8O_{23}) and molybdenum oxides (Mo_8O_{23}) with a blue colour. Briefly, 1.25 ml of Folin-Ciocalteu reactant (previously diluted 1:10 v/v) were added to 250 µl of each aqueous extract and the mixture was left at room temperature for 10 minutes. After that, 1ml of Na_2CO_3 (7,5% p/v) was added and the mixture was left in a water bath at 30°C for 30 minutes, in the dark. At the end, the absorbance of the blue metal oxides was measured in a spectrophotometer at 650 nm. A blank sample was prepared using water instead of aqueous extracts. Gallic acid was used as reference standard for the preparation of a calibration curve and results were expressed as mg GAE equivalents/ 100ml extract. Each extract was analysed in triplicate.

3.2.2.3. Assessment of the properties

3.2.2.3.1. Total antioxidant capacity (TAC)

The antioxidant capacity of the extracts was measured by the following *in vitro* assays.

3.2.2.3.1.1. DPPH assay

Total antioxidant activity through DPPH assay was determined according to a method reported in literature[78], with modifications: 1ml of DPPH solution in methanol (60 µM) was mixed with 1 ml of each extract, in five different dilutions. The mixture was incubated for 30 minutes at room temperature, in the dark and the decreasing in absorbance was measured at 514 nm. A mixture of methanol and DPPH solution was used as blank. Moreover, a standard of ascorbic acid was used to assess a calibration curve to quantify total antioxidant activity. Results were expressed as IC_{50} in mg/ml and as µmol AA equivalents/ml. Mean and standard deviation (n=5) were calculated.
The percentage of DPPH inhibition was calculated as follows:

$$\text{Inhibition percentage (\%)}: \frac{Abs\ DPPH - Abs\ sample}{Abs\ DPPH} * 100$$

Where Abs DPPH is the absorbance of the blank of reactant and Abs sample is the absorbance of the solution when the sample extract has been added at a particular concentration. The Inhibitory Concentration 50 (IC_{50}) value (mg/ml) is the inhibitory concentration at which the DPPH radical was scavenged at 50% and calculated by interpolation from the data.

3.2.2.3.1.2. ABTS assay

The total antioxidant activity of each extract was measured through the assay of the decoloration of the cationic radical 2,2'-azino-bis (3-etilbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$)[74] with some modifications.

The cationic radical $ABTS^{\bullet+}$was produced by adding 7ml of a solution of potassium persulfate ($K_2S_2O_8$)2,45mM to 7ml of a solution of ABTS 7mM. This mixture was kept in the dark, at room temperature for almost 20 hours, before the use. The mixture was then diluted with ethanol until a final absorbance of 0,700 (±0, 02) at 730 nm. After that, four different dilutions of the aqueous extracts were mixed with the solution of the cationic radical in a water bath at 35°C for 30 minutes. A blank was used with water in the place of the extracts. After 30 minutes, the absorbance was measured at 730 nm. To quantify the total antioxidant activity, a calibration curve with Trolox was assessed and results were expressed as IC_{50} (mg/ml) and as µmol Trolox equivalents/ml extract.

Mean and standard deviation (n=4) were calculated.

Calculations were performed using the following formula:

$$\text{Inhibition percentage (\%)}: \frac{Abs\ ABTS\bullet+ - Abs\ sample}{Abs\ ABTS\bullet+} * 100$$

where Abs $ABTS^{\bullet+}$ is the absorbance of the blank of reactant and Abs sample is the absorbance of the solution when the sample extract has been added at a particular concentration. The Inhibitory Concentration 50 (IC_{50}) value (mg/ml) is the inhibitory concentration at which the $ABTS^{\bullet+}$ radical was scavenged at 50% and calculated by interpolation from the data.

3.2.3. Second part of the study

The aim of the work was the selection of an extraction condition suitable for the conversion of glucomoringin into bioactive isothiocyanate inside the selected extracts, preserving the total phenolic content. A further objective was the quantitative determination of glucomoringin and its isothiocyanate inside the extracts, the determination of TPC and the evaluation of the direct antioxidant activity.

This part of the study was carried on with leaves, branches and EDYNEA02 that had been dried in the oven at 40 °C and with the sample of seeds that had been lyophilised, according to the results observed in the first part of the study.

3.2.3.1. Extractions

Aqueous extracts were prepared according to the method reported by Waterman et al.[50], with few modifications. The powders were extracted with deionized and distilled water, at 22°C, for 30 minutes, with the ratio drug/solvent: 1/16. After that, the extracts were subjected to cycles of centrifugation at 3000 rpm for 10 minutes, until the solid particles were separated from the liquid and the liquid was further filtered with 0,45 micron filter. Then the extracts were lyophilized. Lyophilized extracts were stored at -18°C until they were analysed.

Extracts were prepared in triplicate.

Extraction yield was calculated with the following formula:

$$\frac{grams\ of\ lyophilized\ extract}{grams\ of\ M.oleifera\ powder} * 100$$

Results were expressed as mean and standard deviation (n=3).

3.2.3.2. Characterizations

3.2.3.2.1. Isolation and characterization of glucomoringin

To isolate glucomoringin from *M. oleifera* seeds, in order to use it as external standard for HPLC analysis, an intact glucosinolate extraction method, based on Förster et al.[75] was used, with modifications.

20 g powdered *M. oleifera* seeds ("green" variety) were heated to 80°C in 240 ml 70% MeOH for 10 minutes. After centrifugation at 3000 rpm for 10 min, the pellet was re-extracted twice with 80 ml 70% MeOH for 5 min. Combined supernatants were dried in a rotary evaporator at 45°C up to a final volume of 15 ml.

After that, the solution was transferred to a DEAE Sephadex A-25 column that was equilibrated with MilliQ, and the column was washed with 4 volumes of 70% MeOH, followed by 2 volumes of MilliQ, according to the procedure of De Graaf et al[76]. The elution was performed according to a procedure reported by Förster et al.[75], with modifications. Briefly, glucomoringin was slowly eluted with 2 volumes of 0.5 M potassium sulphate and collected into an equal volume of absolute ethanol (99.9%). After centrifugation at 3000 rpm for 10 min, the supernatants were dried in a rotary evaporator at 45°C. Dried residues were re-dissolved in three successive volumes of MeOH, that were equals to 1/25 volume of absolute ethanol added in the previous step. Solutions were centrifuged for 10 min at 3000 rpm. Absolute ethanol (99.9%) was added to the supernatants and solvents were evaporated to dryness on a rotary-evaporator at 45°C.

The dried sample was further treated with 4 ml of MeOH. Then, it was centrifuged at 12000 rpm for 5 minutes. The supernatant was recovered and dried in rotary evaporator.

The identity of glucomoringin was confirmed by NMR spectroscopy (Bruker 400 MHz NMR, sample dissolved in MeOD). The purity was confirmed by inspection of the ^{1}H NMR for traces of other glucosinolates. An additional purity estimate (100% of total HPLC area at 223 nm) was obtained from HPLC of intact glucosinolates under the conditions of Förster et al[75].

The obtained solid representing glucomoringin potassium salt was used as standard for external calibration.

3.2.3.2.2. Enzymatic conversion of glucomoringin into its isothiocyanate

The isothiocyanate of glucomoringin was obtained according to the procedure reported by De Graaf et al.[76], with modifications. Briefly, 86.7 mg of the intact glucosinolate were dissolved in 13ml PBS (pH 7.2, $NaH_2PO_4 \cdot H_2O/Na_2HPO_4 \cdot 2H_2O$). To this solution, 13.4mg of Thioglucosidase from *Sinapis Alba* seeds (25u, T4528, Sigma-Aldrich) were added. The mixture was put in a water bath at 37°C and the reaction was monitored through HPLC, under the conditions of Förster et al.[75] up to complete consumption of the reagent. The isothiocyanate was then recovered from the reaction mixture by washing the solution with diethyl ether for five times. The organic phase was then treated with sodium sulphate and it was dried in a rotary evaporator at 40°C.

Further purification was performed by washing the residue with DCM/H_2O:1/1 for three times. The aqueous phase was at the end washed further with DCM. All the DCM phases were unified and dried in rotary evaporator.

The identity of the isothiocyanate was confirmed by NMR spectroscopy (Bruker 300 MHz NMR, in MeOD). An additional purity estimate (>99% of total HPLC area at 221 nm) was obtained from HPLC analysis under the conditions of Förster et al[75].

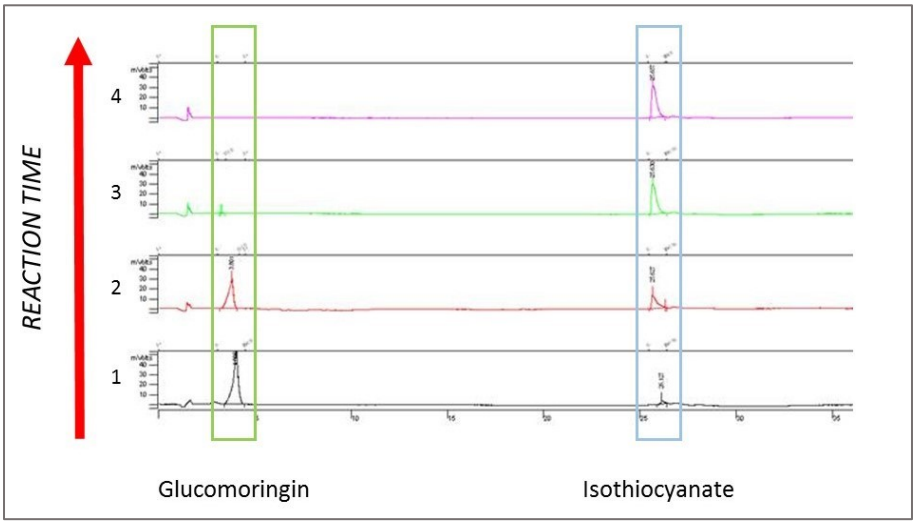


Figure 35: Reaction monitoring chromatograms for the conversion reaction of glucomoringin into its isothiocyanate.(1) Starting point of the reaction (2) 30 minutes reaction (3) 2 hours reaction (4) End point of the reaction.

3.2.3.2.3. HPLC-DAD analysis of glucomoringin and its isothiocyanate in aqueous extracts of *M. oleifera*

Glucomoringin and glucomoringin isothiocyanate determination in aqueous extracts was performed on an Agilent 1260 Infinity HPLC system, according to the method reported by Förster et al.[75], with modifications.

Lyophilised extracts were dissolved in phase A (0.2mg/ml seeds, 2mg/ml leaves, 3 mg/ml EDYNEA02, 12mg/ml branches). Each solution was filtered with a 0.45µm filter and 10 µl sample were injected in a 4.6 x 250 mm 300SB-C18 column (Zorbax, 5µm, Agilent) and separated using the following gradient program: 0-2 min: 0-1%B, 2-20 min: 1-50%B, 20-24 min: 50-100%B, 24-26 min: 100%B, 26-33 min: 100-0%B, 33-38 min: 0%B. Buffered eluents were A: 100% 0.1M ammonium acetate, B: 40% acetonitrile/ 0.1M ammonium acetate. Detection was at 223 nm on a photodiode array detector for glucomoringin and 221nm for the isothiocyanate. Temperature of the column was set at 25°C. Compounds were identified from retention time and UV spectra and they were quantified against external standard (See: *Isolation and characterization of glucomoringin and enzymatic conversion of glucomoringin into its isothiocyanate).*

Analysis were performed in triplicate. Results were reported in µmol/gram of extract and they were expressed as mean ± standard deviation (n=3)

Standard	***Linearity range (M)***	***R^2***
Glucomoringin potassium salt	$1x10^{-5}$ - $5x10^{-3}$	0.9993
Glucomoringin isothiocyanate	$1x10^{-5}$ - $5x10^{-4}$	0.9985

Table 8: Linearity ranges and R^2 values of standards that were used for the external calibration.

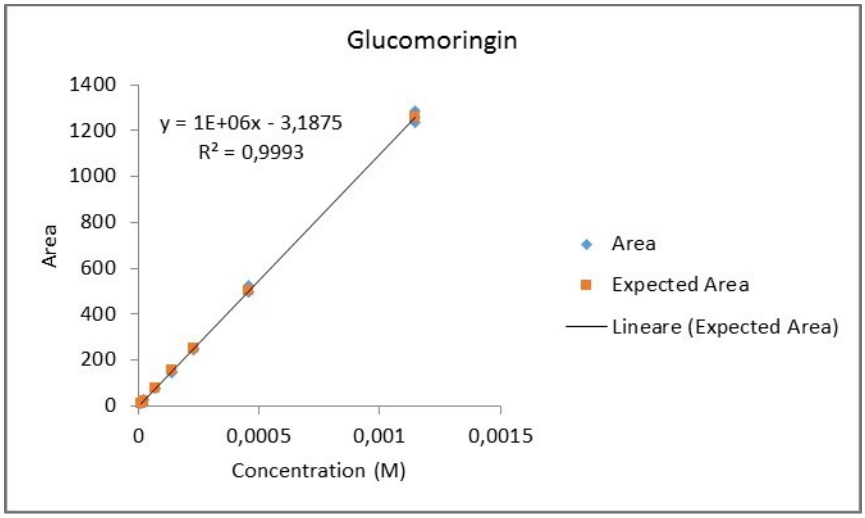


Figure 36: Glucomoringin calibration curve on an *Agilent 1260 Infinity* HPLC system.

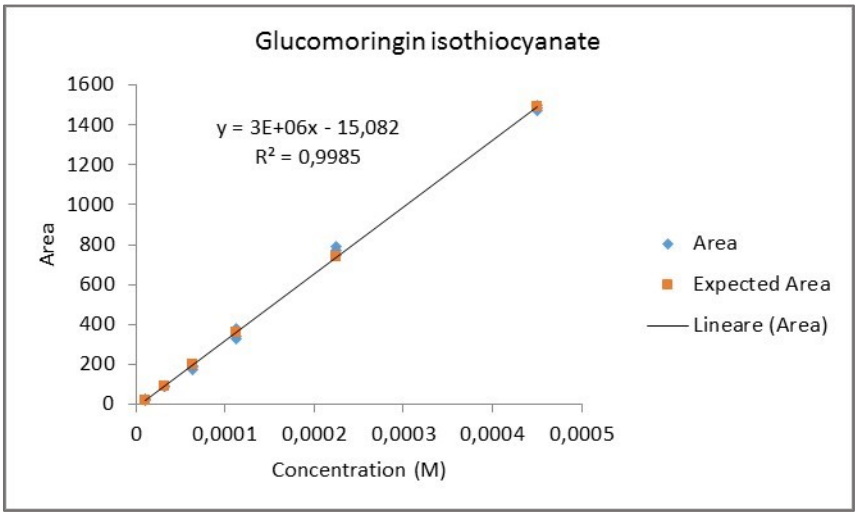


Figure 37: Glucomoringin isothiocyanate calibration curve on an *Agilent 1260 Infinity* HPLC system.

3.2.3.2.4. Determination of total phenolic content (TPC)

The determination of total phenols in the extracts was performed according to the method of Folin-Ciocalteu[77], with modifications.

Briefly, 1.25 ml of Folin-Ciocalteu reactant (previously diluted 1:10 v/v with distilled water) were added to 250 µl of each extract (lyophilized extracts were previously re-dissolved in distilled water in a concentration of about 2mg/ml) and the mixture was left at room temperature for 10 minutes. After that, 1ml of Na_2CO_3 (7,5% p/v) was added and the mixture was left at room temperature for 30 minutes, in the dark. At the end, the absorbance of the blue metal oxides was measured in a spectrophotometer at 650 nm. A blank sample was prepared using water instead of aqueous extracts. Gallic acid was used as reference standard for the preparation of a calibration curve and results were expressed as mg gallic acid equivalents (GAE)/ gram of extract.

Results were expressed as mean ± standard deviation (n=3).

It was also checked the absorbance of single extracts at 650nm: 250 µl of each extract solution were added to 2,25 ml of distilled water. Thus, the absorbance of extracts was read against water and it was subtracted to the absorbance of the samples treated with Folin-Ciocalteu reactant.

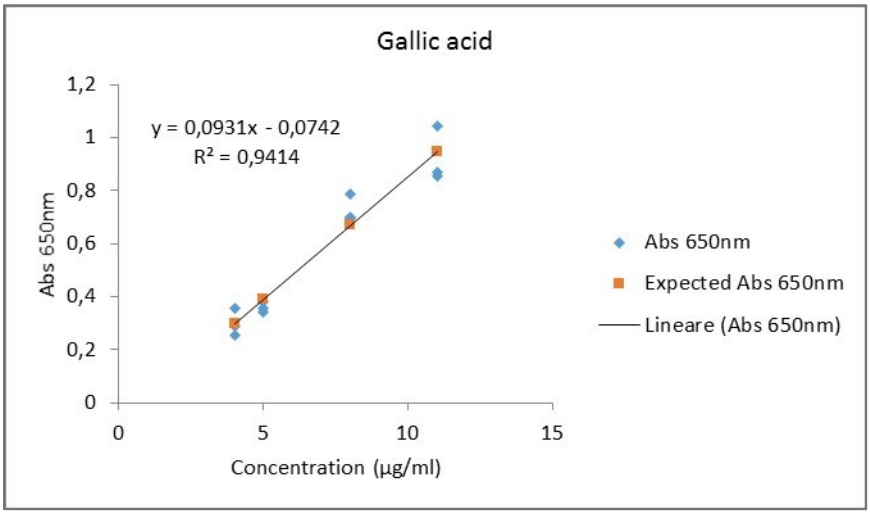


Figure 38: Gallic acid calibration curve.

3.2.3.3. Assessment of the properties

3.2.3.3.1. Total antioxidant capacity (TAC)

The antioxidant capacity of the extracts was measured by the following *in vitro* assays.

3.2.3.3.1.1. DPPH assay

According to the method reported by Povolo et al.[72], 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) was dissolved in methanol at a final concentration of 56 μg/ml. After that, it was prepared a stock solution of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) in methanol at a concentration of 0.1 mg/ml. Eight different dilutions of the stock solution were prepared (1:2; 1:4; 1:6; 1:8; 1:10; 1:15; 1:20; 1:25). For the preparation of the samples, 5 mg of each extract (weighed with the precision of 0.1mg) were dissolved in 1ml distilled water. The aqueous solutions were diluted properly with methanol (see Table 9). Trolox solutions and sample solutions (150 μl) were added to DPPH methanolic solution (750μl) and to pure methanol (750 μl).

sample	***dilution***
Leaves	1:50
Branches	1:100
Seeds	1:5
EDYNEA02	1:20

Table 9: Dilution factors of *M. oleifera* extracts.

According to the method of Szydłowska-Czerniak et al.[73], a control sample was prepared using methanol instead of the extracts.

Absorbance of those solutions was read in a spectrophotometer at 515nm against a blank of methanol, after 5 minutes of reaction.

The scavenging of DPPH was calculated as reported in section 3.1.3.4.1.1.

Therefore, the DPPH values were expressed as μmol Trolox equivalents (TE) per gram of extract and they were reported as mean and standard deviation (n=3).

3.2.3.3.1.2. ABTS assay

See section 3.1.3.4.1.2.

Samples were prepared by dissolving 5 mg (weighed with a precision of 0.1 mg) in 1 ml of distilled water. Leaves and branches samples were diluted 1:200 with distilled water prior to be analysed, while EDYNEA02 sample was diluted 1:50 and seeds sample was diluted 1:20.

Results were expressed as µmol Trolox equivalents/gram of extract.
Mean and standard deviation (n=3) were calculated.

3.2.3.3.2. Antibacterial activity against *Propionibacterium acnes*

The antibacterial activity of aqueous extracts of *M. oleifera* was assessed according to the method of Chomnawang et al., with few modifications[79]. 37 grams of brain heart infusion medium (BHI) was dissolved in 1 L of distilled water and it was autoclaved at 121°C for 15 minutes. 12 ml of the medium were put into a vial and a small aliquot of this amount was used to rehydrate *Propionibacterium acnes* (ATCC®6919™). The bacterium was added into the vial and it was incubated for 72 h, at 37°C under anaerobic conditions. Its concentration was then adjusted to yield approximately $1.0x10^8$ CFU/ml with physiological solution.
Agar was added to BHI medium (15g/L) and the solution was autoclaved. 25 ml of BHI-agar solution was poured over a Petri dish and it was left to solidity.
The streak plate disc diffusion method (SPDD) reported by Othman et al.[80] was followed, with modifications.
One hundred microliters of inoculum, equivalent to 10^8 CFU was then pipetted onto the BHI-agar. A sterile plastic swab was used to inoculate the BHI-agar plate by streaking over the surface with rotation to ensure even distribution of the inoculum.

Concerning the preparation of test samples, it was first tested the activity of the isolated isothiocyanate (0.5-1000 µg/ml) against *P. acnes*, using clindamycin hydrochloride (0.5-1000µg/ml) as positive control and the pure solvent (distilled water) as negative control. These assays were performed in duplicate.

Then extracts were tested: the first time the extracts of branches, leaves, seeds and EDYNEA02 were dissolved in distilled water at two different concentrations (20 mg/ml and 5 mg/ml). Distilled water was used as negative control and clindamycin hydrochloride in distilled water was used as a positive

control. The second time, the extracts were dissolved in DMSO/H_2O:1/1 at the same concentrations indicated above. Therefore DMSO/H_2O:1/1 was used as negative control. A third test was carried out only with the extract of branches, as it showed some activity during the second trial, thus requiring further control. Therefore, the extract of branches was dissolved in DMSO/H_2O:1/1 at the final concentrations of 20 mg/ml and 5 mg/ml. The negative control was DMSO/H_2O:1/1 and the positive control was clindamycin hydrochloride (50-200 μg/ml) in DMSO/H_2O:1/1.

The solutions were sterilized by filtering them with 0.22μm sterile syringe filters. 50 μl of each solution was deposited on a sterile paper disc and the disc was placed on the agar. Plates were then incubated at 37°C for 72h under anaerobic conditions (nitrogen atmosphere). All the operations were performed under a sterile hood.

3.3. Third project line: oilseeds and oily extracts

3.3.1. Plant material and drying

Seeds of *H. sabdariffa* were provided by the company MANTIS AGROPY S.A., located in Guairà Department, Paraguay. The samples were harvested, collected and dried at 59°C for 17 hours in MANTIS AGROPY S.A., Py.

The dried material was then pulverized with a mill and stored in a dark, dry and fresh place.

The following varieties were used in the first part of the study:

1. Fortìm
2. Tenonderà
3. Koape guaré

To perform the second part of the study, the varieties showed below were used:

4. Kibeleza
5. Che la Reina
6. Tenonderà Tempranera

and Tenonderà variety was used as a reference in this case.

Figure 39: *H. sabdariffa* seeds

With the exception of Fortìm (native to Brazil) and Koape guarè (native to the equatorial region of Africa), all other varieties were native to Paraguay. The seeds used in the first part of the study were collected in 2015 while the seeds used in the second part of the study were collected in 2017.

Seeds of *M. oleifera*, "green" variety, air-dried in the dark, were provided by Moringa Guaraní S.A. (YGUA), located in Compañía Colonia Piraretá, Piribebuy District, Cordillera Department, Paraguay. They were pulverized with a mixer and stored in a dry, dark and fresh place.

To perform the second part of the study, *H. sabdariffa* dried leaves were provided by the company MANTIS AGROPY S.A.

The dried material was then pulverized with a mill and stored in a dark, dry and fresh place.

On the other hand, the leaves of *M. oleifera* "green variety" were provided by Moringa Guaraní S.A. (YGUA). They were pulverized with a mixer and stored in a dark, dry and fresh place.

EDYNEA03 was provided by EDYNEA s.r.l, Trissino, Italy.

H. sabdariffa Kibeleza oil and *M. oleifera* oil were extracted from seeds that were provided by the company MANTIS AGROPY S.A. Refined olive oil was purchased at Fagron and deodorized sunflower oil was purchased at a local market.

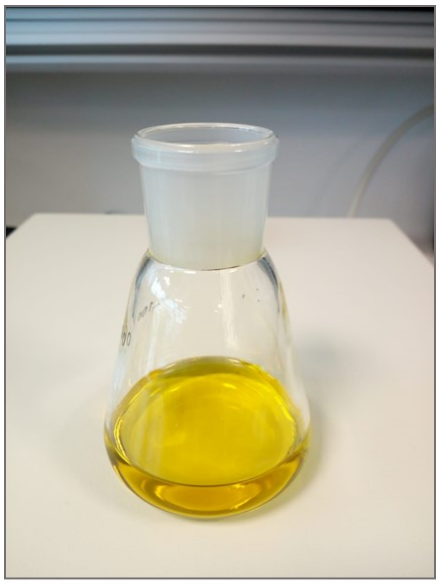


Figure 40*: H. sabdariffa* seed oil

3.3.2. First part of the study

The aim of the work was the selection of a variety of *H. sabdariffa* seeds with a high oil percentage and alfa-tocopherol content, in comparison with *M. oleifera* seeds.

3.3.2.1. Oil content determination in oilseeds

3.3.2.1.1. Determination by NMR

Oil content was quantified by nuclear magnetic resonance (NMR) analysis, according to the method reported by Wang et al.[61], with modifications.

The oil content was measured on a Bruker minispec mq20 NMR analyser that was maintained at 40°C. For the acquisition of the signals, it was used a spin-echo sequence, with a τ of 3.5 ms (except for Tenonderà that had a τ of 10 ms), a recycle delay of 2 s and a total of 16 scans. For establishing an oil standard curve, standards were prepared by weight. For each standard, a pulverized oil-less matrix was added to each sample tube along with a carefully measured mass of oil (*H. sabdariffa or M. oleifera*). For the measurements of the oil percentage, sample of pulverized seeds were carefully weight and added to sample tubes.

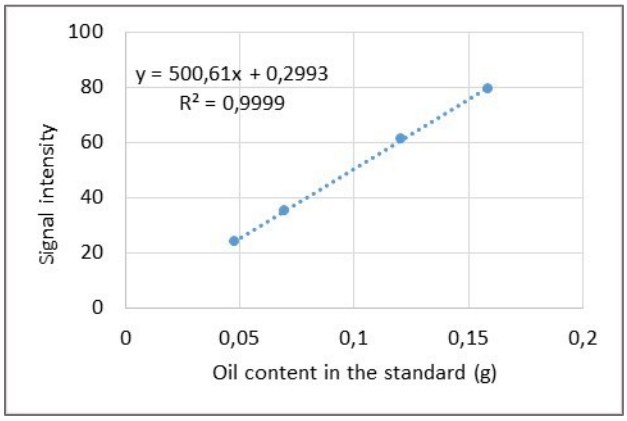


Figure 41: Calibration curve for the determination of the oil percentage in *M. oleifera* seeds.

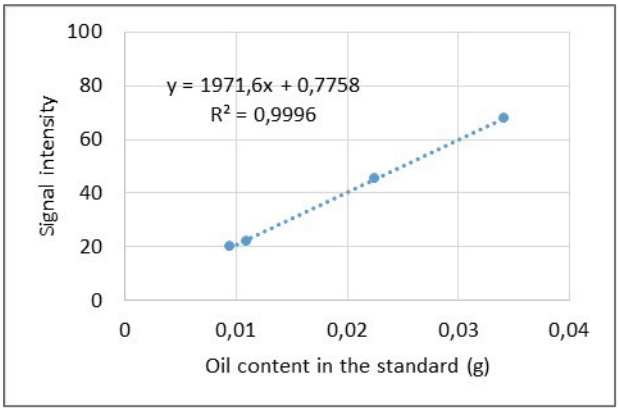


Figure 42: Calibration curve for the determination of the oil percentage in *H. sabdariffa Fortìm* seeds.

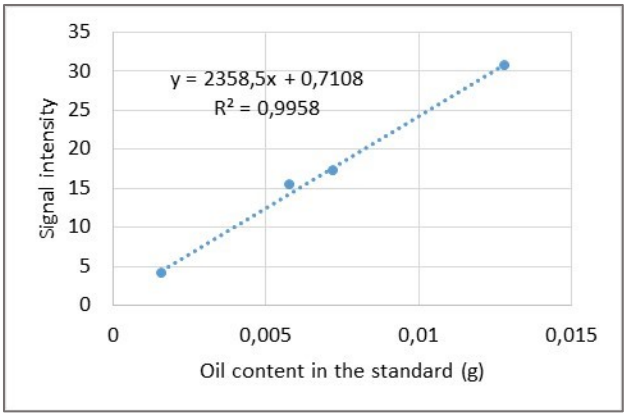


Figure 43: Calibration curve for the determination of the oil percentage in *H. sabdariffa Koape guarè* seeds.

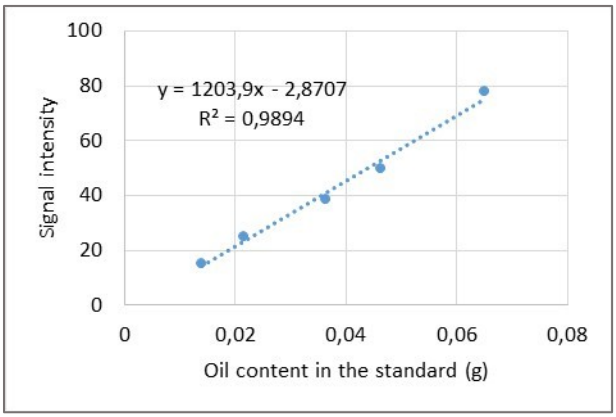


Figure 44: Calibration curve for the determination of the oil percentage in *H. sabdariffa Tenonderà* seeds.

3.3.2.2. Extraction of oil

The extraction of the oils was performed according to a procedure reported by Mohamed et al[81]. Ground seeds were wrapped in filter paper and they were extracted for 8h with n-hexane, by a Soxhlet apparatus. After that, the extract was filtered by filter paper and it was dried in a rotary evaporator at 35 °C.

The oils were stored at +4°C in the dark, for further analysis.

The extractions were done in triplicate.

Extraction yield was calculated with the following formula:

$$\frac{grams\ of\ oil}{grams\ of\ ground\ seeds} * 100$$

Results were expressed as mean and standard deviation (n=3).

3.3.2.3. Characterization

3.3.2.3.1. HPLC-DAD analysis of tocopherols in *M. oleifera* and *H. sabdariffa* seed oil

The determination of tocopherols in the oils was performed according to the method reported by Darnet et al.[82], with modifications.

It was used a Varian ProStar 325-Uv HPLC system, with a Zorbax 300SB-C18 (4.6 x 250 mm; 5µm) column. It was applied an isocratic elution (95:5/MeOH:H_2O), with a flow rate of 1 ml/min. The injection volume was 20 µl.

The quantification of the compounds was performed with the external calibration method: calibration curves were obtained with analytical standards at four different concentrations.

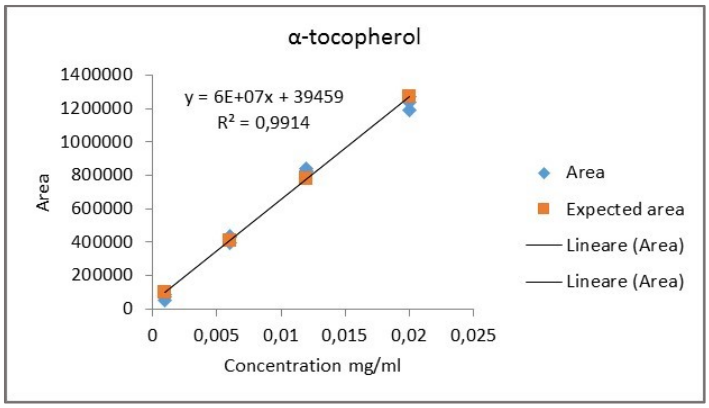


Figure 45: α-tocopherol calibration curve on a Varian ProStar 325-Uv HPLC system.

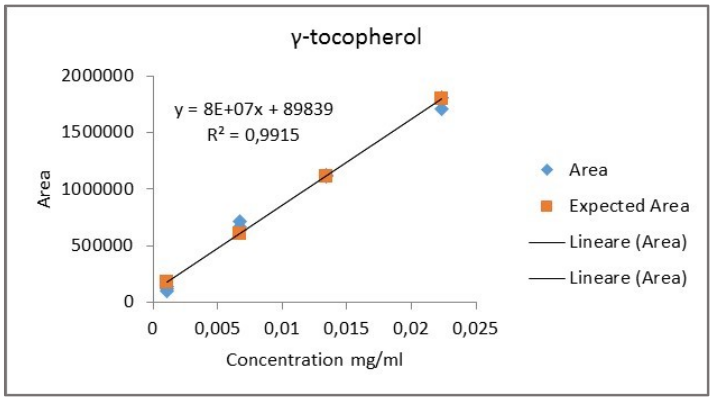


Figure 46: γ-tocopherol calibration curve on a Varian ProStar 325-Uv HPLC system.

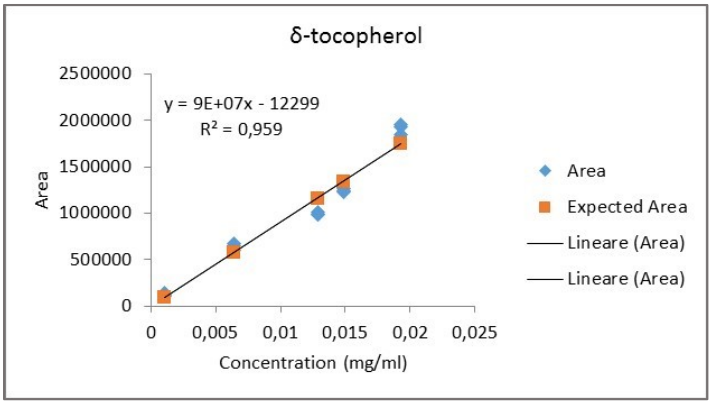


Figure 47: δ-tocopherol calibration curve on a Varian ProStar 325-Uv HPLC system.

For the analysis of the sample, it was followed the method by Gimeno et al[83]. The oil was diluted 1:10 in n-hexane. Then, the hexane solution was added to a mixture of methanol/ethanol in the ratio n-hexane/methanol/ethanol=1/3/1. After vortex mixing and centrifugation at 3000 rpm for 5 minutes, the sample was filtered with a 0.45μm filter and it was injected in HPLC. Compounds were identified from retention time and detection was done at 292nm.

3.3.3. Second part of the study

The first objective of the work was the selection of a variety of *H. sabdariffa* seeds with a high oil percentage and alfa-tocopherol content, compared to Tenonderà variety that had been selected in the first part of the study. The second objective was the extraction of the oil from the selected *H. sabdariffa* variety, the extraction of *M. oleifera* oil and the characterization of their main antioxidant compounds, in comparison with two frequently used oils in nutrition and in cosmetic field. The third objective was the preparation of oil extracts enriched with antioxidants, the characterization and evaluation of the radical scavenging activity.

3.3.3.1. Preliminary selection of a variety of *H. sabdariffa* seeds

3.3.3.1.1. Oil content determination in seeds by NMR

Oil content was quantified by nuclear magnetic resonance (NMR) analysis, according to the method reported by Wang et al.[61], with modifications.

The oil content was measured on a Bruker minispec mq20 NMR analyser that was maintained at 40°C. For the acquisition of the signals, it was used a spin-echo sequence, with a τ of 3.5 ms, a recycle delay of 2 s and a total of 16 scans. For establishing an oil standard curve, eight standards were prepared by weight. For each standard, a pulverized oil-less *H. sabdariffa* matrix was added to each sample tube along with a carefully measured mass of oil of *H. sabdariffa*. For the measurements of the oil percentage, sample of pulverized seeds were carefully weight and added to sample tubes.

Standard	***H. sabdariffa powder (g)***	***Oil (g)***	***Oil (%)***
1	0.3161	0.0285	8.27
2	0.3167	0.0494	13.49
3	0.3355	0.0648	16.19
4	0.3276	0.0926	22.04
5	0.3329	0.1194	26.40
6	0.2821	0.1603	36.23
7	0.2961	0.2033	40.71
8	0.3113	0.2765	47.04

Table 10: Standards used for the preparation of the calibration curve in a minispec mq20 Bruker.

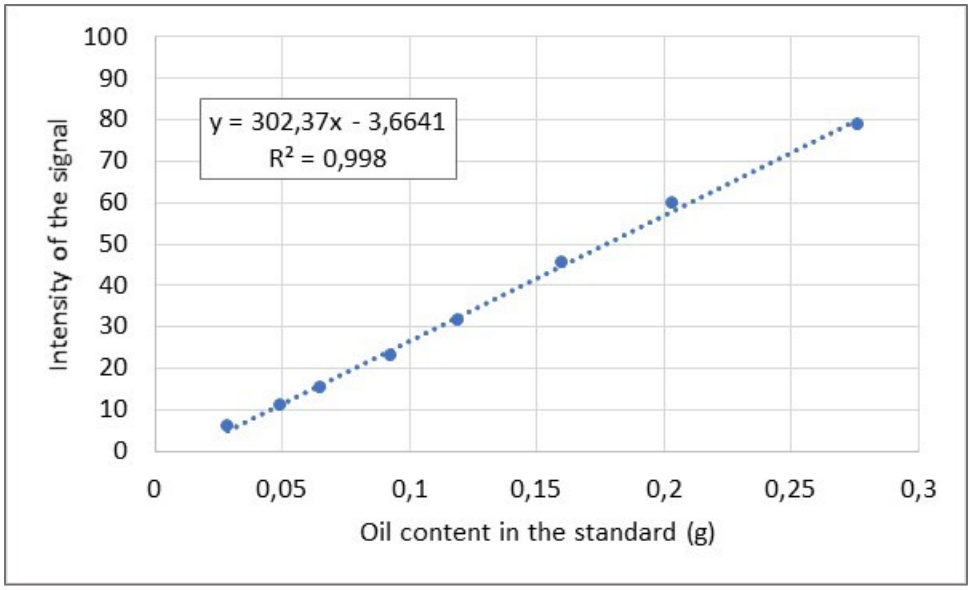


Figure 48: Calibration curve for the determination of the oil percentage in *H. sabdariffa* seeds.

3.3.3.2. Extraction of oil

The extraction of the oils was performed according to a procedure reported by Mohamed et al[81]. Ground seeds were wrapped in filter paper and they were extracted for 8h with n-hexane, by a Soxhlet apparatus. After that, the extract was filtered by filter paper and it was dried in a rotary evaporator at 35 °C.

The oils were stored at-20°C in the dark, for further analysis.

The extractions were done in triplicate.

Extraction yield was calculated with the following formula:

$$\frac{grams\ of\ oil}{grams\ of\ ground\ seeds} * 100$$

Results were expressed as mean and standard deviation (n=3).

3.3.3.3. Characterization

It was performed the determination of tocopherols in *H. sabdariffa* oils, to identify the variety with the highest tocopherol content, especially α-tocopherol, which is the most biologically active isomer.

3.3.3.3.1. HPLC-DAD analysis of tocopherols in *H. sabdariffa* seed oil

The determination of tocopherols in the oil was performed according to the method reported by Gimeno et al., with modifications[83].

It was used an Agilent 1260 Infinity HPLC system, with a Zorbax SB-C18 4.6x 150 mm, 3.5 µm column. It was applied a gradient elution, according to the condition reported in Table 11, with a flow rate of 1 ml/min. The temperature of the column compartment was set at 25°C and the injection volume was set at 20 µl.

Time (minutes)	***% phase A (milli-Q water)***	***% phase B (Methanol)***
0	5	95
10	5	95
12	0	100
55	0	100

Table 11: Timetable of solvents gradient for the elution of tocopherols in vegetable oils.

The quantification of the compounds was performed with the external calibration method: calibration curves were obtained with analytical standards at four different concentrations.

Standard	***Linearity range (mg/ml)***	***R^2***
α-tocopherol	0.0024-0.0243	0.9994
γ-tocopherol	0.0032-0.0325	0.999
δ-tocopherol	0.0019-0.0191	0.8599
α-tocopherol acetate	0.0020-0.0197	0.9964

Table 12: Linearity ranges and R^2 values of tocopherols standards that were used for the external calibration

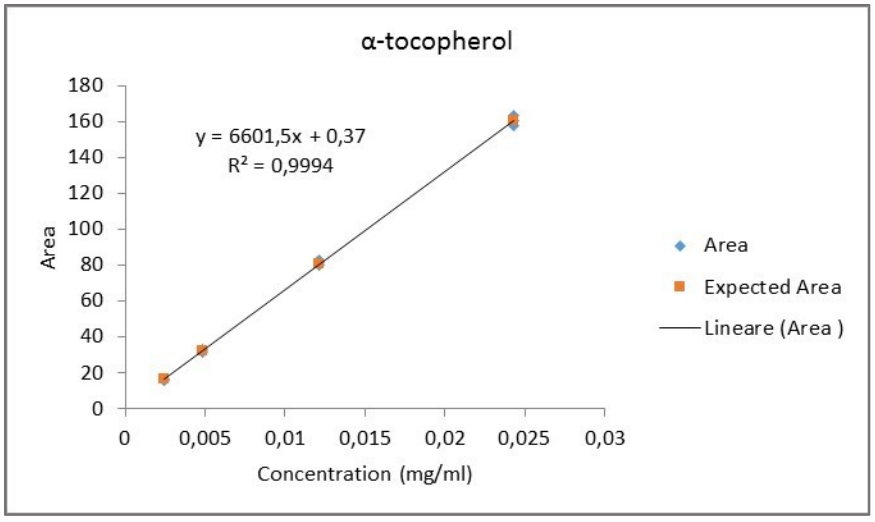


Figure 49: α-tocopherol calibration curve on an *Agilent 1260 Infinity* HPLC system.

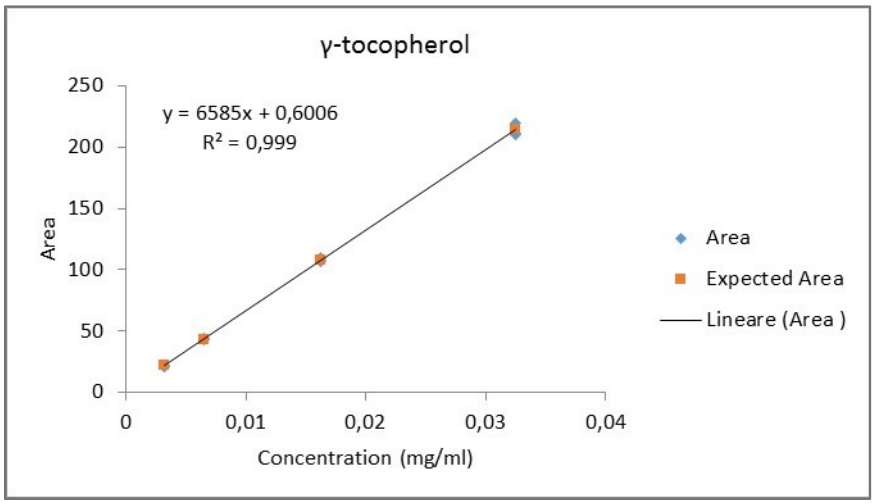


Figure 50: γ-tocopherol calibration curve on an *Agilent 1260 Infinity* HPLC system.

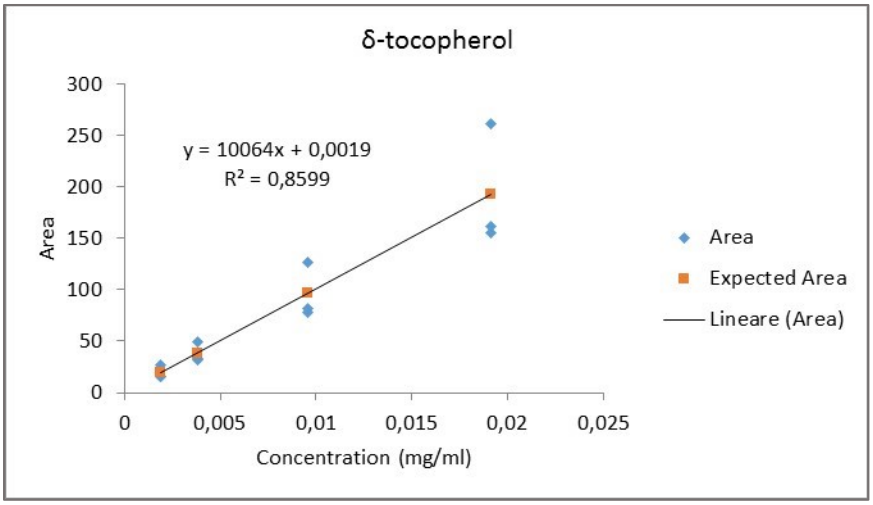


Figure 51: δ-tocopherol calibration curve on an *Agilent 1260 Infinity* HPLC system.

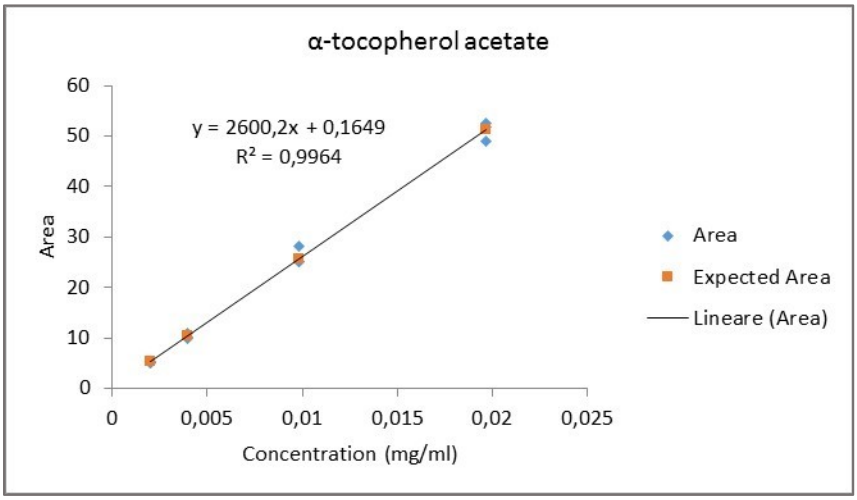


Figure 52: α-tocopherol acetate calibration curve on an *Agilent 1260 Infinity* HPLC system.

For the analysis of the sample, the oil was diluted 1:10 in n-hexane. Then, the hexane solution was added to a mixture of methanol/ethanol in the ratio n-hexane/methanol/ethanol=1/3/1. After vortex mixing and centrifugation at 3000 rpm for 5 minutes, the sample was filtered with a 0.45μm filter and it was injected in HPLC. Compounds were identified from retention time and UV spectra and detection was done with a diode array detector at 292nm for α, (β+γ) and δ tocopherols and at 286nm for α-tocopherol acetate.

Finally, it was measured the recovery of α-tocopherol from a matrix chemically similar to an oil (petroleum ether): a solution of α-tocopherol in petroleum ether was diluted 1:5 with n-hexane. 1ml of this solution was added to 3 ml of methanol and 1 ml of ethanol. The sample was vortex mixed, centrifuged at 3000 rpm for 5 minutes, filtered and injected in HPLC system.

3.3.3.4. Preparation of oily extracts enriched in antioxidant compounds

3.3.3.4.1. Extraction

3.3.3.4.1.1. Extraction of *M. oleifera* and *H. sabdariffa* oil

The extraction of the oils was performed according to a procedure reported by Mohamed et al[81]. Ground seeds were wrapped in filter paper and they were extracted for 8h with n-hexane, by a Soxhlet apparatus. After that, the extract was filtered by filter paper and it was dried in a rotary evaporator at 35 °C.

The oils were stored at + 4°C in the dark, for further analysis.

3.3.3.4.1.2. Maceration

The first trial for the preparation of an oily extract was performed according to a method reported in literature[84] and based on the traditional procedure followed to obtain oleolites.

The extract was prepared by extracting *M. oleifera* ground leaves with refined olive oil, for 30 days, at room temperature, with the ratio drug/solvent=1:/10 The extract was stirred with a magnetic stirrer and it was gently shaken by hand daily. Finally, the extract was centrifuged at 4500 rpm for 1h and the surnatant was separate from the solid powder. The oleolite was stored in an opaque bottle, in a fresh place until further analysis.

3.3.3.4.1.3. Ultrasound assisted extraction (UAE)

The second trial for the preparation of oily extracts was performed according to the method reported by Goula et al.[85], with few modifications. Briefly, the extracts were prepared by dispersing the drug in oil in a final concentration of 0.10 g/ml and soaking the sample tube for 30 minutes, at 51°C, in an ultrasonic bath. The extracts were then filtered by 0.45μm filter and stored at +4°C in the dark until further analysis.

3.3.3.4.2. Characterization

3.3.3.4.2.1. HPLC-DAD analysis of α-tocopherol

The determination of α- tocopherol in the oil was performed according to the method reported by Gimeno et al., with modifications[83].

It was used an Agilent 1260 Infinity HPLC system, with a Zorbax SB-C18 4.6x 150 mm, 3.5 µm column. It was applied a gradient elution, according to the condition reported in Table 13, with a flow rate of 1 ml/min. The temperature of the column compartment was set at 25°C and the injection volume was set at 20 µl.

Time (minutes)	***% phase A (milli-Q water)***	***% phase B (Methanol)***
0	5	95
10	5	95
12	0	100
55	0	100

Table 13: Timetable of solvents gradient for the elution of tocopherols in vegetable oils.

The quantification of the compounds was performed with the external calibration method: the calibration curve was obtained with analytical standard at four different concentrations.

Standard	***Linearity range (mg/ml)***	***R^2***
α-tocopherol	0.0024-0.0243	0.9954

Table 14: Linearity range and correlation coefficient of α-tocopherol standard that was used for the external calibration.

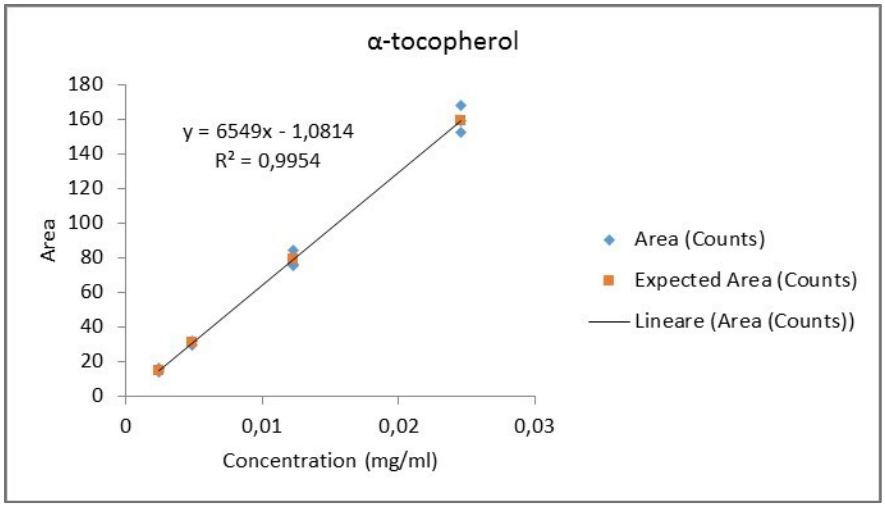


Figure 53: α-tocopherol calibration curve on an *Agilent 1260 Infinity* HPLC system.

For the analysis of the sample, the oils were diluted in n-hexane according to the ratios reported in Table 15. Then, the hexane solution was added to a mixture of methanol/ethanol in the ratio n-hexane/methanol/ethanol=1/3/1. After vortex mixing and centrifugation at 3000 rpm for 5 minutes, the sample was filtered with a 0.45μm filter and it was injected in HPLC. Compounds were identified from retention time and UV spectra and detection was done with a diode array detector at 292nm.
Finally, it was measured the recovery of α-tocopherol from a matrix chemically similar to an oil (petroleum ether): a solution of α-tocopherol in petroleum ether was diluted 1:5 with n-hexane. 1ml of this solution was added to 3 ml of methanol and 1 ml of ethanol. The sample was vortex mixed, centrifuged at 3000 rpm for 5 minutes, filtered and injected in HPLC system.

Sample	***Dilution in n-hexane***
OO, OO-ML, OO-HL, OO-EDYNEA03	1:5
SO, MO, HO and their oleolites	1:10

Table 15: Dilutions of oil samples in n-hexane.

3.3.3.4.2.2. Determination of total carotenoid content (TCC)

Total carotenoid content was determined according to the procedure reported by Szydłowska-Czerniak et al[73].

A stock solution of β-carotene was prepared at a final concentration of 0.1 mg/ml in n-hexane. Four different dilutions (1:50; 1:75; 1:100; 1:200) were prepared in n-hexane and they were used for the preparation of a calibration curve. The absorbance of each solution was read at 450 nm against a blank of n- hexane, in a spectrophotometer.

Standard	***Linearity range (mg/ml)***	***R^2***
β carotene	0.0005-0.002	0.8738

Figure 54: β-carotene calibration curve for the determination of TCC.

For the preparation of the samples, a carefully weight amount of oil or oil extract (0.2-4 g depending on the sample) was dissolved in a final volume of 5 ml of n-hexane. The absorbance of each sample was read in a spectrophotometer at 450 nm against a blank of n-hexane.

The final results of the TCC method were presented as mg β-carotene/100 g of oil or oil extract.

Results are reported as mean and standard deviation of at least four determinations.

3.3.3.4.2.3. Determination of total phenolic content (TPC)

For the preparation of the samples, it was followed the method reported by Szydłowska-Czerniak et al.[73]: test tubes with oils/ oil extracts (3.0 g weighed with precision of 0.1mg) and methanol (5ml) were shaken for 30 minutes at room temperature in the dark. The obtained extracts were separated from the oils in a freezer (- 18 °C, 30 minutes) and transferred quantitatively to a glass bottle. Each sample was extracted three times (total extraction volume 15 ml). All methanolic extracts were stored at 5°C in dark glass bottles, prior to analysis.

The determination of total phenols in the methanolic extracts of the oily samples was performed according to the method of Folin-Ciocalteu[77], with modifications.

Briefly, 1.25 ml of Folin-Ciocalteu reactant (previously diluted 1:10 v/v with distilled water) were added to 250 µl of each extract and the mixture was left at room temperature for 10 minutes. After that, 1ml of Na_2CO_3 (7,5% p/v in distilled water) was added and the mixture was left at room temperature for 30 minutes, in the dark. At the end, the absorbance of the blue metal oxides was measured in a spectrophotometer at 650 nm. A blank sample was prepared using methanol instead of methanolic extracts. Gallic acid was used as reference standard for the preparation of a calibration curve and results were expressed as mg gallic acid equivalents (GAE)/ 100 g of oil or oil extract.

Results were expressed as mean ± standard deviation of at least three determinations.

It was also checked the absorbance of single extracts at 650nm: 250 µl of each extract solution were added to 2,25 ml of distilled water. Thus, the absorbance of extracts was read against 250µl of methanol and 2.25 ml of distilled water and it was subtracted to the absorbance of the samples treated with Folin-Ciocalteu reactant.

Standard	***Linearity range (µg/ml)***	***R^2***
Gallic acid	40-100	0.7318

Table 16: Linearity range and correlation coefficient of gallic acid.

3.3.3.4.3. Assessment of the properties

3.3.3.4.3.1. Total antioxidant capacity (TAC)

Total antioxidant capacity was measured by two different spectrophotometric assays: DPPH and $ABTS^{\bullet+}$

In both the assays, for the preparation of the samples, it was followed the method reported by Szydłowska-Czerniak et al.[73]: test tubes with oils/ oil extracts (3.0 g weighed with precision of 0.1mg) and methanol (5ml) were shaken for 30 minutes at room temperature in the dark. The obtained extracts were separated from the oils in a freezer (- 18 °C, 30 minutes) and transferred quantitatively to a glass bottle. Each sample was extracted three times (total extraction volume 15 ml). All methanolic extracts were stored at 5°C in dark glass bottles, prior to analysis.

3.3.3.4.3.1.1.. DPPH assay

According to the method reported by Povolo et al.[72], 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) was dissolved in methanol at a final concentration of 56 μg/ml. After that, it was prepared a stock solution of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) in methanol at a concentration of 0.1 mg/ml. Eight different dilutions of the stock solution were prepared (1:2; 1:4; 1:6; 1:8; 1:10; 1:15; 1:20; 1:25). Trolox solutions and sample solutions (OK diluted 1:2 and OKFM diluted 1:3) in methanol (150 μl) were added to DPPH methanolic solution (750μl) and to pure methanol (750 μl).

According to the method of Szydłowska-Czerniak et al.[73], a control sample was prepared using methanol instead of the extracts.

Absorbance of those solutions was read in a spectrophotometer at 515nm against a blank of methanol, after 5 minutes of reaction.

The scavenging of DPPH was calculated as reported in section 3.1.3.4.1.1.

The DPPH values were expressed as μmol Trolox equivalents (TE) per 100 g of oil sample and they were reported as mean and standard deviation (n=3).

3.3.3.4.3.1.2. ABTS assay

See section 3.1.3.4.1.2.

Oil samples were properly diluted in methanol. 200 μl of sample were added to 2000 μl of the ABTS solution in ethanol. After 10 minutes, the absorbance was read at 730 nm against a blank of 9 % methanol in ethanol. A control sample was prepared using methanol in the place of the oily extract. Results were expressed as μmol Trolox equivalents/ 100 g of oil sample.

Mean and standard deviation (n=4) were calculated.

sample	***Dilution***
OO, MO	1:2
SO, OO-HL, OO-EDYNEA03,	1:5
HO, SO-ML, SO-HL, OO-ML	1:10
MO-ML, HO-ML	1:50

Table 17: Dilutions of oil samples in $ABTS^{\bullet+}$ assay.

4. RESULTS AND DISCUSSION

4.1 First project line: *Hibiscus sabdariffa* L. calyces

4.1.1. First part of the study: preliminary selection of a variety of calyces rich in anthocyanins

It is known that the ability of a plant to synthesize its metabolites depends both on the plant variety and on the geographical area i.e. the local growing conditions, where the plant is cultivated[32]. Therefore, it is not completely correct to rely on literature data, if we are looking for information concerning the amount of a metabolite in a plant, in order to select the best source of a specific metabolite. In this first part of the study, three different varieties of *H. sabdariffa* L. calyces were studied. All of them were cultivated in the southern part of Paraguay, but the seeds came from three different geographical areas of the earth (Brazil, Paraguay and the equatorial area of Africa).

Therefore, it was important to perform a comparative study among these varieties of calyces in order to assess their anthocyanin content.

4.1.1.1. Extractions

A comparative study by Sindi et al.[67] highlighted that water (with or without the addition of formic acid in a final concentration of 1%) is the best solvent for the extraction of polyphenols, anthocyanins and total antioxidants from *H. sabdariffa* dried calyces, compared to methanol (with or without the addition of formic acid), (see Figure 55). In their study, an extraction temperature of 100°C, an extraction time of 10 minutes and a drug/solvent ratio: 1/100 were identified as the most suitable conditions for a high yield recovery of total phenolics (TPC), total anthocyanins (TMA) and total antioxidants.

Nevertheless, a study by Salazar-González et al.[70] showed that mixtures of water and ethanol (30-50% water) give higher extraction yields of TMA and TPC than pure water, when a lower extraction temperature (e.g. room temperature) and a longer extraction time (e.g. 2 hours) are applied.

Even Camelo-Mendez et al.[86] found out that pure ethanol is the best solvent for the extraction of anthocyanins.

In general, polar pure solvents or mixtures are more suitable for the extraction of anthocyanins than less polar ones (such as ethyl acetate and hexane), and the differences in the observed results depend on the different extraction procedures, too[67].

Since the objective was the preparation of plant extracts with green and biocompatible solvents, it was decided to use water. Even more, aqueous extracts are easier to be used for formulation purposes[87]. Therefore, the conditions reported by Sindi et al.[67] were applied for the preparation of aqueous extracts of the different varieties of calyces.

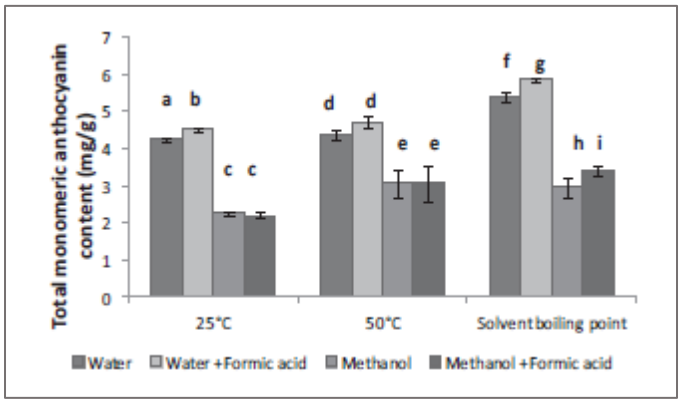


Figure 55: TMA values, measured in aqueous and methanolic extracts of *H. sabdariffa* calyces (with or without formic acid)[67].

In this case, as the extracts were prepared for analytical purposes, it was decided to add 1% formic acid to the extraction medium, as the study reported in literature determined a significant difference in the TMA value, when using water or acidified water. In the last case, the measured value was averagely higher than the TMA value measured without adding formic acid: the addition of acid helps anthocyanins to form the flavylium cation and eliminates degradation, thus increasing the amount of extracted compounds[88].

4.1.1.2. Characterizations

4.1.1.2.1. SPE-mediated enrichment of the extracts

Before starting the analysis of the extracts, it was decided to perform a concentration of the samples (relative to the amount of anthocyanins) by solid-phase extraction (SPE), for an easier determination of the compounds of interest.

SPE is a technique based on the use of a solid stationary phase (packed into a column) to adsorb compounds of interest and/or contaminants inside an extract. The application of a gradient of solvents with increasing solvent strength makes it possible to elute separately the contaminants and the compounds of interest. In this way, it is possible to obtain a final extract more concentrated in the compounds of interest.

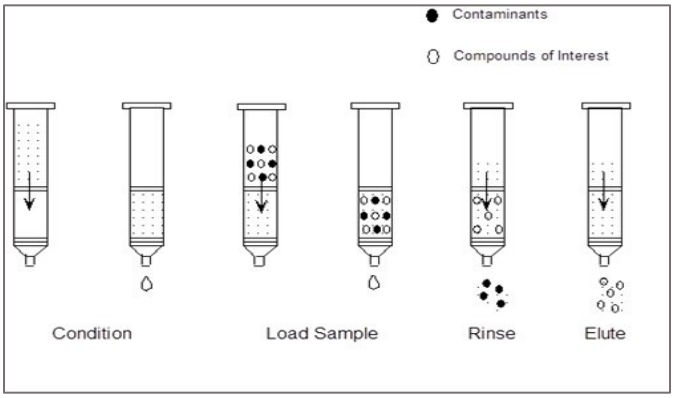


Figure 56: Scheme of an SPE-mediated enrichment of a sample in the compounds of interest[89].

In this work, a hydrophobic stationary phase was used, with a C-18 matrix attached to silica (C-18 Sep-Pak®), according to the method reported by Salazar-González et al[70]. Before inserting the sample inside the SPE column, it was necessary to condition the system with suitable solvents: first of all a strong solvent was used to wet the sorbent phase and then activate it. Without this last passage the aqueous solvent would not be able to penetrate the hydrophobic surface and wet the stationary phase. Subsequently, a weak solvent, such as water or an aqueous solution (e.g. hydrochloric acid 0.01% in water, as used in this case) was made flow through the column. Once this step has been carried out, it was possible to load the sample, so both contaminants and compounds of interest would immediately have been retained by the sorbent phase (by VdW interactions). The rinsing phase

allowed the removal of undesired compounds, by using a weak solvent (0.01% hydrochloric acid in this work). For example, by using C-18 cartridges it was possible to remove sugars and acids from a raw extract[18]. In the elution phase, a stronger solvent (e.g. acidified methanol, 0.01% hydrochloric acid in this case) was used to elute the compounds of interest.

By SPE, it is also possible to separate anthocyanins from other flavonoids, with ethyl acetate elution[18]. Nevertheless, in this case the purpose was only to increase the concentration of anthocyanins in the extracts, and the elution step with water was enough, according to the procedure reported in literature.

4.1.1.2.2. Total monomeric anthocyanin (TMA)

The determination of monomeric anthocyanins is based on the ability of anthocyanins to shift in colour from bright red/bluish at pH 1 to nearly colourless at pH 4.5. In contrast, the polymeric anthocyanin forms retain considerable colour at pH 4.5. Therefore, the absorbance at the absorbance maximum of the sample (520 nm) at pH 4.5 is subtracted from that at pH 1, and the total monomeric anthocyanins are calculated based on the molecular weight and extinction coefficient of the most prevalent anthocyanin, or of an anthocyanin taken as a reference (e.g. cyanidin 3-O-glucoside)[18].

Even more, the absorbance of both the samples (pH 1 and pH 4,5) was read at a wavelength of 700 nm. It might be possible the presence of suspended colloidal material in the samples which could cause light scattering; it is therefore necessary to correct such a possible dispersion by carrying out a sample reading at 700 nm, wavelength in which there is no absorbance of total monomeric anthocyanins[36].

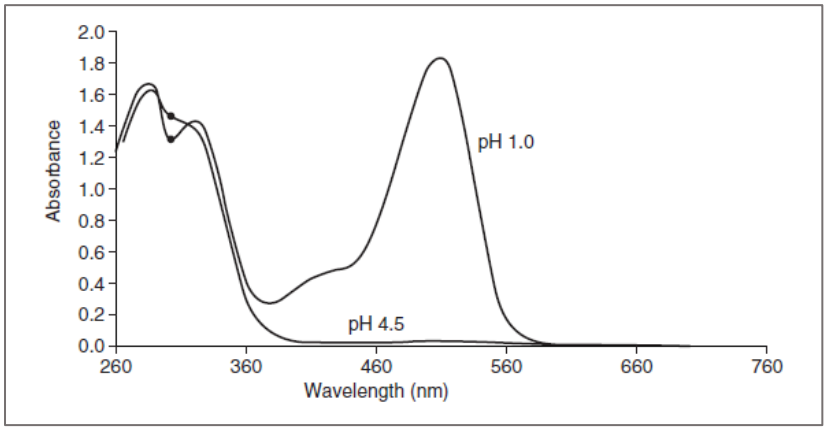


Figure 57: Spectral characteristics of purified radish anthocyanins in pH 1.0 and pH 4.5 buffers[36].

In this first part of the work it was analysed the TMA value of the SPE-enriched extracts of three different varieties of *H. sabdariffa* calyces. The highest value was observed for the extract prepared with Koape guarè calyces that is almost eight times higher than the values observed for the extracts prepared with the other two varieties.

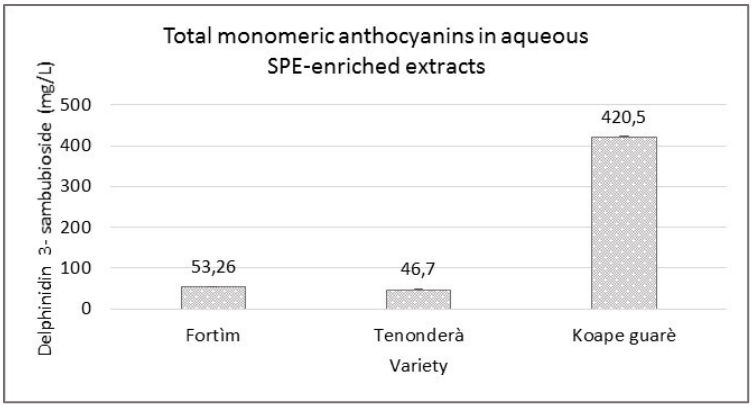


Figure 58: Total monomeric anthocyanin content in aqueous SPE-enriched extracts of *H. sabdariffa* calyces (Fortìm: Brazilian, Tenonderà: Paraguayan, Koape guarè: African).
Results are expressed as mean and standard deviation (n=3)

Koape guarè variety was selected at this point as a good raw material to be used as a source of anthocyanins for nutraceutical applications and its cultivation started in the South of Paraguay, in the property of the company.

However, Koape guarè variety highlighted the existence of some agronomic problems, during its cultivation, i.e. the tendency of this variety to bear fruits too close to winter. For this reason, the calyces are subjected to lowering of the temperature that is responsible for a damage of the calyces and a lower production yield.

Therefore, it was necessary to select another variety of calyces that may be suitable also to be cultivated in the agro-climatic conditions of Paraguay.

4.1.2. Second part of the study

The aim of the work was the selection of the variety of *H. sabdariffa* calyces, with the highest content of anthocyanins, among three new varieties, all native to Paraguay. Koape guarè variety (that had been selected in the previous part of the study related to this first line of the project) was considered the reference variety with which to make comparisons.

A further objective was the optimization of an extraction method to recover anthocyanins in high yield but that could be suitable for the production of extract on large scale, too.

A third objective was the selection of an enrichment method to obtain an anthocyanin-rich aqueous extract, to be exploited in the preparation of nutraceutical products with a higher concentration of the active compounds of *H. sabdariffa*.

In this study, water was chosen as solvent for the preparation of *H. sabdariffa* extracts, as it is a green solvent, biocompatible, universally accepted as a suitable solvent for the preparation of both nutraceutical and cosmetic products (then also accepted in those Islamic States where products deriving from ethanolic extractions are not appreciated) and, last but not least, a solvent with a high affinity for anthocyanins.

The extracts were dried by lyophilisation rather than by evaporation under vacuum at 40 °C using rotary evaporator, because the former technique guarantees greater protection of active principles, by working at much lower temperatures.

4.1.2.1. Selection of a variety

In order to identify a variety of calyces rich in anthocyanins for the substitution of the previously selected variety Koape guarè, three new varieties that are named Kibeleza, Che La Reina and Tenonderà Tempranera were studied. All of them were native to Paraguay.
Koape guarè variety was used as a reference in this part of the study.

The extracts were prepared according to the method reported by Sindi et al.[67], without adding formic acid. The addition of formic acid improves the extraction yield of anthocyanins that can be observed as significantly different. However, TMA value of the final extract could be judged comparable, considering the final objective is the production of an aqueous extract for human consumption, where it is preferable to use just water rather than other solvents.
In this case, the extracts of calyces were only prepared for analytical purposes, i.e. the selection of an anthocyanin rich variety. Nevertheless, since the final objective was the preparation of an aqueous extract for human consumption, it was decided to avoid the use of formic acid, in order to compare these results with the results that were obtained in the other sections of this study.

Finally, total monomeric anthocyanins were measured with the pH differential spectrophotometric method, described above.

In Figure 59, it is possible to observe that the extraction yields of the extracts prepared with the Paraguayan varieties were always higher than that of the reference (represented by the extract prepared with the variety native to the equatorial area of Africa). This may be explained by considering that *H. sabdariffa* calyces contain also polysaccharides in large quantities (e.g. pectin and mucilage) which are water-soluble molecules with a high molecular weight. It is reported in literature that different strains of calyces are characterized by different percentages of total polysaccharides[32] and that varieties from Senegal and Thailand are less rich in polysaccharides than strains from India and Central America[32]. Therefore, we can suppose that the observed differences between the extraction yields are due to different content of water soluble polysaccharides and phytochemicals of the calyces and then to the different geographic origins.
The statistical analysis of the results showed that there is no significant difference in the extraction yields of the three Paraguayan calyces (Tukey's post ANOVA, $p<0.05$)

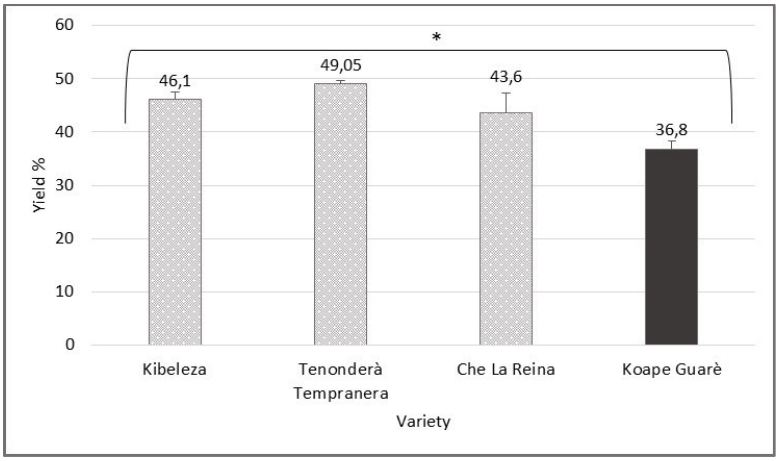


Figure 59: Extraction yields (%) of the extracts prepared from different varieties of *H. sabdariffa* calyces. Grey bars represent samples. Black bar represents the reference. Values are expressed as mean and standard deviation (n=3). (*) indicates a significant difference among the values (ANOVA, p<0.05)

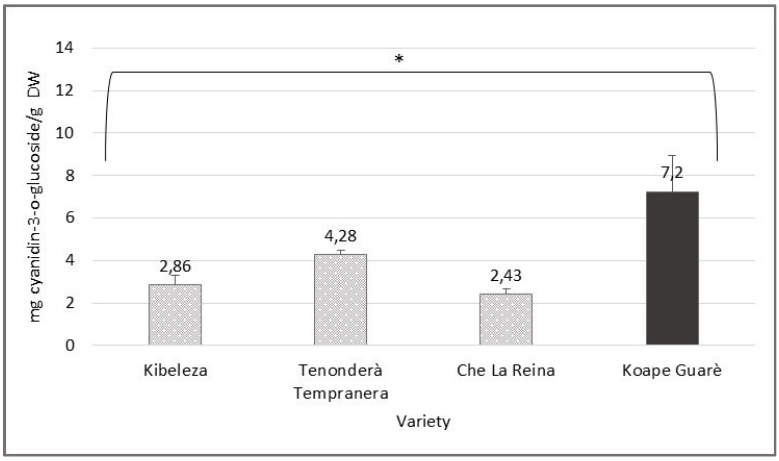


Figure 60: Total monomeric anthocyanin content of different varieties of *H. sabdariffa* calyces, expressed as mg cyanidin 3-O-glucoside equivalents/g DW. Grey bars represent samples. Black bar represents the reference. Values are expressed as mean and standard deviation (n=3). (*) indicates a significant difference among the values (ANOVA, p<0.05)

In Figure 60 are reported the TMA values measured for each extract. Values (expressed as mg cyanidin 3-O-glucoside/g DW) were reported as mean ± standard deviation and they varied from 2,43 ± 0,24 (Che La Reina) to 7,2 ± 1,7 (Koape guarè). The statistical analysis of the results showed that there was a significant difference between the TMA content of the Koape guarè calyces (reference) and the other three varieties of calyces. Nevertheless, there was no significant difference in the TMA content of the three Paraguayan strains of calyces (Tukey's post ANOVA, $p<0.05$).
The differences in the observed TMA values depend on the different geographical origins of the plants, as the ability of a plant to produce antioxidant compounds is also functional to its own protection against UV rays that are related to the latitude where the plant grows.

Since no significant difference was observed in the TMA content of the three Paraguayan varieties, Tenonderà Tempranera was selected as a raw material to be used in the preparation of *H. sabdariffa* aqueous extracts, because of its greater simplicity in cultivation and high rate of flowering, showed in the area of cultivation (Paraguay).

4.1.2.2. Optimization of an extraction method for a large-scale production of aqueous extract of calyces with a high anthocyanin content

In this case the objective was the optimization of extraction parameters suitable for the recovery of anthocyanins in high yield from dried calyces, but also transferable on a large-scale production of extract.

Nowadays, there is few or no literature concerning the preparation of aqueous extracts of *H. sabdariffa* calyces on large-scale, for nutraceutical purposes as a source of anthocyanins. Actually, the calyces of this plant are mainly used as they are in a dried form, for the production of tea bags, or they are used for the preparation of extracts that find later an application in the beverage industry, but without any standardization in anthocyanins[90,91,92]. Therefore, it is not possible to understand if the extraction conditions that others apply can ensure a high recovery of these polyphenols.

In this part of the study, the extraction parameters that are reported in section 4.1.2.1. were taken as a reference. As previously explained, a lower extraction time together with a higher extraction temperature represent a better operating condition when water is chosen as solvent (see section 4.1.1.1.) in terms of anthocyanin recovery. Nevertheless, those conditions are not easily transferable on large-scale.

Therefore, three new extraction experiments were performed, trying to preserve the TMA value that is possible to measure when extracts are prepared according to the reference condition.

Extraction	***Ratio (g/ml)***	***Temperature (°C)***	***Time (minutes)***	***Solvent***	***Technique***
Reference	1:100	100	10	Water	Stirring in a flask
1	1:100	60	60	Water	Stirring in a flask
2	1:100	60	15	Water	UAE, 40 KHz
3	1:100	25	15	Water	UAE, 40 KHz

Table 18: Extraction conditions for the preparation of *H. sabdariffa* aqueous extracts. (UAE) indicates an ultrasounds-assisted extraction.

In the first experiment, extraction time was increased (10-60 minutes) to meet the needs of large-scale production. On the other hand, extraction temperature was reduced (100-60°C) in order to protect anthocyanins from damages due to excessive exposition to high temperatures.

In the second and third experiments, ultrasounds (40 KHz frequency) were applied on the extraction system. Ultrasounds-assisted extractions were performed at two different temperatures (60°C and 25 °C respectively), with an extraction time of 15 minutes. The use of ultrasound waves for anthocyanin extraction from *H. sabdariffa* calyces can also help to improve process efficiency[93], thus justifying a reduction in the extraction time compared to the first extraction experiment. Even more, it was verified the possibility of carrying out industrial extraction with these times, by using ultrasounds.

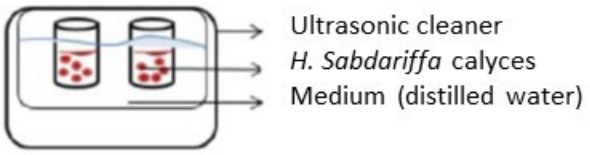


Figure 61. Ultrasonic extraction equipment set[93].

Sonication is widely used for extraction of various substances from plant material. It accelerates the penetration of solvent into cells and the release of components from cells into the solvent, and simultaneously enhances the mass transfer rate significantly[94].

Vibration on the extraction system generated by ultrasonic method provides intensive process agitation. This agitation increases the osmosis between the material and the solvent and as a result, enhance the efficiency of the extraction process[93]. An ultrasonic method with a frequency of 36 kHz can destroy vegetal cells, thus accelerating the process of mass transfer of bioactive compounds from cells to solvents. High frequency results in cavitation in the water medium, and the ultrasonic cavitation generates a breaking force that destroys the cell wall mechanically. Therefore, the transfer of material increase[93].

The analysis of the results showed that there was no significant difference in the extraction yield, calculated for each extraction procedure (ANOVA, Tukey's post ANOVA, $p<0.05$). Values (reported as mean ± standard deviation) ranged from 36,8 ± 1,4% and 39,4 ± 3,7 % yield.

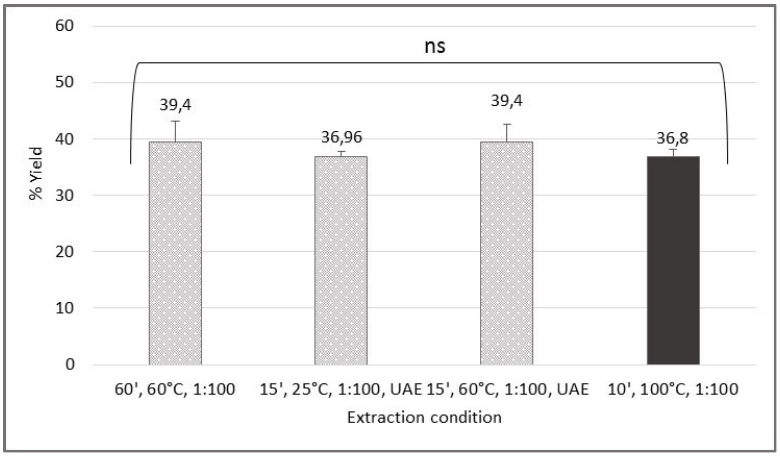


Figure 62: Extraction yields of the extracts prepared by following the conditions reported in Table 18. Grey bars represent samples. Black bar represents the reference. Values are expressed as mean and standard deviation (n=3). (ns) indicates a significant difference among the values (ANOVA, $p<0.05$)

Similarly, no significant difference was observed in the TMA content of the extracts (ANOVA, Tukey's post ANOVA, $p<0.05$). TMA values (expressed as mean ± standard deviation) ranged from 6,17 ± 0,72 to 8,7 ± 1,4 mg cyanidin 3-O-glucoside/g DW.

Therefore, no significant difference in the TMA values of the extracts obtained by ultrasounds at two different temperature was observed.

This result is in contrast with the study of Aryanti et al.[93]: they observed an increasing anthocyanin concentration by increasing the temperature of extraction, for a determined extraction time. They explained their results taking into account the effect of temperature on the diffusion coefficient and solubility of phytochemicals (that increase) and on the viscosity of the solvent (that decrease). Nevertheless, they performed their extractions with a ratio drug/solvent: 1/8 and 1/4[93]. In our case the ratio was 1/100 that means that there was a lot of solvent available for the extraction of the anthocyanins and that the concentration gradient stayed high longer, thus driving the diffusion of the phytochemicals toward the extraction medium, out of the matrix, also maintaining a high extraction rate.

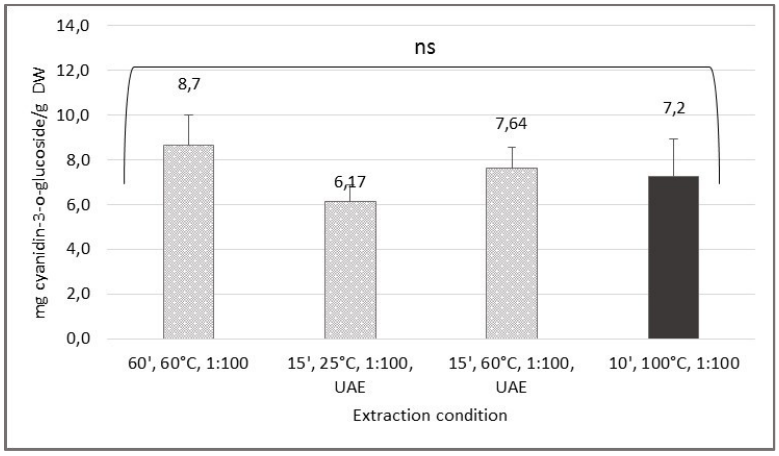


Figure 63: Total monomeric anthocyanin content of the extracts prepared by following the conditions reported in Table 18, expressed as mg cyanidin 3-O-glucoside equivalents/g DW. Grey bars represent samples. Black bar represents the reference. Values are expressed as mean and standard deviation (n=3). (ns) indicates a significant difference among the values (ANOVA, $p<0.05$)

Since there was no significant difference in the TMA content of the extracts depending on the extraction method applied, a choice was made based on economic and marketing reason. Therefore, it was considered suitable for a large-scale production of extract an extraction with water, at 25 °C, for 15 minutes, with the ultrasonic waves. Ultrasonic extraction could be claimed as a plus. Lower temperature during process represents a less expensive industrial operation.

4.1.2.3 Selection of an enrichment condition

In this case, the objective was the preparation of an extract enriched in anthocyanins, to be exploited as a more active ingredient for the nutraceutical or cosmetic field.

Therefore, polyvinylpolypyrrolidone (PVPP) was used to concentrate anthocyanins in the extracts.

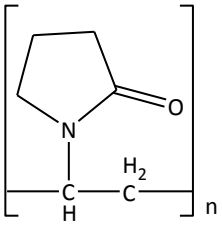


Figure 64: Chemical structure of PVPP

PVPP is a material, available in commerce, obtained by crosslinking polyvinylpyrrolidone (PVP). This polymer is biochemically inert and has no hazards associated, as long as it is known[95].

It is known that PVPP can behave as adsorbent of anthocyanins[96] and other classes of polyphenols[95]. The adsorption is mediated by hydrogen-bonds between the proton-donor from polyphenols (e.g. Hydroxyl groups) and the carbonyl group from PVPP rings[95]. Even more, polar and hydrophobic interactions between the aromatic ring of the polyphenols and the ring of the PVPP are involved[95].

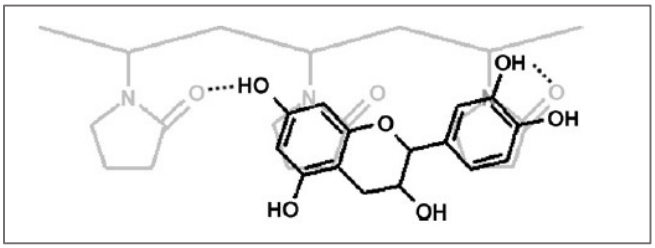


Figure 65: Adsorption of catechin by PVPP through hydrogen bonding between the proton-donor from the polyphenol and the carbonyl group from PVPP, together with π-bond overlap (delocalized electrons) polar and hydrophobic interactions between the aromatic ring of catechin and the PVPP ring[95].

It is reported in literature that the binding of polyphenols to PVPP is more efficient if the compounds are in a pure water solution, with a pH value low enough for the suppression of the polyphenolic hydroxyl groups ionization (indicatively pH<4)[95]. In organic solvents like methanol, PVPP showed a lower recovery efficiency for polyphenols that found an explanation in the strong capacity of the organic solvent to promote the desorption of these phytochemicals from PVPP[95].

In this work, water was chosen as solvent for the preparation of the extracts and the desired pH value was already guaranteed by the presence of a high percentage of organic acids (e.g. citric acid, hydroxycitric acid, hibiscus acid, malic acid) in the calyces. This could be seen also in the brilliant red colour of the extracts, due to the presence of the flavylium cation form of anthocyanins that exist at pH lower than 4. Therefore, the extracts prepared in this study were suitable for the adsorption activity of PVPP.

The extracts from *H. sabdariffa* calyces were treated with PVPP until they became colourless. The concentration of PVPP (30 mg/ml) was chosen according to data reported in literature[95]. After that, different elution systems were applied to the anthocyanin-PVPP complex, in order to recover the compounds of interest.

Sample	***Elution solvent mixture***
Standard	No purification method
Condition 1	Acetone: water(70:30 v/v)
Condition 2	EtOH: water (50:50 v/v)
Condition 3	EtOH: water (50:50 v/v) plus 1% acetic acid
Condition 4	Initial washing with water, followed by elution with EtOH: water (50:50 v/v) plus 1% acetic acid

Table 19: Different solvent systems for the elution of anthocyanins from PVPP.

The PVPP retained a purple colour after elution with each solvent, suggesting some anthocyanins remained in the PVPP-anthocyanin complex. As indicated in literature, these adsorbents, e.g. PVPP, have the disadvantage that have either a high adsorption performance or a high elution performance.

In other words, they do not simultaneously have both a high adsorption performance and a high elution performance[95].

The eluted solutions were then lyophilized and it was calculated both the extraction yield and the TMA values of the solid extracts.

In Figure 66, it is possible to see that the extraction yields obtained by applying different elution conditions were significantly lower than the yield observed when an extract is not subjected to concentration by PVPP and here considered as a standard reference (ANOVA, Tukey's post ANOVA, $p<0.05$). Even more, the extraction yield observed when elution condition 1, 2 and 3 where applied were significantly higher than the yield observed when elution condition 4 was applied (ANOVA, Tukey's post ANOVA, $p<0.05$). This result can be explained by considering that PVPP does not adsorb selectively anthocyanins, but also other polyphenols present in an extract that have affinity for it. Even more, it was proposed in literature the ability of PVPP to bind organic acids too[97]. Therefore, the aqueous washing, additionally applied in condition 4, could remove those compounds giving a lower extraction yield.

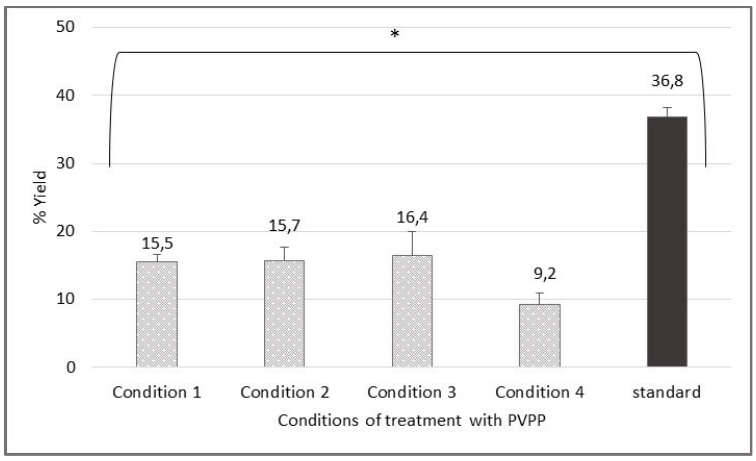


Figure 66: Extraction yields of aqueous enriched-extracts of *H. sabdariffa* calyces. Grey bars represent samples. Black bar represents the reference. Values are expressed as mean and standard deviation (n=3). (*) indicates a significant difference among the values (ANOVA, $p<0.05$)

It was observed that the TMA content of the extract obtained by applying the elution condition 4 was significantly higher than the TMA content observed for the extracts obtained by applying elution condition 1, 2 and 3 (ANOVA, Tukey's post ANOVA, $p<0.05$). Nevertheless, it was not significantly

different from the TMA content of the standard extract (Figure 67). Therefore, further trials have to be done in order to obtain an enriched extract with a higher TMA content than the standard reference, thus justifying such a low extraction yield.

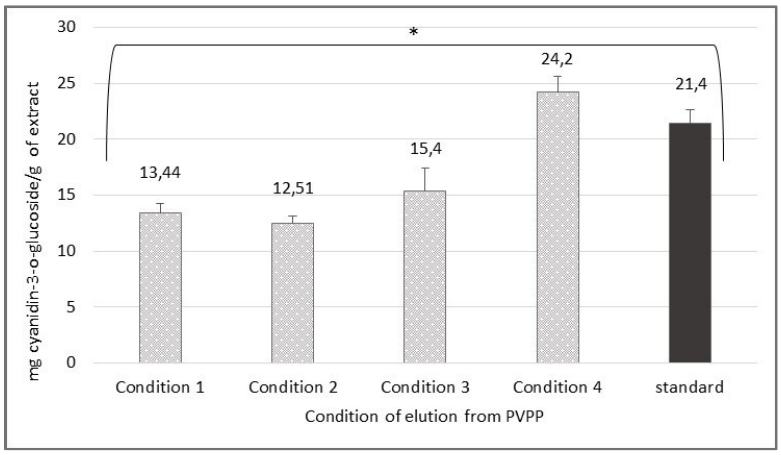


Figure 67: TMA content of enriched extracts of *H. sabdariffa*. Values are expressed as mg cyanidin 3-O-glucoside equivalents/g of extract. Grey bars represent samples. Black bar represents the reference standard. Values are expressed as mean and standard deviation (n=3). (*) indicates a significant difference among the values (ANOVA, $p<0.05$)

The elution condition 1 was chosen based on methods for elution of general flavonoid compounds rather than specifically for anthocyanins[95]. However, it is reported in literature an elution condition more specific for anthocyanins of white wines, based on an extensive washing of the PVPP-anthocyanin complex prior to the elution with ethanol containing 0,1 M ammonia[96]. In our study, it was tried instead an elution with 50% ethanol in water and another elution condition, characterized by the addition of 1% acetic acid, in order to stabilize the eluted anthocyanins. In the end, for condition 4, a preliminary washing with water was introduced: sugars and organic acids (including phenolic acids) can be removed from the PVPP, without removing anthocyanins, by washing with water. This was reported by Passamonti et al.[98] on the purification of the anthocyanin fraction of a grape (*Vitis vinifera*) extract with PVPP. The removal of these organic acids could be used as an explanation for the blue colouring of the anthocyanin extract that was observed in this last case, compared to the red colour of the other extracts. The removal of the acids changes the pH of the environment so that it is more basic, therefore producing a colour change. Passamonti et al.[98] used an acidic mixture of ethanol and water for the elution of the anthocyanins from PVPP, but they chose formic acid. In this study

acetic acid was used because it is safer than formic acid for human consumption, if traces remain in the final product.

A large amount of PVPP is needed to conduct these purifications. If done on an industrial scale, this process could be expensive and produce large amounts of waste. Therefore, it is necessary that the PVPP is recoverable to reduce waste and improve affordability of the process. Following the process described by Magalhães et al.[95] the recovered PVPP was washed with a 4% NaOH in water in a Büchner funnel until the filtrate running off was clear. The recovered PVPP was then washed with distilled H_2O until the pH of the filtrate was neutral to ensure that the PVPP would not be basic when reused for the extraction of anthocyanins that are pH sensitive. The recovered PVPP was then placed in oven at 60°C until dried. This could then be used again for further extractions.

4.1.2.4. Preparation of a model aqueous extract of calyces and characterization of its anthocyanin content, as an ingredient for nutraceutical products

A model aqueous extract of calyces was prepared by using the selected variety of calyces (Tenonderà Tempranera) and the selected extraction method (1:100, 25°C, 15 minutes, UAE). The extract was filtered and lyophilized.

Single anthocyanins of the extract were quantified by HPLC, based on recently developed elution condition[71]. Actually, very poor literature is present about the HPLC-characterization of *H. sabdariffa* anthocyanins, as several research studies focus on anthocyanins of other botanical species like cranberry, grape or blueberry. In general, HPLC-characterization of *H. sabdariffa* anthocyanins is performed on C18 (4.6 x 250 mm; 5µm) columns and the elution systems are based on water, acetonitrile and formic acid[70,40,67]. In this case, a C18 (150 x 4,6 mm; 3.5 µm) column was used with an elution system based on aqueous phosphoric acid and methanol. An analysis of the anthocyanins of cranberry is reported in literature, with such a shorter column, but the mobile phases were aqueous formic acid and acetonitrile[99]. Nevertheless, aqueous phosphoric acid is reported in literature as an alternative to the aqueous formic acid system for the elution of polyphenols in HPLC[18]. Therefore, a suitable gradient elution was set in order to separate the main four anthocyanins of *H. sabdariffa* calyces on that column with mobile phases containing aqueous phosphoric acid and methanol.

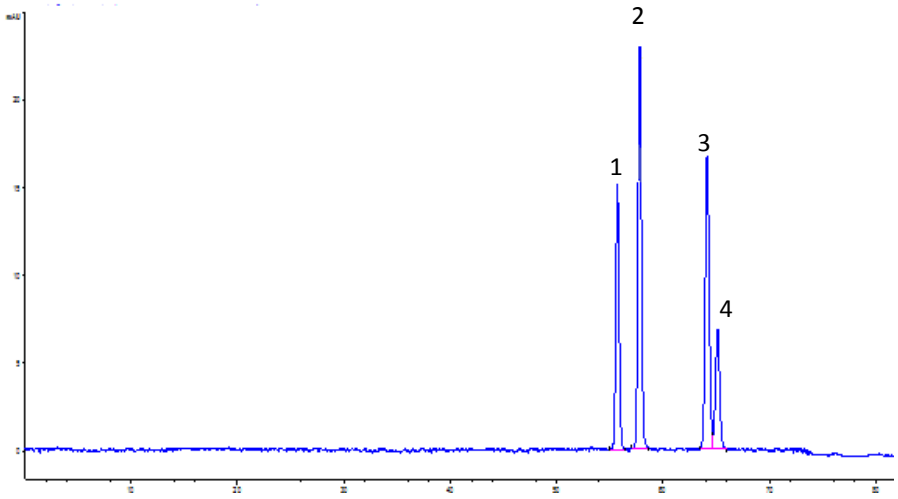


Figure 68: Chromatogram of the four standard of *H. sabdariffa* calyces anthocyanins. (1) delphinidin 3-sambubioside, (2) delphinidin 3-O-glucoside, (3) cyanidin 3-O-glucoside, (4) cyanidin 3-sambubioside.

The analysis of the results (See Table 20) showed that delphinidin 3-sambubioside was the main anthocyanin of *H. sabdariffa* calyces, followed by cyanidin 3-sambubioside. On the other hand, delphinidin 3-O-glucoside was the minor compound present. The peak of cyanidin 3-O-glucoside was not detected in the samples at the maximum concentration used of 26.8 mg/ml. Therefore, a concentration of this compound lower than the lower concentration quantifiable for it with standardized solutions was supposed to be present in the sample extract.

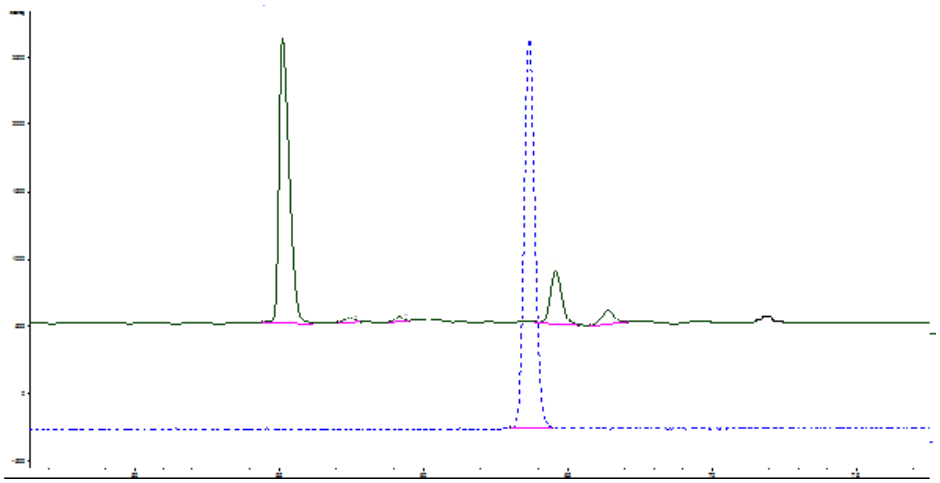


Figure 69: Cyanidin 3-O- glucoside chromatogram (blue) overlying extract chromatogram (black). No peak of cyanidin 3-O-glucoside was detected at 26.8 mg/ml concentration of extract.

These results are in agreement with the study by Sindi et al.[67], the study by Frank et al.[40] and the review by Da-Costa-Rocha et al.[32]

These authors also identified delphinidin 3-sambubioside and cyanidin 3-sambubioside as the main anthocyanins of *H. sabdariffa* calyces and the glucoside derivatives as the minor ones. In their studies, the concentration of delphinidin 3-sambubioside and cyanidin 3-sambubioside in the extracts were comparable, while in our study cyanidin 3-sambubioside was lower than delphinidin 3-sambubioside. This might be due to the different geographical origin of the calyces.

	Delph 3-samb	***Delph 3-O-glu***	***Cyan 3-samb***	***Cyan 3-O-glu***
Tenondrà Tempranera, UAE	*(mg/ g extract)*			
	6,462 ± 0,048	0,0998 ± 0,0060	1,873 ± 0,049	<0,045

Table 20: Concentration of anthocyanins identified by HPLC in a model extract of *H. sabdariffa* calyces. Results are expressed as mean ± standard deviation of n=3 determinations.

Finally, the characterization of the extract was completed by measuring the TMA content and the total antioxidant capacity (See Table 21).

	TMA	***DPPH***	***ABTS***
Tenondrà Tempranera, UAE	*(mg C 3-O-glu/ g extract)*	*(µmol TE/ g extract)*	
	8,82 ± 0,67	569 ± 75	204 ± 14

Table 21: TMA and TAC of a model extract of *H. sabdariffa* calyces.

Results are expressed as mean ± standard deviation.

It was observed that the TMA value of the extract is comparable with the sum of the single anthocyanin determined by HPLC, so it could be used as an alternative method for a faster determination of the anthocyanin content of the extract.

As a final point, it was decided to measure the free radical scavenging activity of this model aqueous extract in order to obtain reference values to be used in the future quality control of this product. For this purpose, two spectrophotometric assays based on the use of DPPH radical and $ABTS^{\bullet+}$ were performed and they will be discussed more in depth in the following sections.

4.2. Second project line: *Moringa oleifera* Lam. tissues

4.2.1. First part of the study

The aim of the work was to prepare and characterize aqueous extracts of different tissues of *M. oleifera*, to identify the best sources of glucomoringin (as a precursor of the bioactive isothiocyanate, with reported indirect antioxidant properties and antibacterial), and polyphenols (known for their direct antioxidant activity), to be exploited in the preparation of extracts for nutraceutical and cosmetic applications.

Even more, the total amount of each vegetal sample, representing a specific part of the plant, was divided into two aliquots, that were subjected to two different drying treatments (oven-drying at 40°C and freeze-drying), in order to verify how the drying treatment could influence the composition of the final extracts.

4.2.1.1. Extractions

As reported by Förster et al.[75], the "desulfo glucosinolates" method is the procedure commonly used for the extraction, determination and quantification of glucosinolates, such as glucomoringin in *M. oleifera*. This procedure is based on a methanolic extraction (MeOH 70%) of glucosinolates, followed by a purification and a desulfation step catalysed by a sulfatase on an ionic exchange resin. A final determination by HPLC completes the procedure[75]. Nevertheless, the desulfation step in the extraction of glucosinolates from *M. oleifera* leaves causes the complete degradation of the natural occurring glucosinolates of this plant, through the conversion of the acetyl isomers into the un-acetylated form[75]. The consequence is an overestimation of the un-acetylated isomer i.e. glucomoringin that is in this study the compound of interest, being the precursor of a bioactive isothiocyante and the major glucosinolate in all the tissues of *M. oleifera*.

It was found in literature a method for the extraction of the intact glucosinolates from *M. oleifera* leaves that is based on a methanolic extraction (MeOH 70%, 80°C), followed by a treatment with barium acetate, in order to purify the sample, and a final HPLC analysis[75].

More in general, glucosinolates from different tissues of *M.oleifera* can be determined by performing an extraction with MeOH 70%, at 70-80°C, followed by a determination by LC-MS or HPLC-DAD-ESI-MS, without any pre-treatment[46,48]. It is also reported in literature an extraction with boiling water on the main glucosinolate of *Barbarea Verna*[100].

On the other side, the extraction of phenolic compounds is efficient when using methanol (also in different percentages with water), ethanol (also in different percentages with water), acetone and water[46]. Indeed, Vyas et al. demonstrated that polar solvents can extract polyphenols better than not polar solvents like chloroform[16].

In this study, water was chosen as solvent for the extraction of the compounds of interest as it has a good affinity for polyphenols and also for glucomoringin, besides being a green solvent, biocompatible and universally accepted as a suitable solvent for the preparation of both nutraceutical and cosmetic products. Moreover, glucomoringin is more hydrophilic than glucosinolates from other Brassicaceae because it has a rhamnose moiety in its molecule and it is an anion.

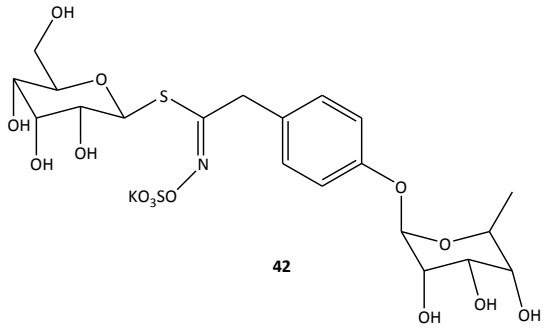
Extractions were performed at 80°C because this temperature can inactivate the endogenous myrosinase, thus allowing the recovery and determination of glucomoringin. An extraction time of 10 minutes was applied because shorter extraction times are better than longer ones for an efficient

recovery of polyphenols, when water is chosen as solvent[67,70]. Even more, glucomoringin stability in aqueous solutions decreases by increasing temperature[75], so a short exposure time to high temperatures was preferred.

4.2.1.2. Characterizations

4.2.1.2.1. Isolation and characterization of glucomoringin

With the aim of obtaining the analytical standard of glucomoringin to perform the quantitative HPLC-analysis of this compound in the extracts, it was performed an extraction from seeds, followed by a purification by using an anionic exchange resins, such as DEAE Sephadex A-25[76].



42

Glucomoringin potassium salt

MW 609.6629

White solid, Yield: 1.14%

MW of the anion: 570.5652

Area % at 223 nm: 100

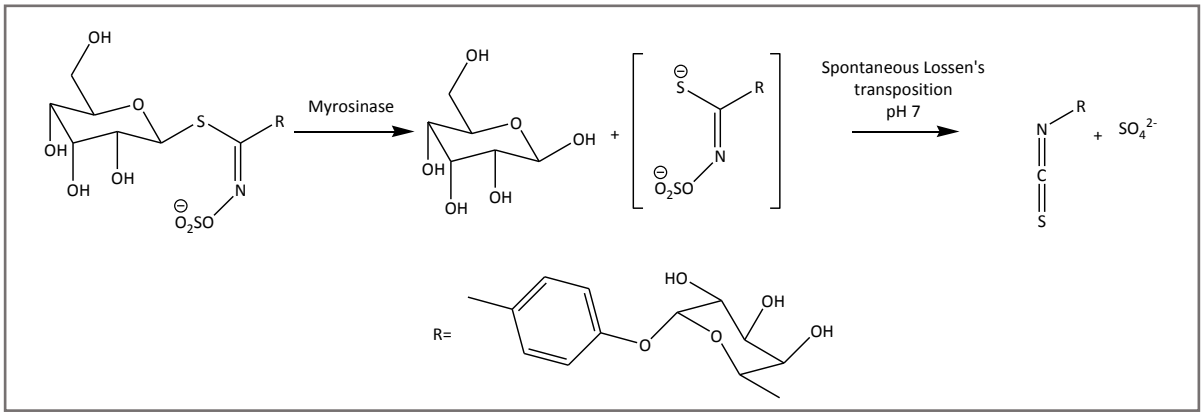
HRMS (ESI-TOF): found $(M-H)^-$ 570.1072, calculated $(M-H)^-$ 570.0957

^{1}H-NMR (400 MHz, CD_3OD) δ: 7,34 (2H,d, J 8.4 Hz, ArH), 7,04 (2H,d, J 8.4 Hz, ArH), 5,40 (1H, d, CH), 4,53 (1H, m, CH), 4,18 (1H, m, CH), 3,99 (2H, m, 2xCH), 3,83 (2H, m, 2xCH), 3,64 (2H, m, 2x CH), 3,45 (1H,m, CH), 3,24-3,11 (4H,m, CH), 1,24 (3H,d, J 6,2 Hz, CH_3).

4.2.1.2.2. Enzymatic conversion of glucomoringin into its isothiocyanate

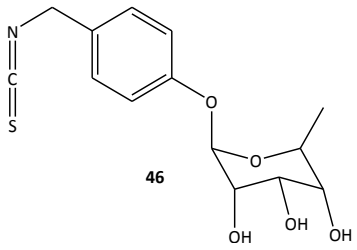
Glucomoringin was converted into the isothiocyante by using an exogenous myrosinase enzyme, available in commerce that was isolated and purified from *Sinapis alba* L.

This enzyme is active up to 60 °C[101]. The reaction catalysed by the enzyme takes place according to the following scheme:



Scheme 6: Conversion of glucomoringin into isothiocyanate, catalysed by the S-glycosidase myrosinase.

In order to obtain the isolated isothiocyanate of glucomoringin or the isothiocyanates deriving from the acetyl isomers of glucomoringin, it is also available a procedure indicated by Cheenpracha et al[66]. This method is based on the preparation of a methanolic extract from ground fresh fruits of *M. oleifera* at room temperature (in another study this procedure is applied to leaves[50]). The obtained extract has subsequently to be subjected to solvent extractions with water/hexane, and then the water phase has to be extracted with ethyl acetate. The latter fraction has to be further purified with specific chromatographic techniques, until the purified compounds are obtained[66]. Nevertheless, this procedure is more long and complex, so it was preferred to isolate the precursor and then convert it into the bioactive isothiocyante, by myrosinase.



46

Glucomoringin isothiocyanate

MW 311.3535

Whitish solid, Yield crude product: 81%

Area % at 221 nm: 92.3

HRMS (ESI-TOF): found $(M-H+2H_2O)^-$ 346.0561, calculated 346.0827; found $(2M-3H+Na+2H_2O+2NH_3)^-$ 356.0878, calculated 356.0136

^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7,25 (2H, d, J 8.4Hz, Ar-H), 7,08 (2H, d, J 8.4Hz, Ar-H), 5,54 (1H, d, J 1.8Hz, CH), 4,66 (2H, s, Ar-CH_2), 4,17 (1H, dd, J 1.8Hz, J 3.3Hz, CH), 4,00 (1H, dd, J 3.3Hz, J 9.2Hz, CH), 3,75 (1H, m, CH), 3,56 (1H, t, J 9.2Hz, CH), 1,30 (3H, d, J 6.2Hz, CH_3).

4.2.1.2.3. HPLC-DAD analysis of glucomoringin in aqueous extracts of *M. oleifera*

The HPLC analysis of glucomoringin was performed according to the conditions reported by Forster et al.[75], with modifications. In particular, ammonium acetate buffers were used as eluents, in order to minimise the conversion of the acetyl-derivatives of glucomoringin to glucomoringin itself, by loss of the acetyl group[75]. This event would have caused an overestimation of glucomoringin inside those samples that contained also the acetyl derivatives of glucomoringin.

Results of the quantitative analysis of glucomoringin (GMG) were expressed as µmol anion per 100 ml extract and were reported as mean ± standard deviation.

The analysis of the results showed that the extract obtained from seeds had the highest concentration of glucomoringin (6,841±0,097 µmol/100 ml) among those extracts obtained from freeze-dried materials, with values ranging from 3,36±0,16 µmol/100 ml to 6,841±0, 097 µmol/100 ml. Among the extracts obtained from the oven-dried matrices, the extract obtained from branches showed the highest content of glucomoringin (6,13±0,18 µmol/100 ml), together with EDYNEA01 (5,43±0,47 µmol/100 ml) and EDYNEA02 (5,66±0,29 µmol/100 ml) as there was no significant difference between them (ANOVA, Tukey's post ANOVA, $p<0.05$). Extracts of the oven-dried matrices present values ranging from 4,46±0,12 µmol/100 ml to 6,13±0,18 µmol/100 ml.

It was possible to observe that there was a significant difference in the amount of glucomoringin of extracts obtained from freeze-dried materials (ANOVA, Tukey's post Anova, $p<0.05$), with the exception of the extracts obtained from EDYNEA01 and EDYNEA02. This may be due to the fact that they represent extracts of the same part of the plant, even of different varieties ("green" and "purple" varieties). Even more, there was neither significant difference among the extracts obtained from lyophilized leaves and branches. Bennett et al.[48] reported lower glucomoringin content in branches compared to leaves, in the case of a freeze-drying treatment of the plant samples, but they only analysed the outer bark of branches instead of the entire branch, as it has been done in this study. Even more, Bennett et al.[48] also observed that in the case of a freeze-drying treatment of different tissues of *M. oleifera*, seeds represent the tissue with the higher concentration of glucomoringin.

A t-Student test ($p<0, 05$) was performed to compare glucomoringin content in extracts of the same part of the plant, dried under different treatments. In the case of extracts obtained from leaves, it was observed that an oven-dried treatment does not cause any significant difference in the content of glucomoringin, compared to an extract prepared with the same material, but freeze-dried. In all

the other cases, it was observed a significant difference among treatments, but in the case of branches, EDYNEA01 and EDYNEA02 derived extracts, it was the oven-dried material that gives the extract with the higher amount of glucomoringin, while the opposite happened for seeds and bark derived extracts. A possible explanation is that branches contain, as well as leaves and EDYNEA tissues, the acetylated isomers of glucomoringin. The higher temperature of the oven compared to a freeze-drying might promote the conversion of the isomers into glucomoringin itself, by losing the acetyl group. In fact, the conversion reaction depends on temperature as reported by Förster et al[75]. Even more, Sriwichai et al.[102] reported an increase in the releasable nutrients of *M. oleifera* subjected to processing, especially drying by oven at mild temperatures. On the other hand, seeds and bark have never been reported to contain acetyl isomers. Therefore, the effect that may prevail is a loss of glucomoringin in the oven-dried material, according to the fact that it is a thermo sensitive compound[75]. Further studies are needed to confirm these hypotheses.

Finally, the peak of glucomoringin isothiocyanate was qualitatively detected in the extracts of bark, seeds, EDYNEA01 and EDYNEA02, suggesting that some parts of the plant may already contain the bioactive compound in the activated form. As reported in literature[12], isothiocyanates can be present also in intact plant tissues in a very low or undetectable concentration.

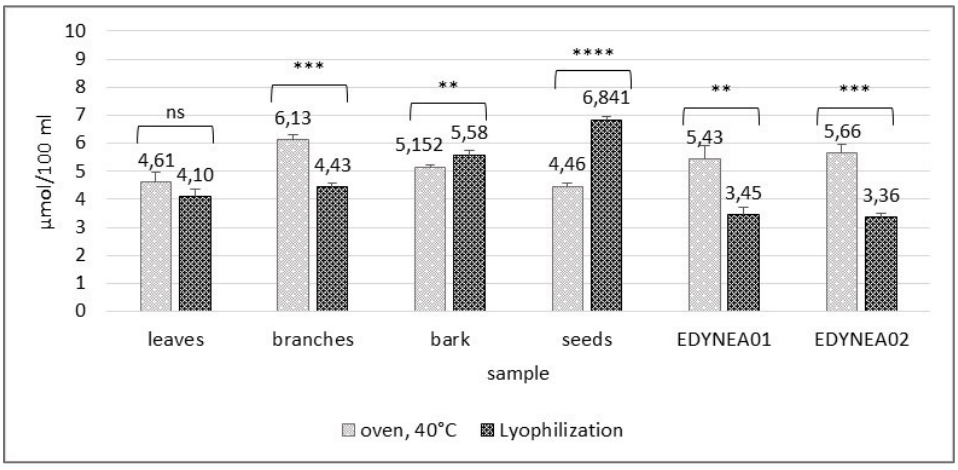


Figure 70: Glucomoringin concentration in extracts of different tissues of *M. oleifera* subjected to two different drying treatments (oven at 40°C and freeze-drying). Results are expressed as mean and standard deviation of n=3 determinations. (*) indicates significant differences among means (t-Student, $p<0.05$)

4.2.1.2.4. Determination of total phenolic content (TPC)

The determination of total phenols in the extracts was performed according to the method of Folin-Ciocalteu. In this method, the reactant of Folin-Ciocalteu (a mixture of phosphomolybdate and phosphotungstate) reacts at a basic pH with phenolic compounds, producing tungsten (W_8O_{23}) and molybdenum oxides (Mo_8O_{23}) with a blue colour that is proportional to the concentration of polyphenols[103]. The reaction is based on electron transfer between the antioxidant and the Folin-Ciocalteu reactant.

More precisely, this assay measures the reducing capacity of a sample, that is expressed in terms of phenolic content, as it was demonstrated its ability to react also with non-phenolic reducing substances that here behave like interfering compounds[103].

It was observed that even if the assay measures all the polyphenols inside a sample, not all the single phenolics respond in the same way[103].

Since nowadays the Folin-Ciocalteu reactant is commercially available, this assay is widely used to quantify polyphenols in plant extracts, as well as wines and other foods[103]. In Europe it has been adopted as the official procedure for measuring total phenolic levels in wines (European Community 1990)[103].

So, it was also adopted in this study for the determination of total polyphenols in the extracts of *M. oleifera.*

Phenolic compounds can directly contribute to the antioxidant activity of an extract[104]. For this reason, we decided to investigate the polyphenol content in each aqueous extract.

The analysis of the results showed that among those extracts obtained from oven-dried tissues the extract obtained from leaves had the higher content of polyphenols (158,0±2,9 mg GAE/100 ml). On the other hand, the extract obtained from branches had the higher total polyphenols content (139,2 ±1,6 mg GAE/100 ml) among those extracts obtained from freeze-dried material.

In both treatments bark and seeds derived extracts showed the lowest amount of total polyphenols, with values ranging from 3,562±0, 049 mg GAE/100 ml to 9,76±0, 11 mg GAE/ 100 ml.

In general, it was observed a significant difference in TPC among extracts of the same part of the plant (t-Student, $p<0,05$) and in particular extracts obtained from oven-dried material showed a higher content in polyphenols than extracts obtained from freeze-dried material. Branches represented the only exception: in this case, the extract from the freeze-dried aliquot of the sample had the highest content in total polyphenol. This situation might be due to the presence of thermosensitive compounds in branches that could be lost because of the oven drying treatment. In all the other

samples, the higher TPC value found for extracts of the oven-dried series may be due to an increasing permeability of the solvent in the vegetal tissue subjected to ruptures when dried in an oven at mild temperatures, according to Sriwichai et al[102].

A significant difference in total polyphenol content was observed among extracts of different parts of the plant, that were dried by the same treatment (ANOVA, $p<0,05$).

According to data reported by Godinez-Oviedo et al.[44] and considering the study by Vyas et al.[16], the leaves of *M. oleifera* are the tissue with the highest concentration of polyphenols. A possible explanation of the high polyphenol content and, the high antioxidant activity of leaves or leaves extracts is that leaf has a longer life span compared to other parts of the plant. Moreover, it is the site of energy production. So, it may be exposed to a great oxidative damage[16]. Indeed, the highest TPC value observed in this part of the study was related to the extract obtained from oven-dried leaves (158,0 ± 2,9 mg GAE/100 ml).

Up to now, there has not been studies comparing the total phenolic content of aqueous extracts of different part of *M. oleifera* cultivated in Paraguay and dried with two different methods (oven 40 °C and freeze-drying).

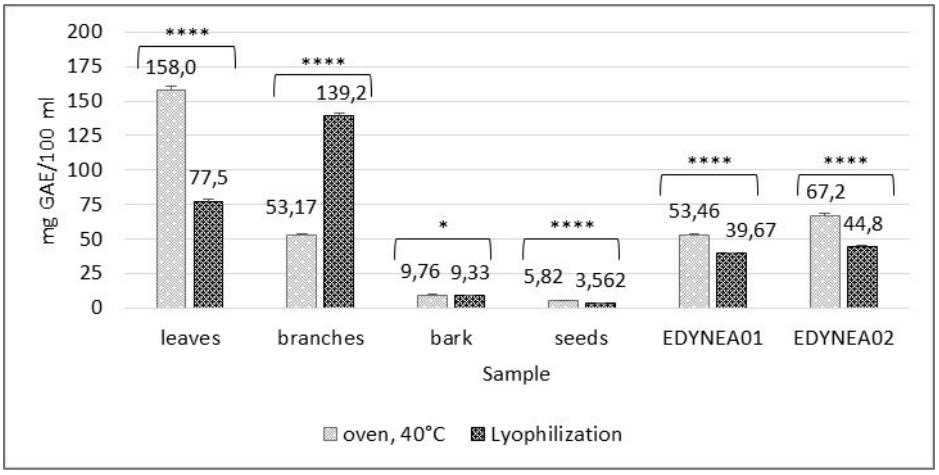


Figure 71: Total phenolic content in extracts of different tissues of *M. oleifera*, subjected to two different drying treatments (Oven, 40°C and freeze-drying). Values are expressed as mean and standard deviation of n=3 determinations. (*)indicates significant differences among means (t-Student, $p<0.05$)

4.2.1.3. Assessment of the properties

The total antioxidant capacity of the extracts was measured by two spectrophotometric assays (DPPH and $ABTS^{\bullet+}$), based on the capacity of antioxidant compounds (i.e. polyphenols and other antioxidants) to donate a hydrogen atom or electrons to a free radical, which has a specific colour when it is in the oxidized form, but then loses the colour after reduction. In both assays, a standard compound was used as reference to make comparisons with the antioxidant capacity of the aqueous extracts. Results are expressed as Inhibitory Concentration 50 (IC_{50}) in mg/ml, which is the concentration of the extract that is necessary to reduce the activity of the radical at 50%. The lower is the IC_{50} value of an extract, the higher is its antioxidant capacity, also compared to the standard reference. Both these spectrophotometric assays measure a direct radical-scavenging capacity.

4.2.1.3.1. ABTS assay

In the ABTS assay, the reference was Trolox, a hydrosoluble analogue of alfa-tocopherol. Its IC_{50} is 0,028 mg/ml. About the extracts, the lower IC_{50} was observed for the extract obtained from leaves (0,80 ± 0,03 mg/ml), in the oven-drying treatment, and for the extract obtained from branches (0,71 ± 0,03 mg/ml), in the freeze-drying treatment. Nevertheless, both those extracts had an IC_{50} value higher than Trolox. In the statistical analysis of the results, it was observed a significant difference among different drying treatments for the same part of the plant in all extracts (t-Student, $p<0, 05$). Even more, there was a significant difference in the antioxidant capacity of extracts obtained from different tissues, with the same drying treatment (ANOVA, $p<0, 05$).

The IC_{50} values ranged from 0,80±0,03 mg/ml (leaves extract) to 17,5±0,4 mg/ml (seed extract) in the oven-dried series, and from 0,71±0,03 mg/ml (branches extract) to 20,9±0,4mg/ml (seed extract) in the freeze-dried series.

Moyo et al.[105] reported as 0,69 mg/ml the IC_{50} value for an aqueous extract of *M. oleifera* leaves, cultivated in South-Africa and air-dried. That value is comparable with the value that was found for our aqueous extract of *M. oleifera* leaves, cultivated in Paraguay and dried in an oven at 40°C (0,80±0,03 mg/ml). This can be explained by the fact that the production of antioxidant compounds in a plant depends on the latitude where the plant grows, among other factors.

4.2.1.3.2. DPPH assay

In the DPPH assay, the reference was ascorbic acid, with an IC_{50} of 0,0042 mg/ml. Results were in agreement with the results observed in the ABTS assay. Values of IC_{50} ranged from 0,16±0,04 mg/ml (leaves extract) to 0,86±0,05 mg/ml (EDYNEA01 extract) in the oven-dried series, and from 0,20±0,03 mg/ml (branches extract) to 12,5±0,3 mg/ml (bark extract) in the freeze-dried series.

Therefore, the extracts obtained from oven-dried leaves and lyophilized branches showed the best total antioxidant capacity (0,16±0,04 mg/ml and 0,20±0,03 mg/ml respectively), although their IC_{50} values were higher than that of the reference. Those two extracts were the same that presented the higher total phenolic content, in the oven-drying and freeze-drying treatments respectively. As a matter of facts, total antioxidant activity measured by $ABTS^{\bullet+}$ and DPPH was correlated with total phenolic content (Pearson r ABTS=0,9119 and Pearson r DPPH=0,8667).

Oven, 40°C			***Lyophilisation***		
Extract	***Total antioxidant capacity***		***Extract***	***Total antioxidant capacity***	
	ABTS IC_{50} (mg/mL)	**DPPH** IC_{50} (mg/mL)		**ABTS** IC_{50} (mg/mL)	**DPPH** IC_{50} (mg/mL)
Leaves	0,80 ± 0,03	0,16 ± 0,04	***leaves***	1,22 ± 0,03	0,54 ± 0,03
bark	0,81 ± 0,05	0,50 ± 0,08	***bark***	6,86 ± 0,03	12,5 ± 0,3
branches	1,40 ± 0,09	0,41 ± 0,02	***branches***	0,71 ± 0,03	0,20 ± 0,03
seeds	17,5 ± 0,4	*	***seeds***	20,9 ± 0,4	*
EDYNEA01	1,42 ± 0,07	0,86 ± 0,05	***EDYNEA01***	1,91 ± 0,08	0,79 ± 0,04
EDYNEA02	1,56 ± 0,06	0,73 ± 0,04	***EDYNEA02***	1,90 ± 0,04	0,73 ± 0,03

Table 22: Total antioxidant activity of aqueous extracts of different parts of *M. oleifera* dried by oven or freeze-drying. Values are expressed as mean ± standard deviation. (*) indicates the impossibility to measure the IC_{50} value due to the method used. The reference compound for the $ABTS^{\bullet+}$ was Trolox (IC_{50}: 0.028 mg/ml). The reference compound for DPPH assay was ascorbic acid (IC_{50}: 0.0042 mg/ml).

4.2.2. Second part of the study

The aim of the work was the selection of an extraction condition suitable for the conversion of glucomoringin into the bioactive isothiocyanate inside the selected extracts, preserving the total phenolic content. A further objective was the characterization of glucomoringin- isothiocyanate inside the extracts, the determination of TPC and the evaluation of the direct antioxidant capacity (DPPH and $ABTS^{\bullet+}$ assays).

The isothiocyanates of *M. oleifera* are more stable and hydrosoluble compounds compared to the isothiocyanates from other Brassicaceae, thanks to the rhamnose moiety in their molecule[50]. Therefore, the preparation of *M. oleifera* extracts already containing active isothiocyanates, offers the advantage of administering a titrated dose of these compounds, thus providing a better alternative to the administration of the precursor, which could be bio-activated by the intestinal microflora, but in an undefined measure.

Also in this part of the study, the attention was focused on glucomoringin and its isothiocyanate, without focusing on the other three acetyl-isomers. As it was previously explained, glucomoringin is present in all *M. oleifera* tissues and it is the most abundant of the four glucosinolates.

We continued this part of the study with leaves, branches and EDYNEA02 that had been dried in the oven at 40 °C and with the sample of seeds that had been lyophilized, according to the results observed in the first part of the study. In fact, they were considered good sources of both polyphenols and glucomoringin as precursor of the bioactive isothiocyanate.

4.2.2.1. Extractions

The conversion of glucomoringin into its isothiocyanate was achieved by applying the extraction conditions reported by Waterman et al.[50] that were based on an extraction with distilled water at 22°C for 30 minutes[50].

In fact, the practice of preparing *M. oleifera* extracts containing isothiocyanates is very recent and the literature available on this subject is very poor.

According to a study reported in literature[106], dried powders of leaves and seeds were extracted both with water at 60°C for 45 minutes and with ethanol (50% and 95% in water) for 48 hours at room temperature. Those extracts were dried and the content of astragalin (as representative of the polyphenolic class) and total isothiocyanates (expressed as mg of phenethyl isothiocyanate equivalent/g extract) were determined. Results of that study highlighted that the maceration with 50 % ethanol gave the best results in terms of isothiocyanates recovery (7,52 ± 0,44 mg of phenethyl isothiocyanate equivalent/g extract compared to 2,46±0.11 for the aqueous extract and to 0,72±0.10 for the 95% ethanolic extract), but it was not the best option for the recovery of astragalin[106]. Even more, the use of ethanol does comply with the purpose of this study to use water as a green solvent, for biocompatible and easily formulable extracts.

On the other hand, Waterman et al.[50] used fresh leaves to prepare an extract with distilled water, at room temperature, for 30 minutes, with the help of the grinder Vitamix. The grinder helped the rupture of the vegetal cells thus favouring the reaction between the endogenous myrosinase and the precursor glucosinolate. According to Waterman et al.[50] fresh leaves are a better starting material compared to dried leaves, as the drying process can destroy the endogenous myrosinase, thus lowering the final extraction yield of isothiocyanates[50].

The aqueous extract prepared by Waterman et al. was characterized in glucomoringin-isothiocyanate at 1.15% that is a higher value than that reported by Engsuwana et al.[106] for their aqueous extract. This is due to the fact that Waterman et al. used both fresh material and the grinder.

Moreover, the extraction method developed by Waterman et al. was able to capture the majority of polyphenols present in the starting material[50].

Since it was one of the objectives to use water as solvent, and since another objective was to select the extraction conditions based on the ability to give an efficient recovery of polyphenols from the tissue, the parameters of Waterman et al. were applied on the dried powders object of this study. In fact, it was expected a higher concentration of glucomoringin-isothiocyanate in the final extract than the concentration reported by Engsuwana et al.[106] for their aqueous extract: these authors used a

temperature of 60°C that is really closed to the inactivation temperature of myrosinase, while Waterman et al.[50] suggested to work at room temperature.

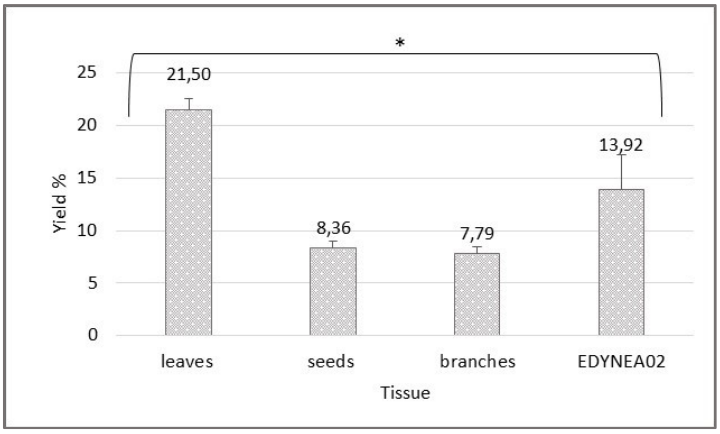


Figure 72: Extraction yields of *M. oleifera* extracts, obtained by performing an extraction with water, at 22°C, for 30 minutes, with different powders dried tissues. Results are expressed as mean and standard deviation of n=3 determinations. (*) indicates a significant difference in the observed values (ANOVA, $p<0.05$)

The highest extraction yield was observed for the extract obtained from oven-dried leaves (21,50±1,09%). The lower extraction yields were observed for the extracts obtained from lyophilized seeds and oven-dried branches (8,36±0,63% and 7,79±0,70%), and no significant difference was observed between them (ANOVA, Tukey's post ANOVA, $p<0.05$). An intermediate extraction yield was observed for the extract obtained from EDYNEA02 (13,92±3,26%).

Figure 73: Lyophilised aqueous extracts of *M. oleifera* tissues.
Leaves (dark brown), branches (light brown), seeds (whitish) and EDYNEA02 (pink).

4.2.2.2. Characterizations

4.2.2.2.1. Isolation and characterization of glucomoringin

In order to get some more glucomoringin to proceed with the experiments, it was performed the extraction and isolation of glucomoringin from *M. oleifera* seeds one more time. The crude compound was dissolved in methanol. Then it was centrifuged at 12000 rpm for 5 minutes. After that, it was dried in a rotary evaporator. It was obtained a white solid with a yield of 0.6 % that was almost half of the yield obtained when glucomoringin was isolated the first time. This is probably because it was necessary to discard some eluted fractions, as they had some impurities together with the compound of interest.

Area % at 223 nm: 100

^{1}H-NMR (300 MHz, CD_3OD) δ: 7,34 (2H,d, J 8.4 Hz, ArH), 7,04 (2H,d, J 8.4 Hz, ArH), 5,40 (1H, d, CH), 4,53 (1H, m, CH), 4,18 (1H, m, CH), 3,99 (2H, m, 2xCH), 3,83 (2H, m, 2xCH), 3,64 (2H, m, 2x CH), 3,45 (1H,m, CH), 3,24-3,11 (4H,m, CH), 1,24 (3H,d, J 6,2 Hz, CH_3).

4.2.2.2.2. Enzymatic conversion of glucomoringin into its isothiocyanate

It was repeated also the reaction to obtained glucomoringin-isothiocyanate, to get more compound for further analysis.

The reaction between glucomoringin and myrosinase yielded 33, 4 mg of a whitish solid (80% yield)

Area % at 221 nm: >99%

^{1}H-NMR (300 MHz, CD_3OD) δ: 7,31 (2H, d, J 8.7Hz, Ar-H), 7,10 (2H, d, J 8.7Hz, Ar-H), 5,44 (1H, d, J 1.7Hz, CH), 4,70 (2H, s, Ar-CH_2), 4,00 (1H, dd, J 1.9Hz, J 3.4Hz, CH), 3.84 (1H, dd, J 3.4Hz, J 9.4Hz, CH), 3,62 (1H, m, CH), 3,45 (1H, t, J 9.4Hz, CH), 1,22 (3H, d, J 6.2Hz, CH_3).

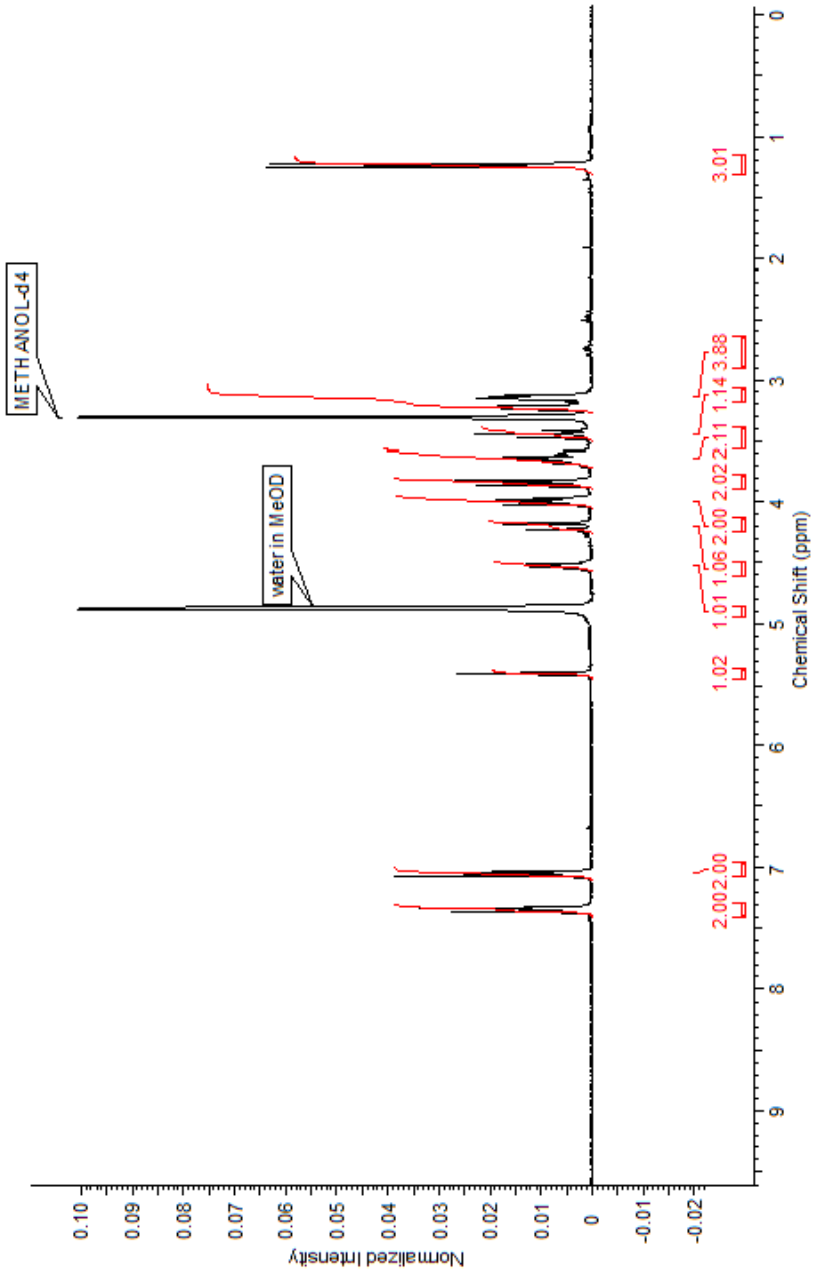


Figure 74: ^{1}H NMR spectrum of glucomoringin. Solvent CD_3OD.

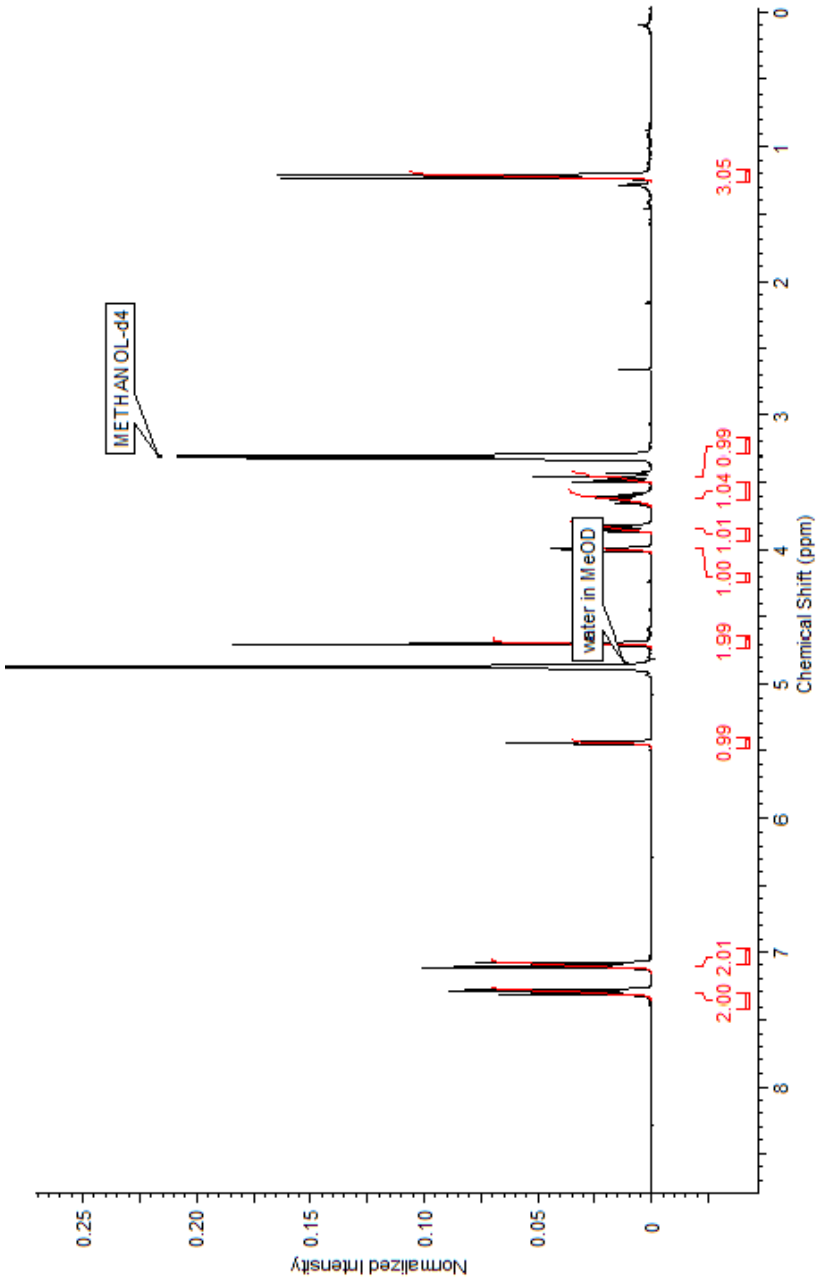


Figure 75: ^{1}H NMR spectrum of glucomoringin-isothiocyanate. Solvent CD_3OD

4.2.2.2.3. HPLC-DAD analysis of glucomoringin and its isothiocyanate in aqueous extracts of *M. oleifera*

The quantification of glucomoringin-isothiocyanate inside the extracts of *M. oleifera* was performed according to the method reported by Forster et al.[75], with modifications. Indeed this method consider the use of ammonium acetate buffered eluents to minimise the conversion of the acetyl-derivatives of glucomoringin isothiocyanate into the un-acetylated from, thus causing an overestimation of the compound of interest[75].

As it is possible to see in Figure 76, it was observed that the concentration of glucomoringin isothiocyanate in the extract from seeds (664±77 µmol/g) was significantly higher than the concentration measured in the other three extracts (ANOVA, Tukey's post ANOVA, $p<0.05$). It was also observed that the average content of isothiocyanate in the extract of EDYNEA02 (71,7±3,5 µmol/g) was higher than that of leaves extract (21,3±3,3 µmol/g). Finally, it was observed that the average content of isothiocyanate in the extract from branches (3,03±0,25 µmol/g) was lower than that of leaves, even if the previous analysis of the precursor had showed that branches were a better source of glucomoringin than leaves (with the same drying condition). This might be due to the fact that branches powder is harsher than the dry powders of other tissues. Therefore, its extraction at room temperature it may not have been so efficient as it was at 80°C.

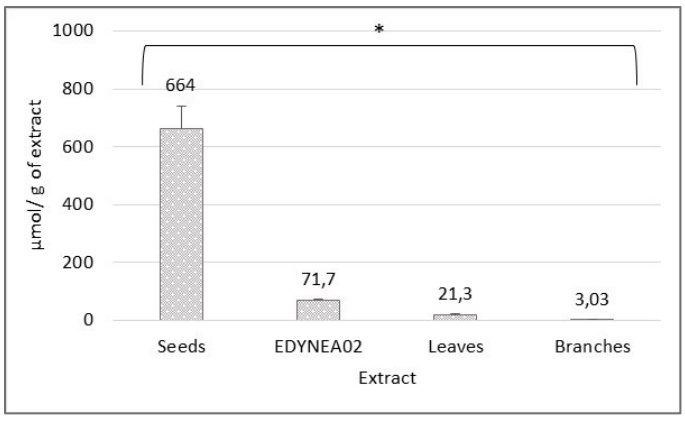


Figure 76: Glucomoringin isothiocyanate in *M. oleifera* extracts. Results are expressed as µmol/g of lyophilised extract and are reported as mean ± standard deviation (n=3). (*) indicates a significant difference among values (ANOVA, $p<0.05$)

Engsuwana et al.[106] indicated a total isothiocyanate content of their aqueous leaves extract equal to 2,46±0,11 mg of phenethyl isothiocyanate equivalent/g extract. The aqueous leaves extract prepared in the present study, by following the conditions of Waterman et al.[50] yielded 6,6 ±1,0 mg of glucomoringin isothiocyante/g extract. The higher amount found in our extract may be due to the lower extraction temperature applied (22°C *versus* 60°C) that is more suitable for the hydrolytic activity of myrosinase. Even more, it may be also due to the different geographical origin of the powders, since our leaves came from Paraguay and the leaves used by Engsuwana et al. came from Thailand[106].

It was also observed that the concentration of glucomoringin isothiocyante in our extract from seeds (207 ± 33 mg/g extract) was almost eight times higher than that found by Engsuwana et al. in their extract from mature dried seeds (27,8 ± 0,34 mg of phenethyl isothiocyanate equivalent/g extract)[106]. A possible explanation, besides the different geographical origins of the powdered material, may be found in the different drying process that were used: in our study, that seed sample was lyophilized and in the study by Engsuwana et al. the seeds were oven-dried[106]. Therefore, there may have been a loss of myrosinase due to a higher temperature of the oven-drying process than that applied in the freeze-drying process.

It is noteworthy that the extracts from seeds and EDYNEA02 showed a higher content of the isothiocyanate. This could be due to the presence of the isothiocyanate in some intact tissues, as observed in the first part of the study of this second research line, in addition to isothiocyanate obtained by enzymatic conversion during the extraction process.

Finally, no glucomoringin peak was detected in the extracts. Therefore, we can conclude that the amount of glucomoringin extracted was effectively converted into the isothiocyanate by the endogenous enzyme.

4.2.2.2.4. Determination of total phenolic content (TPC)

The determination of total polyphenols was performed according to the Folin-Ciocalteu method, previously described (see section 4.2.1.2.4.), on the aqueous extracts that were obtained from the matrices that had been selected in the first part of the study of this second research line.

The purpose was the application of extraction conditions suitable also for the efficient recovery of polyphenols from the vegetal matrices. In Table 23, results were expressed as mg GAE/ g DW and it was possible to observe that extraction yields of polyphenols obtained by applying the new extraction conditions (Waterman et al.[50]) were lower but comparable with the yield measured when the determination of polyphenols was performed in the first part of the study.

	New extraction condition	Old extraction condition
	mg GAE/g DW	mg GAE/g DW
leaves	13,4 ± 0,8	20,6 ± 0,4
seeds	1,46 ± 0,08	0,53 ± 0,01
branches	4,8 ± 0,6	7,31 ± 0,05
EDYNEA02	3,7 ± 0,9	8,7 ± 0,2

Table 23: TPC values of *M. oleifera* extracts. New extraction condition (22°C, 30 minutes, 1:16, water). Old extraction condition (80°C, 10 minutes, 1:12, water, repeated twice for 5 minutes with a ratio 1:4). Results are expressed as mg GAE /g DW as mean ± standard deviation of three determinations.

The analysis of the results showed that there was a significant difference in the TPC value measured for the four extracts (ANOVA, Tukey's post ANOVA, $p<0.05$), with the lowest value observed for the extract obtained from seeds (17,49±0,94 mg GAE/g extract). The highest values were observed for the extracts from leaves and branches (62,38±0,72 and 62,0±4,7 mg GAE/g respectively), with no significant difference between them (See figure 77).

It was interesting to observed that, in these new extraction conditions, the branches (that have a lower TPC content than leaves) can bring to an extract with an equal TPC amount. This means that, in the case of branches, the extraction yield is lower than that of leaves, but the extraction is more

selective for the class of phenolic compounds bringing to a final product more concentrated in polyphenols.

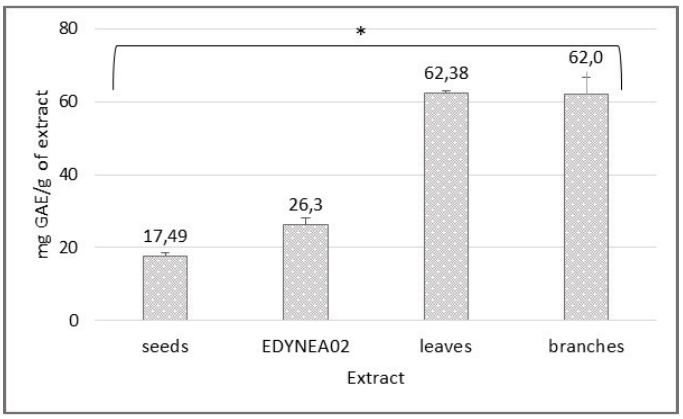


Figure 77: TPC content of *M. oleifera* extracts prepared according to the conditions reported by Waterman et al. (49). Results are expressed as mg GAE/ g extract and they are reported as mean and standard deviation of n=3 determinations. (*) indicates a significant difference in the observed values (ANOVA, $p<0.05$).

Normally, branches represent a waste product in the collection and processing of *M. oleifera* leaves, so the result observed in this study highlight this raw material as a good source of polyphenols to be considered together with the leaves for further investigation.

4.2.2.3. Assessment of the properties

The direct antioxidant activity of *M. oleifera* extracts was evaluated by the previously described *in vitro* spectrophotometric assays that are based on the use of DPPH and ABTS radicals.

As showed in Figure 78, the lower TAC was observed for the extract obtained from seeds, while the higher TAC was observed for the extract obtained from branches. It was also observed that the results obtained by the DPPH assay are comparable with the results obtained by the ABTS radical assay. Therefore, the second assay just confirmed that the aqueous extract obtained from oven-dried branches at room temperature was the best for total antioxidant capacity.

A significant difference was seen between the TAC of branches extract and TAC of leaves extract in both the assays (ANOVA, Tukey's post ANOVA, $p<0.05$).
This result highlights again the potential of branches for the preparation of extracts rich in antioxidant compounds, when suitable extraction conditions are applied.

In the DPPH assay, TAC values varied from 6,0±1,1 µmol TE/ g of extract (seeds) to 322±37 µmol TE/ g of extract (branches). In the $ABTS^{\bullet+}$ assay TAC values varied from 25,7±3,1 µmol TE/ g of extract (seeds) to 416±58 µmol TE/ g of extract (branches).

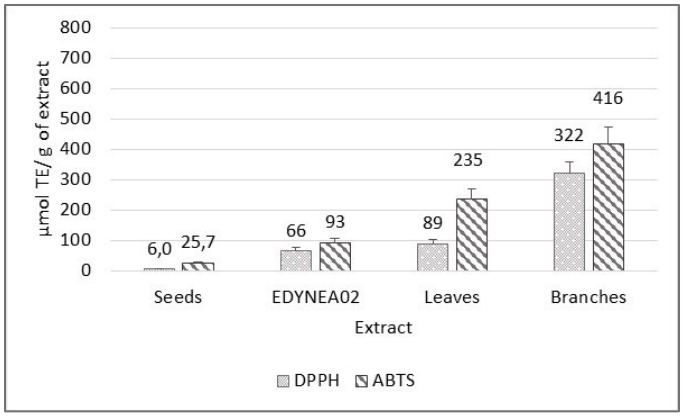


Figure 78: TAC of *M. oleifera* aqueous extracts measured by DPPH and ABTS radicals *in vitro* assays. Results are expressed as µmol TE/ g extract and they are reported as mean± standard deviation.

4.2.2.4. Summary of the results

In conclusion, extracts with a high concentration of glucomoringin isothiocyanate were characterized by a lower content in TPC and a lower TAC. On the other hand, where there was a higher concentration of total polyphenols, it was measured a higher radical-scavenging capacity, too (Figure 79).

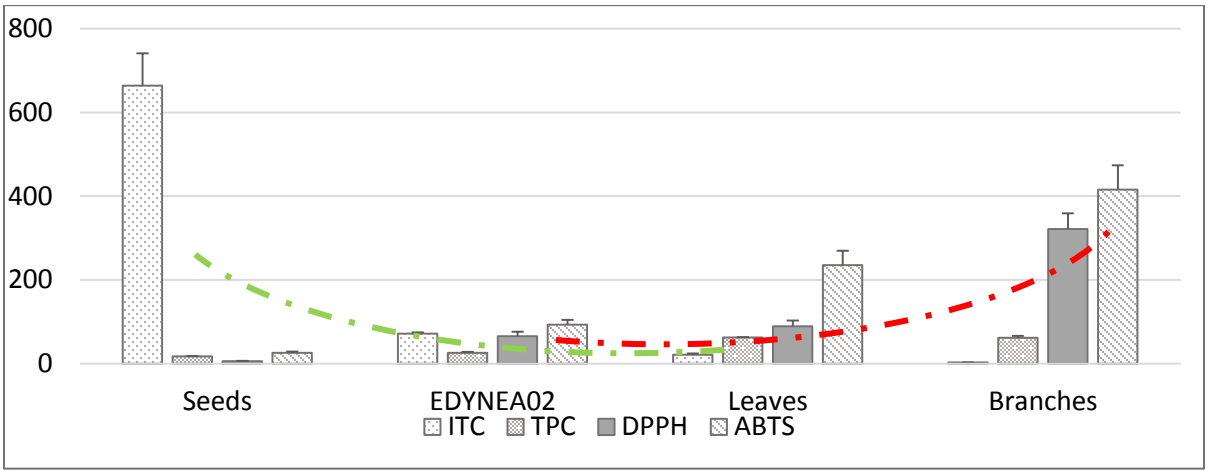


Figure 79: Summary of the glucomoringin isothiocyanate content (µmol/g extract), total phenolic content (mg GAE/ g extract), DPPH and ABTS radical assays (µmol TE/ g extract) for *M. oleifera* aqueous extracts, obtained at 22°C, for 30 minutes, 1:16 and then lyophilised.

This was demonstrated also by the correlation analysis between TPC, ITC ABTS and DPPH values. The correlation matrix in Table 24 showed that TPC and TAC (DPPH and ABTS) were positively correlated with a high correlation coefficient (r_{DPPH}=0.702032, r_{ABTS}= 0,890126). On the other hand, isothiocyanate content (ITC) and TAC were negatively correlated (r_{DPPH}= -0,59888, r_{ABTS}= -0,69226). This means that if the concentration of the glucomoringin isothiocyante increases, the TAC measured by DPPH and ABTS radicals assays decreases. The opposite happens if TPC in the extracts increase. Therefore, the direct radical-scavenging activity measured for the extracts was mainly due to the contribution of total phenolics, rather than to the isothiocyanate.

This result is in agreement with the study of Tumer et al.[12] in which it was observed a strong direct antioxidant activity for a polyphenols-rich fraction of a *M. oleifera* leaves extract. For the isothiocyanates-rich fraction of the same extract it was measured instead a high indirect antioxidant activity.

	TPC	*DPPH*	*ABTS*	*ITC*
TPC	1,0000			
DPPH	0,7020	1,0000		
ABTS	0,8901	0,9100	1,0000	
ITC	-0,7478	-0,5989	-0,6923	1,0000

Table 24: Correlation matrix of TPC, ITC and direct antioxidant activity measured by DPPH and ABTS radical assays.

4.2.2.5. *M. oleifera* and *Propionibacterium acnes*

The isothiocyanate of *M. oleifera* and the aqueous extracts of leaves, seeds, branches and EDYNEA02 (characterized in glucomoringin-isothiocyanate, total phenolics and antioxidant capacity) were assayed for their ability to inhibit the growth of *P. acnes*, *in vitro*, by the disc diffusion assay. A positive control of clindamycin hydrochloride was used together with a negative control represented by the solvent.

It was not observed any inhibition zone nor in the plates where it had been applied the purified isothiocyanate of glucomoringin, neither in the plates where the extracts had been applied. On the other hand, an inhibition zone was observed in the plates containing the positive control, with an inhibition diameter that increased with the increasing concentration of clindamycin. This confirmed that the applied procedure was correct.

The result observed in this study was in disagreement with the result reported by Rattanasena[59] who observed an inhibitory activity on the growth of *P. acnes* by testing an aqueous extract obtained from a mixture of different tissues of *M. oleifera* including leaves. Since Rattanasena tested aqueous extracts obtained at room temperature, like the extracts tested in this study, the differences in the results may depend on the differences in the methods used for the assessment of the inhibitory activity. It is reported in literature that the well-in agar method used by Rattanasena in his study is characterized by a low level of reproducibility, so it can be considered not completely reliable[107].

4.3. Third project line: oilseeds and oily extracts

4.3.1. First part of the study

The aim of the work was the selection of a variety of *H. sabdariffa* seeds with a high oil percentage and α-tocopherol content, in comparison with *M. oleifera* seeds.

4.3.1.1. Oil content determination in oilseeds

4.3.1.1.1. Determination by NMR

The determination of the oil content in the seed samples was performed by a low resolution NMR instrument (NMR Bruker minispec mq20), using the *spin-echo* sequence.

It was observed that the oil content of *M. oleifera* seeds (27,6 ± 1,8%) was higher than the oil content of all the three varieties of *H. sabdariffa* seeds. The percentage of oil in the varieties of *H. sabdariffa* seeds ranges between 2,91±0,15% (Koape guarè) and 15,4 ± 1,1% (Tenonderà). It was observed an intermediate oil percentage for the variety Fortìm (8,01 ± 0,34%).

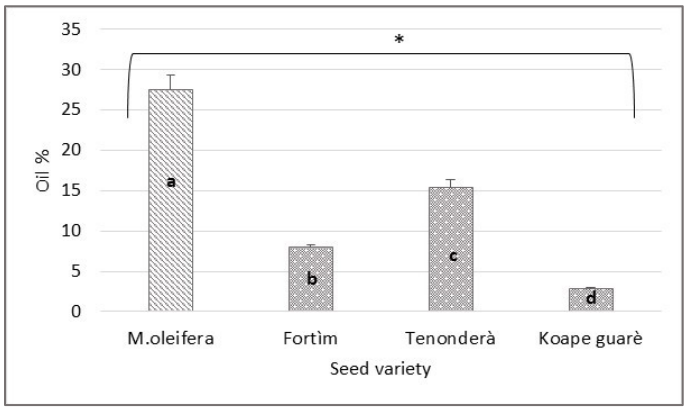


Figure 80: Oil percentage in different varieties of *H. sabdariffa* seeds, compared to *M. oleifera* seeds. Results are expressed as mean ± standard deviation of n=6 determinations.
(*) indicates an overall significant difference among the values (ANOVA, $p<0.05$). Letters indicates significant differences among each value (Tukey's post ANOVA, $p<0.05$)

Concerning the oil percentage of *M. oleifera* seeds, it was observed that the value measured for our sample that was cultivated in Paraguay (southern region) was lower than the values reported by Nadeem et al[64]. These authors indicated an oil percentage of 38-42% for seeds cultivated in the temperate regions of Pakistan. In fact, the production of oil depends on the geographical area where the plant is cultivated and on the genetic of the plant.

On the other side, the oil percentage measured for Tenonderà variety (15,4 ± 1,1%) was comparable with the values generally reported in literature for *H. sabdariffa* oil content in seeds, even in the case of different geographical origins (See Table 25).

It could be observed that the oil percentage of the other two analysed varieties of *H. sabdariffa* (Koape guarè and Fortìm) was very low compared to the reference values and to the value found for Tenonderà variety. Also in this case, this may be due to the agro-climatic conditions in which those varieties were grown and on the genetic of the plants[108]: those two varieties had been imported in Paraguay from Brazil and the equatorial area of Africa, while Tenonderà was native to Paraguay.

Oil content	***Geographical origin of the seeds of H. sabdariffa***
20 %	Nigeria[109]
14,6%	Malaysia[62]
18%	n.r.[61]
19%	Spain[81]
15,4%	Paraguay (Tenonderà)

Table 25: Oil content of *H. sabdariffa* seeds, cultivated in different geographical areas of the world.

(n.r.): not reported

4.3.1.2. Extraction of oil

The extraction of the oils was performed by Soxhlet, with n-hexane, according to several procedures reported in literature[109]. Solvent extraction, though being a complex operation, constitutes the most efficient method for the recovery of oil from any oleaginous matrix according to Akinoso et al[109].

4.3.1.3. Characterization

4.3.1.3.1. HPLC-DAD analysis of tocopherols in *M. oleifera* and *H. sabdariffa* seed oil

It is a widespread procedure in literature the quantification of tocopherols in vegetable oils by normal-phase HPLC (NP-HPLC) rather than reverse-phase HPLC (RP-HPLC)[62,81]. The use of a polar stationary phase allows the separation of all the four isomers of tocopherols (α, β, γ, δ). On the other hand, using a not polar stationary phase causes the simultaneous elution of (β+γ)tocopherols, that are determined together[83]. The lack of separation of β and γ tocopherols in the case of edible plant oils introduces rather small error in quantification of these isomers. This is because plant oils do not contain β-tocopherol or contain it in relatively small amounts, as reported also in literature studies[22]. For this reason and because a normal-phase chromatographic column was not available in our laboratory, it was applied a reverse-phase chromatographic method for the determination of tocopherols.
Concerning the preparation of the samples, it was reported in literature that saponification (a common procedure for the preparation of samples) causes significant losses of tocopherols even in protective conditions such as darkness and high nitrogen[83]. For this reason, it was preferred a method based on the simple dilution of the oil in a proper mixture of solvents[83].

In the analysis of our samples, we focused the attention firstly on the concentration of α-tocopherol, as kinetic studies confirmed that α-tocopherol is the most efficient antioxidant toward peroxyl radicals, especially in *vivo*[18]. Then we determined also the amount of (β+γ) tocopherols that are less active than α- as antioxidant, but that were present in our samples. γ-tocopherol showed a strong activity in the seed, protecting compounds like fatty acids, according to data reported in literature[110]. The percentage of (β+γ)-tocopherols was here reported only for the seed oil with the highest percentage of α-tocopherol (Tenonderà).

It was observed that the percentage of α-tocopherol in *M. oleifera* oil (0,00813 ± 0,00018%) was lower than the percentage measured in the oils of *H. sabdariffa* seeds. Among the three varieties of *H. sabdariffa* oils (from seeds cultivated in Paraguay) the percentage of α-tocopherol ranged from 0,0384 ± 0,0012% (Koape guarè) to 0,0519 ± 0,0016 % (Tenonderà), with an intermediate value of 0,04862 ± 0,00055 % (Fortìm). Therefore, Tenonderà variety showed the highest amount of α-tocopherol. It also showed the highest percentage of (β+γ) tocopherols (reported in the box in Figure X), that was also higher than α-tocopherol. δ-tocopherol was not detected in the oils.

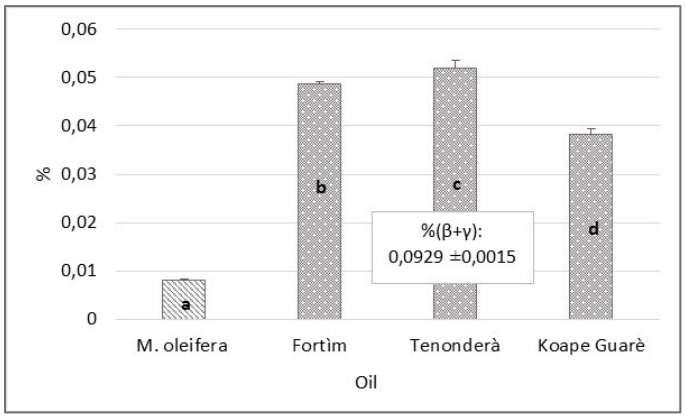


Figure 81: α-tocopherol content (%) of *H. sabdariffa* seed oils, compared to *M. oleifera* seed oil.
Results are expressed as mean ± standard deviation of n=3 determinations.
Different letters indicate significant difference among the values (ANOVA, Tukey's post ANOVA, $p<0.05$).
In the box it is indicated the percentage of (β +γ) tocopherols in the oil Tenonderà, that has the higher percentage of α-tocopherol.

If we consider *H. sabdariffa* oils, the results of this study are in agreement with the study by Mohamed et al[81]. These authors found that the percentage of α-tocopherol in *H. sabdariffa* oil was almost 0,05% and γ-tocopherol almost 0,15%. In their study, β-tocopherol was not detected and δ-tocopherol was just a minority component[81]. Nevertheless, Nyam et al.[62] reported a much lower percentage of α-tocopherol in their oil (extracted from Malaysian seeds), while the percentage of γ-tocopherol was comparable with the one of Tenonderà oil (≈0,07%).
Considering values reported in literature about the percentage of α-tocopherol in *M. oleifera* seed oil (0,0013%)[64], it was possible to note that Tenonderà oil has a higher content of this compound, thus confirming our experimental observation. Therefore, Tenonderà oil is a better source of α-tocopherol than *M. oleifera* oil, to be exploited by a company for commercial purposes.

4.3.2. Second part of the study

This study was continued by performing a new selection of a variety of *H. sabdariffa* seeds with a high oil percentage and α-tocopherol content. Actually, Tenonderà variety that had been selected in the first part of the study revealed some agronomic problems that required its substitution with another variety.

Subsequently, it was performed the extraction of the oil from the newly selected *H. sabdariffa* variety and from *M. oleifera* seeds. These two oils together with two frequently used oils in nutrition and in cosmetic field (olive and sunflower oils) were used for the preparation of oily extracts enriched in compounds with known radical-scavenging activity (carotenoids, polyphenols and α-tocopherol). The oleolites and the respective pure oils were characterized and the radical-scavenging activity was evaluated.

4.3.2.1. Preliminary selection of a variety of *H. sabdariffa* seeds

4.3.2.1.1. Oil content determination in seeds by NMR

It was observed that the oil percentage of the three new varieties of *H. sabdariffa* (Che La Reina, Kibeleza and Tenonderà Tempranera) was significantly lower than the oil percentage of the previously selected variety Tenonderà. Even more, a significant difference was observed for the oil percentage of Tenonderà Tempranera and Kibeleza (ANOVA, Tukey's post ANOVA, $p<0.05$). However, the mean values of the oil content of the three new varieties were comparable.

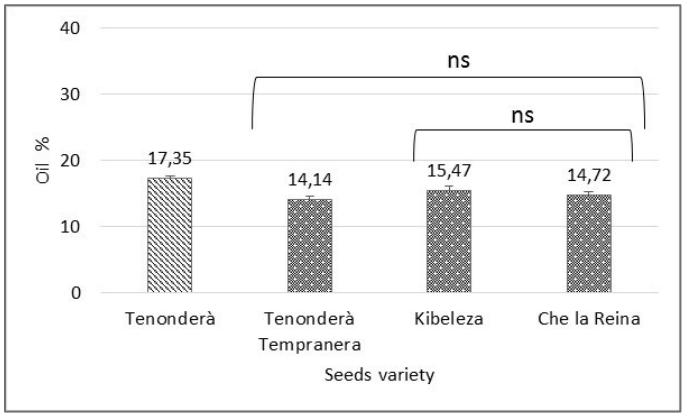


Figure 82: Oil percentage in different varieties of *H. sabdariffa* seeds, compared to Tenonderà seeds. Results are expressed as mean ± standard deviation of n=6 determinations.
(ns) indicates no significant difference among the values (ANOVA, Tukey's post ANOVA, $p<0.05$)

In this case, it was observed that the oil percentage of the seeds of each variety (14,14 ± 0,51%: Tenonderà Tempranera; 15,47 ± 0,71%: Kibeleza; 14,72 ± 0,57 %: Che La Reina) was comparable with the values reported in literature about the oil percentage of seeds of *H. sabdariffa* cultivated in other geographical areas of the world (Table 25). In this case all the three new varieties were native to Paraguay.

4.3.2.1.2. Characterization

4.3.2.1.2.1. HPLC-DAD analysis of tocopherols in *H. sabdariffa* seed oil

The results of this analysis are showed in Table 26.

Variety	***% α-tocopherol***	***% (β+γ)-tocopherol***	***% δ- tocopherol***	***% tocopherol acetate***
Tenonderà	0,0746 ± 0,0060	0,0768 ± 0,0053	n.d.	n.d.
Tenonderà tempranera	0,0645 ± 0,0070	0,0641 ± 0,0078	n.d.	n.d.
Che La Reina	0,0452 ± 0,019	0,0556 ± 0,019	n.d.	n.d.
Kibeleza	0,0579 ± 0,0038	0,0648 ± 0,0034	n.d.	n.d.

Table 26: Percentages of tocopherols in samples of different varieties of *H. sabdariffa* oils, extracted from seeds that were cultivated in Paraguay. Values are expressed as mean ± standard deviation of n=3 determinations.

The analysis of the results showed that there was no significant difference in the percentage of α-tocopherol between Tenonderà oil, Tenonderà Tempranera and Kibeleza oils. Nevertheless, the percentage of α-tocopherol in the oil of Che La Reina variety was significantly lower than that of Tenonderà variety (ANOVA, Tukey's post ANOVA, $p<0,05$).

Even more, no significant difference was observed in the percentages of (β+γ)-tocopherols of all the samples. Finally, neither δ-tocopherol nor α-tocopherol acetate were detected in the samples, using this method.

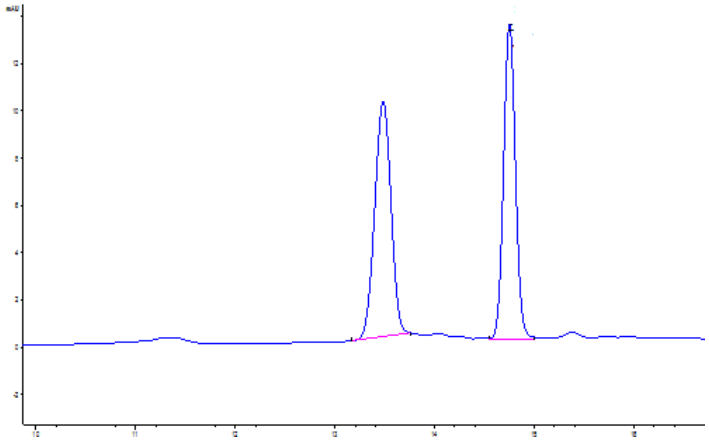


Figure 83: Chromatogram of Tenonderà oil at 292 nm. The first eluting peak corresponds to (β+γ)-tocopherols. The second eluting peak corresponds to α-tocopherol.

Because of the significantly lower content of α-tocopherol in Che La Reina oil compared to the reference oil Tenonderà, Che La Reina variety was discarded. In order to choose a variety of seeds to replace Tenonderà, it was then considered the oil percentage of the remaining varieties. Both Kibeleza and Tenonderà Tempranera could replace Tenonderà but Kibeleza was chosen because of the higher oil content than Tenonderà Tempranera on average.

4.3.2.2. Preparation of oily extracts enriched in antioxidant compounds

4.3.2.2..1. Extraction of *M. oleifera* and *H. sabdariffa* oil

Before starting the preparation of the oily extracts, it was necessary to extract the oils from the seeds of *M. oleifera* and from the selected variety of *H. sabdariffa* seeds (Kibeleza). It was applied the procedure based on the use of n-hexane with Soxhlet.

These two oils were used together with refined olive oil and organic sunflower oil for the preparation of the oleolites. Refined olive oil and organic sunflower oil are normally used for the preparation of cosmetic products as they are odourless and almost colourless or with a pale yellow colour, so they are suitable to be used as a base in the preparation of cosmetics. The oils of *H. sabdariffa* and *M. oleifera* have a pale yellow colour and they smell slightly of nuts, so it may be an option to use them as they are without refining.

In this part of the study, the oily extracts prepared with olive oil were obtained by extracting three different kinds of vegetal matrices (*M. oleifera* leaves, *H. sabdariffa* leaves and the patented matrix EDYNEA03). On the contrary, due to a low amount of *M. oleifera* and *H. sabdariffa* oils available, oily extracts prepared with MO and HO were obtained only by using M. oleifera leaves. Indeed, M. oleifera leaves have a higher carotenoids content than *H. sabdariffa* leaves. Even more, they are known to be richer in polyphenols and to be tenderer than *H. sabdariffa* leaves, hence easier to be extracted.

4.3.2.2.1.1. Maceration

The traditional method for the preparation of oily extracts of vegetal matrices, also called oleolites, is based on the maceration for a long time (30-90 days) at room temperature under stirring[84].

Extraction using edible oils can be considered as a green process (e.g. applying environmentally friendly solvents, reducing the energy consumption, and producing the non-denatured extract without contaminants)[85].

Nevertheless, the viscosity of the oils and the low diffusion coefficient of the phytochemicals due to the low extraction temperature have negative effects on the extraction yields of the compounds of interest[85].

This was observed in the oily extract that was prepared as a trial following the traditional method, by extracting *M. oleifera* leaves with olive oil and measuring compounds of interest (e.g. α-tocopherol).

4.3.2.2.1.2. Ultrasound assisted extraction (UAE)

Recently, ultrasound-assisted extraction (UAE) of compounds with antioxidant properties has been widely applied in order to overcome problems such has the high viscosity of the oily solvents and their scarce efficiency of penetration of the vegetal sample[85] responsible for low extraction yields in the compounds of interest.

Extraction enhancement by ultrasounds has been attributed to the propagation of ultrasound pressure waves and resulting cavitation forces, where bubbles can explosively collapse and generate localized pressure causing plant tissue rupture and improving the release of intracellular substances into the solvent[85].

When using ultrasounds, the efficiency of the extraction depends on the intensity of ultrasounds: the higher is the intensity, the higher is the extraction efficiency[85]. The extraction yield depends also on the extraction time and temperature.

Extraction yield of antioxidant compounds increases from 10 to 30 minutes but decreases from 30 to 60 minutes according to a study reported in literature about the extraction of carotenoids from a fruit waste with vegetable oils and the ultrasounds[85]. This could be explained by the fact that the extraction process occurs in two phases: the first one involves the penetration of the solvent into the cellular structure followed by the dissolution of the soluble constituents in the solvent. The second phase involves the external diffusion of the soluble constituents through the porous structure of the solid tissue and the transfer to the solution[85]. Since ultrasounds accelerates the penetration of the solvent in the vegetal tissue (by breaking vegetal cells), shorter extraction times are suitable to perform the extraction process. Otherwise, due to longer extraction times with ultrasounds, there might be a loss of the compounds of interest, because of their degradation in the solution medium.

Concerning temperature, too higher temperatures may facilitate the dissolution of impurities and the degradation of thermal labile compounds[85]. In a study using sunflower oil for the preparation of ultrasound- assisted extraction of carotenoids from a fruit waste, an ideal temperature of 51 °C was identified, so we decided to apply it also to our extraction systems, as our interest was also focused toward the recovery of antioxidant compounds such as carotenoids and polyphenols, also using sunflower oil.

4.3.2.2..2. Characterization

4.3.2.2..2.1. HPLC-DAD analysis of α-tocopherol

On average, it was not observed a significant variation in the content of α-tocopherol in the oily extracts compared to the relative pure oil (ANOVA, Tukey's post ANOVA, p< 0,05).

The only exceptions were represented by the oleolite prepared by extracting *M. oleifera* leaves with olive oil (from 7,42 ±0,23 to 13,20 ± 0,61 mg/100 g) and *M. oleifera* leaves with *M. oleifera* oil (from 17,24 ± 0,52 to 19,61 ± 0,23 mg/100g). These two oils showed a similar behaviour towards the extraction of *M. oleifera* leaves. (See Figure 84)

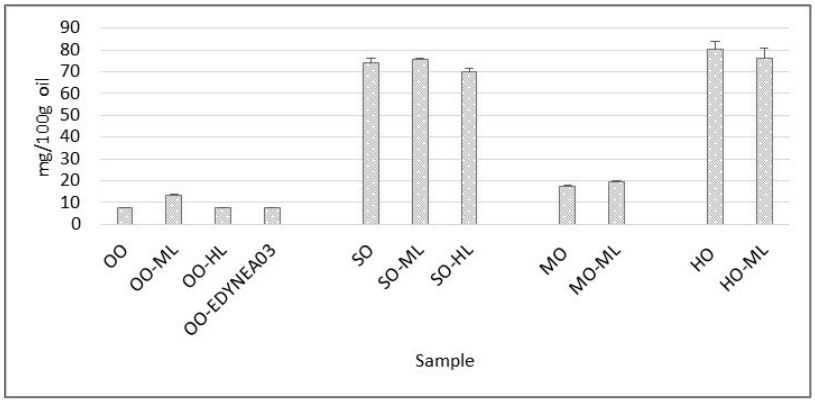


Figure 84: α-tocopherol content (mg/100 g oil) of different vegetable oils and their oleolites.

Results are reported as mean ± standard deviation of n=3 determinations.

OO: olive oil; OO-ML: olive oil-Moringa leaves; OO-HL: olive oil-Hibiscus leaves; OO-EDYNEA03: olive oil-EDYNEA03; SO: sunflower oil; SO-ML: sunflower oil-Moringa leaves; SO-HL: sunflower oil-Hibiscus leaves; MO: Moringa oil; MO-ML: Moringa oil-Moringa leaves; HO: Hibiscus oil; HO-ML: Hibiscus oil-Moringa leaves.

The increasing in the concentration of α-tocopherol observed for the oleolite prepared with olive oil and *M. oleifera* leaves, confirmed that the application of the ultrasound technique is a better option for an effective extraction of phytochemicals when using oil as solvent than traditional methods for the preparation of oleolites. In fact, a decrease in the content of α-tocopherol had been observed in the oily extract that was prepared by traditional maceration using *M. oleifera* leaves and olive oil (See Figure 85)

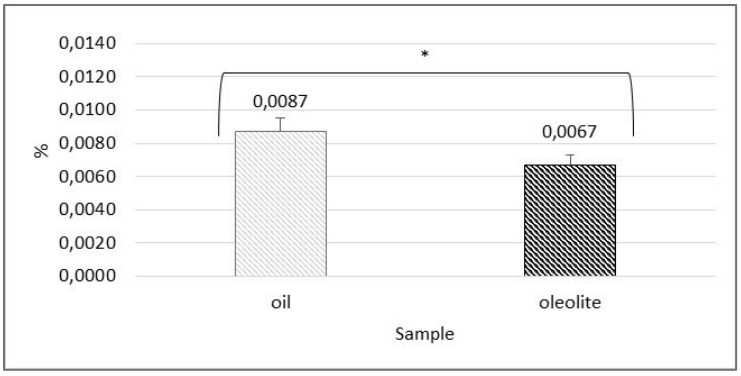


Figure 85: α-tocopherol content olive oil and its oleolite, prepared by extracting *M. oleifera* leaves. A traditional extraction method was applied, based on maceration.

Finally, the highest concentration of α-tocopherol were observed in the oils of *H. sabdariffa* and sunflower, and their values were comparable. Our findings were in agreement with the study by Tuberoso et al.[110] because also these authors reported a higher content of α-tocopherol in sunflower oil (494,2 ± 15,1 mg/1Kg) than in olive oil (212,1 ± 4,1 mg/ 1Kg).

4.3.2.2.2.2. Determination of total carotenoid content (TCC)

Total carotenoids were determined by a spectrophotometric method that is based on the preparation of a calibration curve with a standard carotenoid reference compound, like β-carotene. The curve is used to quantify total carotenoids in the sample that are expressed as mg equivalents to β-carotene per 100 g of oil or oily extract.

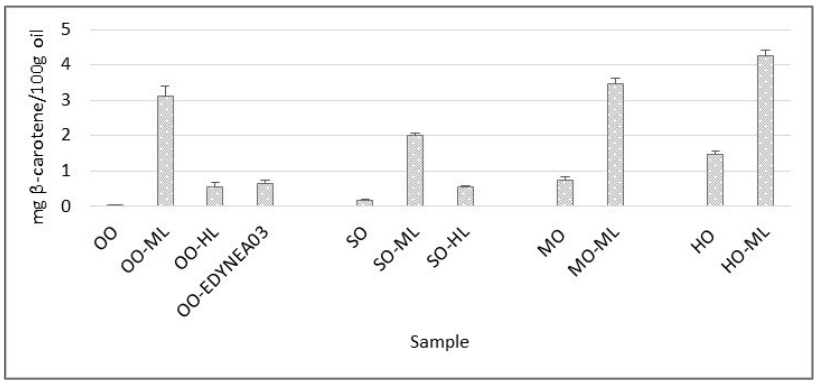


Figure 86: Total carotenoid content of different vegetable oils and their oleolites.

Results are reported as mean ± standard deviation of at least four determinations.

OO: olive oil; OO-ML: olive oil-Moringa leaves; OO-HL: olive oil-Hibiscus leaves; OO-EDYNEA03: olive oil-EDYNEA03; SO: sunflower oil; SO-ML: sunflower oil-Moringa leaves; SO-HL: sunflower oil-Hibiscus leaves; MO: Moringa oil; MO-ML: Moringa oil-Moringa leaves; HO: Hibiscus oil; HO-ML: Hibiscus oil-Moringa leaves.

It was observed a significant increase in the concentration of total carotenoids in all the oily extracts compared to the respective pure oil (ANOVA, Tukey's post ANOVA, $p<0,05$).

As showed in Figure 86, the increase is much higher when the oleolite was prepared by extracting *M. oleifera* leaves, rather than *H. sabdariffa* leaves or the patented matrix EDYNEA03.

In a study carried out on the leaves of *H. sabdariffa*, *C. asiatica*, *M. oleifera* and *M. koenigii* it was showed that the highest carotenoid content was found in *M. oleifera* compared to the other three plants[111].

The result obtained from the EDYNEA 03 matrix is similar to that obtained from the analysis of the oleolites obtained from the extraction of the leaves of *H. sabdariffa* and this could be due to a low total carotenoid content in that patented matrix.

It was possible to observe that the highest amount of total carotenoids was present in the oily extract obtained by the extraction of *M. oleifera* leaves with *H. sabdariffa* oil (4,27 ± 0,16 mg β-carotene/100

g) and *H. sabdariffa* Kibeleza oil had the highest concentration of total carotenoids (1,45 ± 0,12 mg β-carotene/100 g) among the four pure oils used. The value that it was found in this study related to a Paraguayan oil was much more lower than the value reported by Zoué et al.[112] that was equal to 120,0 ± 0,0 mg β-carotene/100 g). Their seeds were from Ivory Coast, so this difference may be due to the different geographical origin of the seeds.

4.3.2.2.2.3. Determination of total phenolic content (TPC)

The analysis of the total polyphenols was performed on the methanolic extracts of the oils, with the Folin-Ciocalteu method, previously described in section 4.2.1.2.4.

It was observed on average a significant increase of the TPC in the oleolites compared to the corresponding pure oil, with the only exception of the oily extract obtained by an extraction of *M. oleifera* leaves with *H. sabdariffa* oil (ANOVA, Tukey's post ANOVA, $p<0,05$). The latter was interested by a decrease in the TPC that may be explained by the fact that such a high content of phenols in the starting pure oil does not favour the further extraction of this class of compounds. Therefore, the extraction conditions caused even a loss of phenolic compounds.

For the oily extracts obtained by using olive oil, TPC values ranged from 124 ± 11 mg GAE/100 g oil (OO-EDYNEA03) to 161 ± 24 mg GAE /100 g oil (OO-HL).
For the oily extracts obtained by using sunflower oil, TPC values ranged from 79,3 ±4,0 mg GAE/ 100g oil (SO-HL) to 105,1 ± 4,5 mg GAE/100 g oil (SO-ML).
The TPC value of the oleolites obtained by using *M. oleifera* oil and *H. sabdariffa* Kibeleza oil were 447 ± 34 mg GAE/100 g oil and 640 ± 14 mg GAE/100 g oil, respectively.

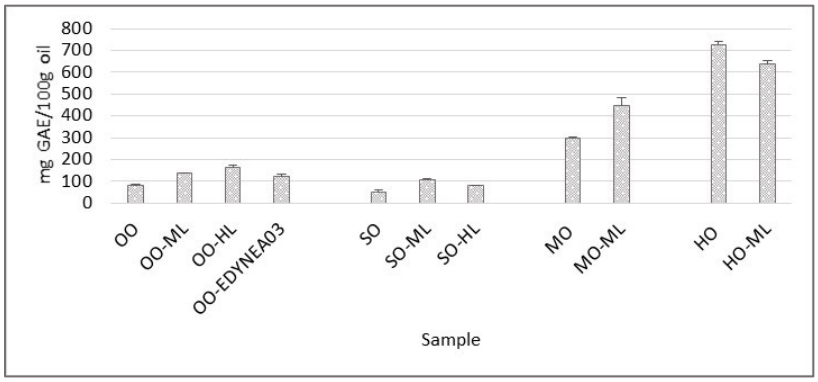


Figure 87: TPC of different vegetable oils and their oleolites.

Results are reported as mean ± standard deviation of at least three determinations.

OO: olive oil; OO-ML: olive oil-Moringa leaves; OO-HL: olive oil-Hibiscus leaves; OO-EDYNEA03: olive oil-EDYNEA03; SO: sunflower oil; SO-ML: sunflower oil-Moringa leaves; SO-HL: sunflower oil-Hibiscus leaves; MO: Moringa oil; MO-ML: Moringa oil-Moringa leaves; HO: Hibiscus oil; HO-ML: Hibiscus oil-Moringa leaves.

It was observed that the oils of *M. oleifera* and *H. sabdariffa* Kibeleza and their oleolites had a higher TPC than the oils of olive and sunflower and their oleolites, with the higher value observed for *H. sabdariffa* Kibeleza oil. The TPC value that it was found in this study for *H. sabdariffa* Kibeleza oil was sharply higher than the value reported in literature for an oil of *H. sabdariffa* seeds, cultivated in Ivory Coast (6,6 ± 0,0 mg GAE/100 g)[112].

4.3.2.2.3. Assessment of the properties

4.3.2.2.3.1Total antioxidant capacity (TAC)

The analysis of the results showed that there was no significant difference in the antioxidant activity of the oleolites compared to the corresponding pure oil (ANOVA, Tukey's post ANOVA, $p<0.05$). Therefore, the production of an oily extract higher in antioxidant components had not lead to a better antioxidant activity, at least in the extraction conditions used. Among the oils, the more active was the one from the selected variety of *H. sabdariffa* seeds (DPPH: 173 ± 20 µmol TE/100 g oil and $ABTS^{\bullet+}$: 169 ± 12 µmol TE/100 g oil).

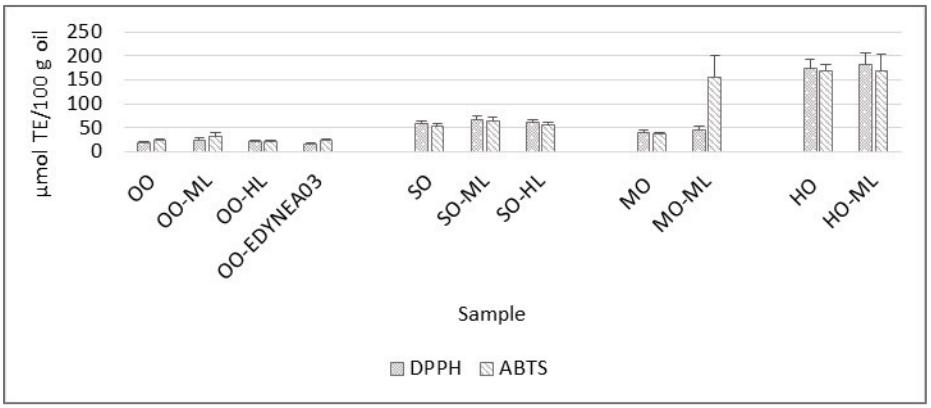


Figure 88: radical scavenging activity of different oils and oleolites, measured by DPPH and $ABTS^{\bullet+}$ assays. Results are expressed as mean ± standard deviation of n=3 determinations and n=4 determinations for DPPH and $ABTS^{\bullet+}$ respectively.

OO: olive oil; OO-ML: olive oil-Moringa leaves; OO-HL: olive oil-Hibiscus leaves; OO-EDYNEA03: olive oil-EDYNEA03; SO: sunflower oil; SO-ML: sunflower oil-Moringa leaves; SO-HL: sunflower oil-Hibiscus leaves; MO: Moringa oil; MO-ML: Moringa oil-Moringa leaves; HO: Hibiscus oil; HO-ML: Hibiscus oil-Moringa leaves.

The correlation analysis (see Table 27) showed that polyphenols were mainly involved in the direct antioxidant activity of the extracts. Nevertheless, a positive correlation was observed also between the radical-scavenging activity and the content of total carotenoids and α-tocopherol, even if lower than that calculated for polyphenols.

Tuberoso et al.[110] reported that the free radical scavenging activity is influenced by the tocopherols content in oils. Nevertheless, they considered that the variability of the correlation between the antioxidant activity and the composition of the oils could be attributed to the differences in the

chemical composition of the oils (e.g. carotenoids, and phenols contents) and their mutual interactions[110].

	TCC	*TPC*	*α-tocopherol*	*DPPH*	*ABTS*
TCC	1,0000				
TPC	0,5793	1,0000			
α-tocopherol	0,1661	0,3322	1,0000		
DPPH	0,4679	0,8244	0,7680	1,0000	
ABTS	0,6595	0,8918	0,5487	0,8399	1,0000

Table 27: Correlation matrix of TPC, TCC, α-tocopherol and direct antioxidant activity, measured by DPPH and $ABTS^{\bullet+}$ assays.

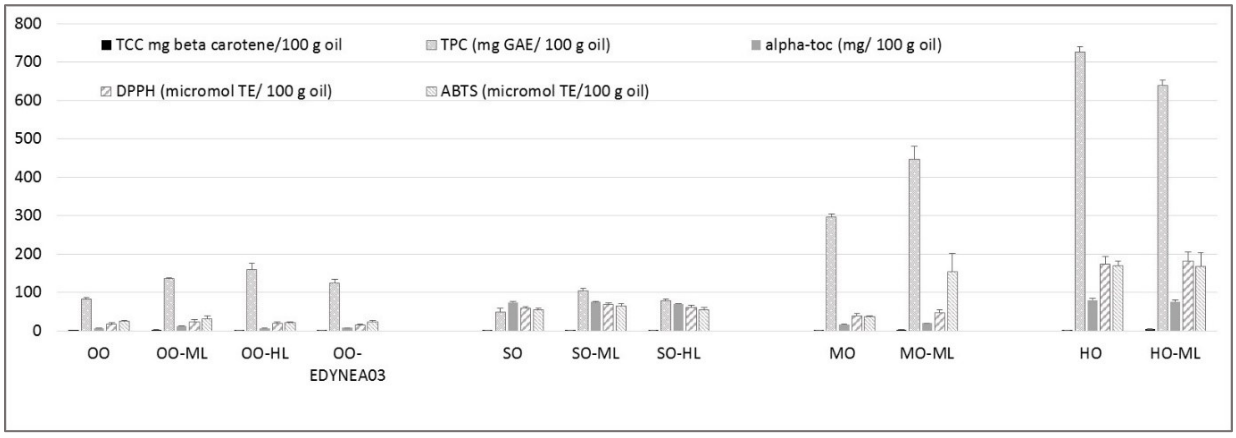


Figure 89: summary of the results obtained from the characterization of different vegetable oils and their oleolites that were produced by extracting M. oleifera leaves or H. sabdariffa leaves or EDYNEA03. OO: olive oil; OO-ML: olive oil-Moringa leaves; OO-HL: olive oil-Hibiscus leaves; OO-EDYNEA03: olive oil-EDYNEA03; SO: sunflower oil; SO-ML: sunflower oil-Moringa leaves; SO-HL: sunflower oil-Hibiscus leaves; MO: Moringa oil; MO-ML: Moringa oil-Moringa leaves; HO: Hibiscus oil; HO-ML: Hibiscus oil-Moringa leaves.

5.CONCLUSIONS

5.1. First project line: *Hibiscus sabdariffa* L. calyces

In the first part of the study, it was concluded that Koape guarè variety was the richest in anthocyanins among the varieties considered and cultivated by EDYNEA in the southern part of Paraguay; however, the cultivation of Koape guarè in Paraguay showed some agronomic problems due to climate and day length. Indeed, Koape guarè originated from the equatorial area of Africa. For this reason, it has been necessary to look for another variety of *H. sabdariffa*, more suitable for the cultivation in that area. After all, Tenonderà Tempranera variety was selected as the best one among the new varieties analysed. All the new varieties were native to Paraguay and their cultivation showed to be suitable for the agro-climatic conditions of that area.

The study, performed to optimize extraction parameters suitable for the extraction of anthocyanins from calyces in high yields but also transferable on a large scale production of extract, highlighted that Tenonderà Tempranera calyces can be used to prepare aqueous extracts at room temperature, assisted by ultrasounds (40 KHz) and with an extraction time of 15 minutes. As no significant differences were observed in the TMA values of extracts prepared according to different extraction methods, the choice of the best extraction parameters relied on economic and marketing reasons. Actually, ultrasonic extraction could be claimed as a plus and a lower temperature during process represent a less expensive industrial operation.

The enrichment method based on the use of PVPP, for the production of aqueous extracts enriched in anthocyanin for nutraceutical and/or cosmetic applications, needs to be improved in order to increase the TMA value despite the lower extraction yield. As far as we know, it is not reported in literature an enrichment method for the anthocyanins of *H. sabdariffa*, based on the use of PVPP.

Finally, it was prepared a model extract using the selected variety of calyces and the selected extraction method. This extract was lyophilised and characterized and in this way it was possible to refine the chromatographic and spectrophotometric methods that could be used in the future to carry out quality controls, in case this extract would be produced and used as a source of anthocyanins in the formulation of a food supplement. The antioxidant activity of standardized *H. sabdariffa* extracts from calyces has already been reported in literature, *in vivo*, too. However, further studies can be carried out to assess the in *vivo* antioxidant activity of this particular extract. Studies concerning the formulation of *H. sabdariffa* extracts in nutraceutical and cosmetic products are recommended, too.

5.2. Second project line: *Moringa oleifera* Lam. tissues

Concerning *M. oleifera*, the aqueous extracts studied (obtained from matrices native to Paraguay, subjected to oven-drying or freeze-drying) represent a good source of polyphenols, as direct antioxidants, especially the extracts obtained from oven-dried leaves and freeze-dried branches (leaves>branches). Even more, they are a good source of glucomoringin as precursor of its bioactive isothiocyanate with reported indirect antioxidant properties. The best extracts in terms of glucomoringin content were the ones from oven-dried branches, EDYNEA01, EDYNEA02 and the one obtained from freeze-dried seeds (seeds>branches, EDYNEA01, EDYNEA02). Particular interest assume the extracts obtained from EDYNEA01, EDYNEA02 (oven-dried) and the extract obtained from the seeds (freeze-dried) because they showed to already contain the bioactive isothiocyanate. The extract obtained from EDYNEA02 (oven-dried) is the extract that combines a good glucomoringin content and the presence of its isothiocyanate with a significant phenolic content, so a correlated direct antioxidant activity.

Therefore, it was decided to continue the second part of the study relative to this second project line with the most interesting matrices: oven-dried leaves (as a good source of polyphenols), freeze-dried seeds (as a good source of glucomoringin), oven-dried branches and EDYNEA02 (both considered interesting sources of these classes of compounds).

Then, it was possible to select an extraction condition suitable for the recovery of total polyphenols from the vegetal matrices and to convert glucomoringin into the bioactive isothiocyanate. Through the application of the selected extraction parameters, the branches (that have a lower phenolic content and total antioxidant capacity than leaves) can bring to an extract with an equal TPC amount but a higher antioxidant capacity than the extract of leaves, thus becoming a valid alternative to leaves in the preparation of extracts rich in antioxidant compounds.

This observation is particularly important considering that branches are generally a waste product in the processing of *M. oleifera*. Moreover, the lighter colour of the extract obtained from branches and its lighter fragrance make it more suitable as ingredient of cosmetic products with antioxidant properties. The extract obtained from EDYNEA02 showed to be a valid alternative to leaves as a source of glucomoringin-isothiocyanate, instead.

As stated by Tumer at al.[12] there is a unique combination of direct (polyphenols) and indirect (isothiocyanates) antioxidant compounds in *M. oleifera*. This justifies and supports the use of polyphenol- and isothiocyanates-enriched *M. oleifera* products, both in nutraceutical and in cosmetic

field. Nevertheless, further in vitro studies have to be carried out to assess the indirect antioxidant activity of the extracts and to evaluate if there is a correlation with the concentration of the isothiocyanate. Moreover, further studies are recommended to assess the nutrikinetics properties of the extracts in case of an oral administration or their *in vivo* antioxidant properties for both nutraceutical and cosmetic applications.

Finally, no inhibitory activity on the growth of *P. acnes* was observed, nor due to the isolated isothiocyanate, neither due to *M. oleifera* extracts.

5.3. Third project line: oilseeds and oily extracts

In the first part of the study related to this third line of the project, it was concluded that Tenonderà variety of *H. sabdariffa* seeds was the best one because of its oil content and tocopherols content in the oil. Nevertheless, Tenonderà showed some agronomic problems during its cultivation in Paraguay, despite being a local variety. For this reason, it was performed a new selection of *H. sabdariffa* seeds.

Kibeleza variety was then selected as a suitable variety for seeds. Its oil showed an interesting profile of total carotenoids, total phenolic compounds, alpha-tocopherol and antioxidant capacity compared to *M.oleifera* oil and to other two oils commonly used in nutrition and cosmetic applications (refined olive oil and sunflower oil.)

The subsequent production of oily extracts, although showed a higher content of antioxidants compared to pure oils, has not lead to a better antioxidant activity, at least in the extraction conditions used. These conditions were based on high extraction temperature in order to reduce the viscosity of the oily solvent and on the application of ultrasounds with a frequency of 40 KHz in order to facilitate the extraction process by inducing the rupture of the plant cells.

In the future, further tests should be done to obtain an oily extract enriched in antioxidant phytochemicals with a higher antioxidant activity compared to the pure oil, to be used as ingredient of cosmetic antioxidant products or as a vehicle of antioxidant compounds in nutraceutical or nutri-cosmetic products.

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