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REVIEW ARTICLE

Plant-Derived Polyphenols in Human Health: Biological Activity, Metabolites and Putative Molecular Targets

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Abstract: ***Background:*** *Hibiscus sabdariffa*, *Lippia citriodora*, *Rosmarinus officinalis* and *Olea europaea*, are rich in bioactive compounds that represent most of the phenolic compounds' families and have exhibited potential benefits in human health. These plants have been used in folk medicine for their potential therapeutic properties in human chronic diseases. Recent evidence leads to postulate that polyphenols may account for such effects. Nevertheless, the compounds or metabolites that are responsible for reaching the molecular targets are unknown.

Objective: data based on studies directly using complex extracts on cellular models, without considering metabolic aspects, have limited applicability. In contrast, studies exploring the absorption process, metabolites in the blood circulation and tissues have become essential to identify the intracellular final effectors that are responsible for extracts bioactivity. Once the cellular metabolites are identified using high-resolution mass spectrometry, docking techniques suppose a unique tool for virtually screening a large number of compounds on selected targets in order to elucidate their potential mechanisms.

Results: we provide an updated overview of the *in vitro* and *in vivo* studies on the toxicity, absorption, permeability, pharmacokinetics and cellular metabolism of bioactive compounds derived from the abovementioned plants to identify the potential compounds that are responsible for the observed health effects.

Conclusion: we propose the use of targeted metabolomics followed by *in silico* studies to virtually screen identified metabolites on selected protein targets, in combination with the use of the candidate metabolites in cellular models, as the methods of choice for elucidating the molecular mechanisms of these compounds.

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1. INTRODUCTION

The interest in dietary polyphenols has increased over the past 10 years due to their large abundance in plants and fruits and their multiple beneficial properties for human health. These molecules are synthetized by plants as secondary metabolites and are involved in diverse functions, such as growth, lignification and structure, pigmentation, pollination or defense against pathogens, predators and ultraviolet radiation [1, 2]. These compounds are structurally characterized by the presence of one (phenol) or more (polyphenol) hydroxyl substituents and can be classified into different groups, depending on their carbon skeleton: phenolic acids, flavonoids, stilbenes and lignans. Flavonoids are the most abundant polyphenols in diet and can be divided into flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols. The complexity of polyphenols increases since these compounds can be associated with several carbohydrates or organic acids and with other polyphenols, which widely increases their diversity and their ability to reach multiple molecular targets [3-5].

Plant-derived polyphenols have been found to possess antitumor, antimicrobial, antiviral, anti-inflammatory, antiatherogenic, antihypertensive, anti lipogenic and antioxidant activities [6-13]. Over the last decades, polyphenols have been widely designated as strong antioxidant agents, and it has been considered that the supported biological effects are derived from this antioxidant capacity [12]. Recently, a new perspective is emerging, as polyphenols have exhibited a pleiotropic character, so there is an increasing consideration of polyphenols in the prevention and treatment of multifactorial diseases, such as cancer or obesity-related pathologies [14].

Although pharmacological potency strongly influences the *in vivo* activity, polyphenols biological activity (efficacy) depend not only on their potency but also on their bioavailability. Some polyphenols are rapidly absorbed by the gut barrier and reach the circulating plasma in their native form, while others are poorly absorbed and may be highly metabolized or rapidly excreted. Accordingly, the metabolites that reach the circulating blood and target tissues may differ from their native forms and the gastrointestinal tract plays a crucial role in this. These compounds may be first hydrolyzed by gastric fluid in the stomach and later metabolized by the enzymes of intestinal cells or catabolized by the colonic microflora, which may drastically affect the absorption of these molecules through the gut barrier (Fig. **1**). In addition, polyphenols may also undergo important Phase I and Phase II reactions in the liver, promoting their excretion through urine or bile and reducing their bioavailability. The most frequent conjugation reactions are methylation, sulfation and glucuronidation. In the

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circulating plasma, polyphenols or their metabolites may circulate when bound to albumin and may be able to penetrate tissues where they can exert their potential systemic effects. Once in the target tissues, these molecules can also be accumulated into tissue cells or can undergo new biotransformations into other compounds [1, 3, 4] (Fig. **1**).

Therefore, understanding the bioavailability, absorption and metabolism of plant polyphenols from diet is essential in order to clarify their mode of action, and determine the final active metabolites. With this purpose, many researchers have focused their attention on the study of the absorption of polyphenols by measuring plasma concentrations and/or examining urinary and fecal excretions from animal models or humans after consumption of a single dose of the compound or a complex plant extract or beverage [15, 16]. Pharmacokinetic measurements may include several variables: maximum plasma concentration (Cmax), time to reach Cmax (Tmax), area under the plasma concentration-time curve (AUC) and the elimination half-life ($t_{1/2}$) (Fig. **1**). Several factors, such as administration route, gender, age, genetic polymorphisms, hormonal status and food interaction, seriously influence the pharmacokinetics and bioavailability of polyphenols [17]. Nevertheless, studies that consider these influential factors are scarce.

The purpose of this review is to offer a summary, built on our own experience of the bioavailability, absorption, distribution, metabolism and excretion of polyphenols, that are present in four selected edible plants, bearing the most representative polyphenols' families, namely, *Hibiscus sabdariffa*, *Lippia citriodora*, *Rosemary officinalis* and *Olea europaea* (Figs. **2-4**), as well as to propose some of their putative molecular targets. These plants are commonly consumed as beverages, such as teas or juices or food seasonings and some of them have been used in folk medicine. Within the last decade, we have accumulated enough evidence to postulate that compounds derived from these plants may contribute to the prevention and/or the treatment of several metabolic pathologies, such as cancer, obesity, diabetes or cardiovascular diseases. It is postulated that these beneficial effects are mainly due to the presence of phenolic compounds, and studies on their pharmacological activities as pure compounds are extensive and consistent [18]. On the other hand, little is known about the pharmacokinetic behavior of these phenolic compounds after the consumption of plant extracts, and further investigations are required to identify the active metabolites that are responsible for such effects. Furthermore, the toxicological profile and tolerability are also important points that deserve more attention to understand possible side effects and to establish a safe dose to be administered as a dietary supplement in humans.

1.1. *Hibiscus sabdariffa*

1.1.1. Description and Composition

Hibiscus sabdariffa L. (HS) is a tropical plant belonging to the Malvaceae family, a wide family that comprises more than 4000 species. This plant is commonly known as Roselle, Karkade or Jamaica sorrel and is native to India and Malaysia. Currently, it is widely cultivated in the tropics and subtropics of both hemispheres and its flowers are normally consumed throughout the world. HS is an annual, erect and herbaceous sub-shrub that can grow up to 2.4 m in height with a typical red flower (calyx) consisting of five large sepals [19]. Anthocyanins are the natural pigments responsible for the red color of HS calyces, and they make HS a profitable product as a coloring ingredient in drinks. Furthermore, ingredients based on HS calyces have been traditionally used in folk medicine for the treatment of hypertension [20, 21], pyrexia [22] and inflammation [21, 23], kidney [24, 25] and liver [26-28] disorders and even obesity [29, 30]. Likewise, HS extracts have been shown to possess antioxidant [15, 27, 31-33], antitumor [34], anti-atherosclerotic [35, 36] and antimicrobial [37] properties.

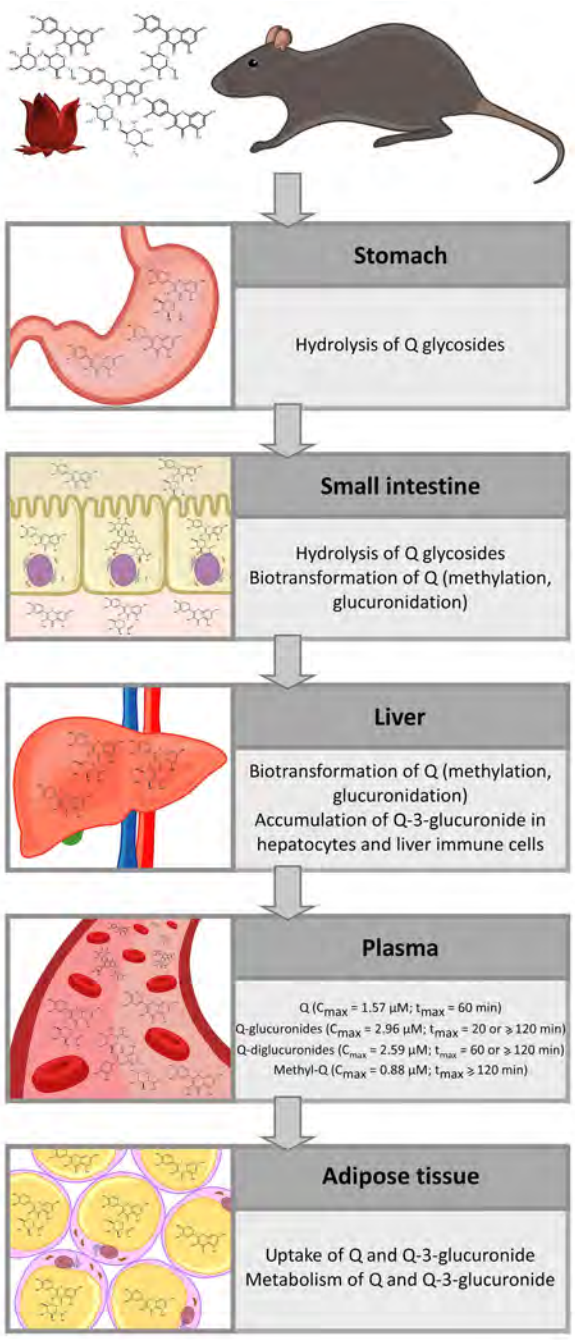

Fig. (1). Schematic flow diagram showing the bioavailability and metabolism of polyphenolic compounds along the different steps involved from the oral ingestion to their arrival to target tissue (adipocytes) in a rat model. Quercetin (Q) and its derivatives from HS extract have been selected to illustrate this process. After oral ingestion, glycosides derivatives are likely hydrolyzed in the stomach. Then, in the small intestine, both in lumen and inside enterocytes, additional hydrolysis reactions occur along with methylation and glucuronidation. Once absorbed, most probably by passive diffusion, first pass effect introduces additional methylation and glucuronidation moieties on liver along with hepatic accumulation of some of the metabolites such as Q-3-glucuronide. After phase I and II metabolism reactions, metabolites reach plasma and exhibit different pharmacokinetic behavior (maximum plasma concentration, C_{max}; time to reach Cmax, t_{max}) depending of their individual characteristics. Finally, metabolites arrive to their target, adipose tissue in this particular case, where not all but only selected compounds (Q and Q-3-glucuronide) are transported into adipocytes and suffer additional intracellular metabolism such as glucuronidation or deglucuronidation. The diagram has been constructed based on the evidence accumulated by our group, and others [15, 28, 46-48].

The qualitative characterization [38] and quantitation [31] of the compounds present in the aqueous extract of HS calyces have been carried out by high-performance liquid chromatography with diode array detection coupled to electrospray time-of-flight mass spectrometry or ion trap tandem mass spectrometry (HPLC-DAD-ESI-TOF-MS or HPLC-DAD-ESI-IT-MS). In these first studies, a total of seventeen compounds were found and quantified in the aqueous extract (Fig. **2A**). Among them, the main constituents were organic acids, phenolic acid derivatives, flavonol derivatives, phenylpropanoids and anthocyanins (Figs. **3** and **4**). Low molecular weight polysaccharides are another abundant group of compounds present in HS [39], but the biological activity of HS extract lies in the polyphenolic content, mainly quercetin and kaempferol derivatives, which are concentrated after ethanol precipitation or affinity chromatography [33]. In this later study, a total of 37 polyphenolic compounds were determined.

1.1.2. Biological Activities

A large amount of *in vitro*, *in vivo* and clinical studies have been published in order to explain the beneficial effects and potential mechanism of HS bioactive compounds. Most of these effects seem to be associated with the potent antioxidant capacity of HS extracts, which may take place through their direct strong scavenging effects on reactive oxygen species (ROS) [31], and also by their capacity to increase the activity/expression of antioxidant enzymes, such as glutathione (GSH), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in liver [27]. The relationship between the antioxidant capacity of anthocyanins and their antiatherogenic activity, through the inhibition of *in vitro* low-density lipoprotein (LDL) oxidation and prevention of oxLDL-induced apoptosis, has also been reported [36].

The antioxidant activity of HS has been well correlated with an anti-inflammatory effect since inflammation is normally related to oxidative stress in several chronic diseases as confirmed previously in the liver of Sprague-Dawley rats treated with bacterial lipopolysaccharide (LPS). The inflammatory and oxidative effects induced by LPS were re-established after pretreatment with an extract of HS [40], suggest an important implication of HS extract on the treatment of chronic inflammatory diseases. We have also reported the anti-inflammatory effects of HS polyphenolic extract through the means of reduced pro-inflammatory cytokines in a hypertrophic adipocyte cell model [33] and in humans trials [21, 23].

The vast majority of clinical trials assayed with HS extracts show the antihypertensive effectiveness of this extract in patients with metabolic syndrome and hypertension. According to most studies, the antihypertensive effect of HS extract is related to the inhibition of angiotensin I-converting enzyme (ACE) and a reduction in serum sodium levels [41, 42]. Nevertheless, Joven *et al*. suggested that the inhibition of ACE activity contributes in a lesser degree and that the antioxidant, anti-inflammatory and endothelium-dependent effects are, most likely, the mechanisms involved in the hypotensive effect of HS [21].

Several studies have confirmed the anti-obesity effect of HS, proposing the use of this plant in the treatment of obesity and metabolic syndrome [29, 30, 43, 44]. Previously, a direct effect of compounds from HS extract on an adipose tissue model has been reported. Our research group has evaluated a polyphenol-enriched HS extract in a model of adipogenesis from 3T3-L1 cells and in hypertrophic and insulin resistant adipocytes [33]. The polyphenolic extract of HS extract showed potent activity in inhibiting adipogenesis, triglyceride accumulation, ROS generation and pro-inflammatory cytokine secretion, suggesting that the complex mixture of HS polyphenolic compounds may interact with numerous endogenous molecular targets.

As a sign of the multitargeted action of polyphenols, the capacity of HS polyphenols to modulate gene expression in a hyperlipidemic mouse model has been reported. Mice deficient in LDL receptor (LDLr-/-) were fed a high-fat diet to induce fatty liver disease and were fed HS polyphenols. The results of this study showed that a treatment with HS polyphenols reduced weight gain, ameliorated liver steatosis and modified the composition of liver tissue compared to the control. Furthermore, these changes were associated with a differential expression of liver miRNAs and lipogenic genes and an activation of 5'-adenosine monophosphate-activated protein kinase (AMPK) [28]. All these data support the pleiotropic character of the polyphenols of HS and their potential involvement on multifactorial diseases.

1.1.3. Pharmacokinetics and Toxicology

Despite abundant research on the pharmacology of HS, studies about its pharmacokinetics are scarce, and the main compounds or metabolites responsible for the bioactivity of the plant remain unclear. Furthermore, the final pharmacological effect of HS depends on the composition and dosage of the extract. Therefore, the identification of its bioactive components, its bioavailability in humans and its safety are important issues in order to estimate a suitable formulation and dosage to reach the desired therapeutic effect.

The potential therapeutic superiority of an HS polyphenolic extract enriched in flavonols and its synergic effect has been suggested in adipocyte cell model [33]. Nevertheless, we should be cautious when extrapolating *in vitro* data to an *in vivo* situation. Compounds may be metabolized by the intestinal flora and enzymes in the wall of the intestine, influence the absorption and bioavailability of these compounds and modify their biological activity *in vivo*. Plasma samples from healthy volunteers administered an acute dose of HS aqueous extract (10 g) revealed a significant decrease in monocyte chemoattractant protein-1 (MCP-1) at 1.5 and 3 h after ingestion, suggesting fast absorptions and high circulating concentrations of the bioactive compounds from the extract [23]. A pharmacokinetic study of a single oral dose of HS extract in healthy volunteers revealed very low concentrations of anthocyanins in plasma, which reached the maximum levels at 1.5 h after ingestion, indicating the poor absorption and fast urinary excretion of intact anthocyanins [45]. Several studies have proposed anthocyanidin-3-glucosides as candidates for the beneficial effects of HS. However, the rapid and poor absorption of anthocyanidins and recent evidence in animal models are increasingly pointing to the fact that flavonols, such as quercetin, may also be considered as candidates for such effects [23, 28, 33].

The bioavailability and pharmacokinetics of the HS polyphenol-enriched extract has been evaluated in Wistar rats after an acute oral dose of 1200 mg/kg [15]. A total of seventeen compounds were detected in rat plasma (Fig. **3A**). Several glucoronides of quercetin and kaempferol were found in rat plasma, which most likely were derived from the aglycone forms of these flavonoids after pre- or post-absorption deglycosylation. The organic acids and phenolic acid derivatives not only reached higher concentrations in plasma than flavonols, but also exhibited lower initial elimination half-life values, indicating the lack of accumulation of these compounds in the tissue. Among all the quercetin and kaempferol derivatives found in plasma, the highest concentrations were found for quercetin glucuronide and quercetin aglycone, compounds that showed larger elimination values, revealing a tissue accumulation and probable long-term effects (Fig. **1**). In this study, a correlation between the presence of polyphenols in plasma and antioxidant status was also observed.

Interestingly, the presence of quercetin-3-glucuronide was also detected in the liver and intestinal mucosa of hyperlipidemic LDLr-/- mice fed a polyphenolic-enriched HS extract for 10 weeks [28]. This glucuronide metabolite was also found in immune cells surrounding the surface of lipid droplets in the liver (Fig. **1**). The findings of this study suggested that intestinal mucosa exerts an important enzymatic activity through glucuronyl conjugation of the aglycone form of quercetins derived from HS. Thus, quercetin-3-

glucuronide could be one of the major metabolites from HS to account for the changes in the composition of liver tissue through the modulation of expression of liver miRNAs and lipogenic proteins.

The permeability of the polyphenolic extract from HS has also been evaluated in Caco-2 human cell monolayers, a model of human intestinal absorption [46, 47]. Analysis of the extract by ultra-high-performance liquid chromatography coupled with ultra-high-resolution quadrupole time-of-flight mass spectrometry (UHPLC-ESI-UHR-Qq-TOF-MS) identified most of the compounds that were previously described [31, 33]. In this study, absorption of the major HS metabolite, quercetin-3-glucuronide, and of isolated compounds, which are present at important levels in HS extract, were also studied in Caco-2 cell monolayers (quercetin, quercetin-3-glucoside and N-feruloyltyramine) [15, 28]. Nevertheless, neither phenolic acids nor anthocyanins were selected for their reported poor absorption, which reinforces the hypothesis that these compounds have little or no contribution to HS biological effects. The study revealed a significant absorption in the cell monolayer for all the compounds, especially for quercetin, most likely by passive diffusion and a high basolateral-apical permeability, suggesting a mechanism of transport efflux of these compounds (Fig. **1**).

Intestinal mucosa and liver are not the only tissues where the glucuronidation reactions occur. Recently, Herranz-López *et al*. found glucuronyltransferase and glucuronidase activities in hypertrophied 3T3-L1 adipocytes that were treated with quercetin and its metabolite quercetin-3-glucuronide [48]. In this study, an assessment of the absorption of quercetins was monitored. Both compounds were absorbed by hypertrophied adipocytes, and they were partially metabolized to quercetin-3-glucuronide and quercetin, respectively. Likewise, quercetin absorption was more efficient and faster than its metabolite, most likely indicating a passive diffusion through the plasmatic membrane of adipocytes; this was proposed as the main mechanism responsible for the observed decrease in ROS (Fig. **1**).

In a human intervention study, a systemic antioxidant potential was evaluated in eight healthy volunteers 24 h after the ingestion of 10 g of an aqueous HS extract. A significant increase in plasma and urine antioxidant potential and a reduction in oxidative stress was observed, in agreement with animal studies [49]. Furthermore, the main hibiscus anthocyanins and one glucuronide conjugate were detected in the urine of volunteers. The significant increase in hippuric acid in urinary excretion indicated a high biotransformation of HS polyphenols, which suggested a role of the colonic microbiota in this biotransformation [49].

Reports on the toxicology data of HS are limited. Nevertheless, infusions and aqueous extracts of this plant have been traditionally used in food and folk medicine and are generally considered safe. No acute toxicity was observed within seven days of an oral administration of 15 g/kg of ethanol and aqueous HS extracts in mice [22]. In contrast, liver injury was reported in rats when an aqueous-methanolic extract of HS was administered in at least 15 successive doses of 250 mg/kg/d [50]. Furthermore, total mortality, preceded by a severe loss of weight and diarrhea, was observed in rats after a chronic 90-day oral administration of aqueous and ethanol extracts of HS at 2000 mg/kg [51]. In contrast, neither acute nor chronic toxicity was observed in female rats after a single oral administration of extract at 5000 mg/kg or chronically at doses of 50, 100 and 200 g/kg for 270 days [52]. The discrepancy among different studies is most likely due to the different compositions of the utilized HS extracts. Although no side effects have been reported in human trials to date [29, 41, 42], further studies with well-characterized extracts are required to ensure the safety and tolerability of HS extracts in humans.

1.2. *Lippia citriodora*

1.2.1. Description and Composition

Lippia citriodora (LC) (syn. *Lippia triphylla*, *Aloysia triphylla*), commonly known as lemon verbena, belongs to the genus *Lippia* (Verbenaceae), which includes approximately 200 species of herbs, shrubs and small trees. LC is a deciduous shrub native to South America, however, it is also cultivated in Southern Europe and North Africa, since it was introduced to Europe at the end of the 17th century [53]. The leaves and stems of LC are rich in essential oils; geranial (citral), neral and limonene represent the main components of the total essential oil of the plant and are responsible for its lemony flavor. Furthermore, the individual percentages of these three compounds, especially for geranial and limonene, change depending on the developmental stage, which may be related to flowering [53].

LC extracts are also characterized by the presence of polyphenolic compounds, with phenylpropanoids as the main class of polyphenols of this plant (Fig. **2** and **3B**). Verbascoside (also known as acteoside) is the most abundant among all the phenylpropanoids [54], and it has been proposed as the main compound responsible for the biological activity of LC, especially its potent antioxidant capacity [55-57]. In addition, two verbascoside isomers (isoverbascoside and forsythoside A), two verbascoside derivatives (β-hydroxy-verbascoside and β-hydroxy-isoverbascoside), eukovoside and martynoside are other phenylpropanoids found in LC extracts [54, 55, 58] (Fig. **3B**). Flavones are another class of polyphenols that are present in minor quantities in LC extract. All of them are present in their diglucuronide form, such as luteolin-7-diglucuronide, apigenin-7-diglucuronide, chrysoeriol-7-diglucuronide and acacetin-7-diglucuronide [55, 58]. Other constituents identified by HPLC-DAD-ESI-MS are two iridoid glycosides (gardoside and theveside), verbasoside, cistanoside F and campneoside I and its isomer [58] (Figs. **2** and **3B**). Furthermore, asperuloside, tuberonic acid glucoside (or 5'-hydroxyjasmonic acid 5'-O-glucoside), shanziside and ixoside were also identified when the LC extract was analyzed by capillary electrophoresis-electrospray ionization-mass spectrometry [59].

1.2.2. Biological Activities

The leaves from LC are used as a spice for beverages and food preparations because of their lemony flavor. Additionally, a decoction and infusion of LC have traditionally been taken for the treatment of asthma, colds, fever, stomach ache, indigestion and other gastrointestinal disorders and skin diseases. In addition, it has been used as a diuretic, digestive, analgesic, antispasmodic and anti-inflammatory remedy [60]. In particular, the activity of LC is attributed to verbascoside since this phenylpropanoid represents 0.5 to 3.5% dry weight of the LC leaves, and it has been demonstrated to possess potent antioxidant capacity [55-57] and anti-inflammatory [61], antimicrobial [62] and anti-tumor [63] properties.

Verbascoside exhibited a higher capacity to scavenge free radicals within a hydrophobic environment than other antioxidants, such as hydroxytyrosol and caffeic acid. Furthermore, this phenylpropanoid was much stronger than these compounds and as potent as quercetin in inhibiting lipid peroxidation [55], a fact that may be related to its affinity for phospholipid membranes [64]. Nevertheless, the LC extract tested by Funes *et al*. [55], which contained 25% verbascoside, showed a higher antioxidant capacity than expected, based on the antioxidant capacity of pure verbascoside, suggesting a putative synergistic effect of verbascoside with other minor components, such as the flavones, in agreement with previous suggestions [54].

1.2.3. Pharmacokinetics and Toxicology

In spite of the widespread use of this plant in folk medicine, studies about its pharmacological effects are relatively recent. The capacity of verbascoside and LC extract to alleviate high-glucose

A

Hibiscus sabdariffa

Compound	Family
Hibiscus acid	Organic acids/ Dicarboxylic acids
Delphinidin-3-sambubioside Cyanidin-3-sambubioside	Flavonoids/ Anthocyanins
Chlorogenic acid Methyl digallate Coumaroylquinic acid 5-O-Caffeoylshikimic acid	Phenolic acids
Myricetin-3-arabinogalactose Quercetin-3-sambubioside Quercetin-3-rutinoside Leucoside Quercetin-3-glucoside Kaempferol-3-O-rutinoside Myricetin Quercetin	Flavonoids/ Flavonols
Methyl epigallocatechin	Flavonoids/ Flavanols
N-Feruloyltyramine	Others/ Tyramines

Lippia citriodora

Compound	Family
Shanziside Gardoside Theveside	Iridoid glycosides
Verbascoside Cistanoside F β-Hydroxyverbascoside/ β-Hydroxyisoverbascoside Campenoside I Isoverbascoside Eukovoside Martynoside	Phenylpropanoids
Luteolin-7-diglucuronide Chrysoeriol-7-diglucuronide Acacetin-7-diglucuronide	Flavonoids

Rosmarinus officinalis

Compound	Family
Apigenin Hispidulin Cirsiliol Diosmetin Cirsimaritin Genkwanin	Flavonoids
Rosmanol Epiisorosmanol Epirosmanol Miltipolone Carnosol Rosmadial Rosmaridiphenol Carnosic acid 12-Methoxy carnosic acid Hinokione	Diterpenes
Anemosapogenin Augustic acid Benthamic acid Micromeric acid Betulinic acid Ursolic acid	Triterpenes
9-Shogaol	Phenylpropanoid derivatives

Olea europaea

Compound	Family
(Epi)loganic acid isomers Oleoside/ Secologanoside isomers Hydroxytyrosol-glucoside isomers Hydroxytyrosol Tyrosol glucoside Elenolic acid glucoside/methyloleoside isomers Oleuropein aglycone Demethyloleuropein Oleuropein glucoside/neonuezhenide isomers Hydroxyoleuropein isomers Hydro-oleuropein Oleuropein/oleuroside isomers Methoxyoleuropein Dimethyl hydroxy octenoyloxi secologanoside isomers Ligstroside isomers Oleuropein methyl ether Piperchabaoside/(epi)frameroside/ ligustalisode dimethylacetal	Secoiridoids
Verbascoside	Phenylpropanoids
p-Coumaric acid glucoside Calceolarioside isomers Caffeoylglucoside	Hydroxycinnamic acids
Glucosyl rhamnosylquercetin (rutin) isomers Luteolin rutinoside/luteolin neohesperidoside/apigenin diglucoside Luteolin glucoside isomers Apigenin rutinoside/apigenin neohesperidoside Diosmetin rhamnoside glucoside (diosmin) isomers Apigenin glucoside Diosmetin glucoside Luteolin Quercetin Resinoside Apigenin	Flavonoids
Phenethyl primeveroside Ethyl-glucopyranosyloxy-oxopropyl-cyclohexaneacetic acid	Others
Olivil Olivil glucoside	Others/ Lignans
Esculin	Others/ Hydrocoumarins
Trihydroxystearic acid Trihydroxy-octadecenoic acid Dihydroxyhexadecanoic acid	Others/ Fatty acid derivatives
Sucrose	Others/ Disaccharides
Quinic acid	Others/ Carboxylic acids

Fig. (2) contd….

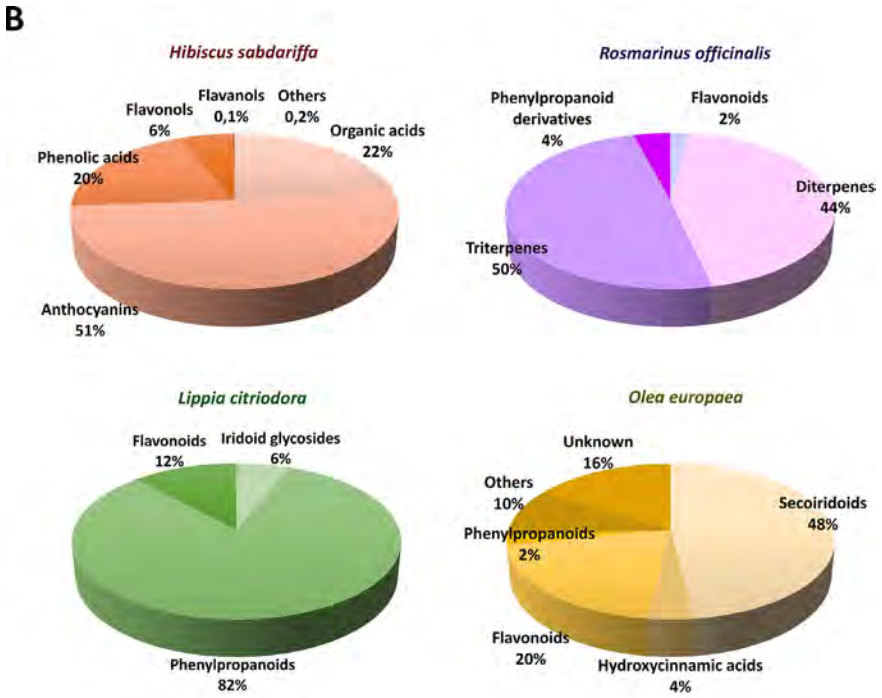

Fig. (2). Major bioactive compounds identified in *H. sabdariffa, L. citriodora, R. officinalis* and *O. europaea* extracts by liquid chromatography coupled to high resolution mass spectrometry (A). Compounds are grouped in families according to their chemical structure. (B) Percentage (w/w) of the different families of identified compounds on each extract. Identified compounds not included in families are shown as "others". Non-identified but quantified compounds are shown as "unknown". The complete characterization of the extracts has been previously reported [33, 65, 100, 181].

induced metabolic stress in hypertrophic adipocytes through AMPK-dependent mechanisms and to improve fat metabolism in hyperlipidemic mice has been recently reported [65]. Nevertheless, the data on the bioavailability and pharmacokinetics of phenolic compounds derived from LC extract are scarce, and little is known about the metabolites that could contribute to the biological activity of this plant. The studies carried out to date have focused on the bioavailability and pharmacokinetics of the main candidate for exerting the beneficial effects of LC, verbascoside. Nonetheless, as postulated from previous findings, other metabolites derived from LC compounds could also contribute to its bioactivity [16], which deserves further studies.

The pharmacokinetics of verbascoside derived from an LC extract studied in an animal model was reported [55]. In this study, Wistar rats were orally treated with a high acute dose of LC extract that contained a 25% of verbascoside, and blood samples were taken at different times after ingestion. Verbascoside was the only metabolite found in plasma samples, and its maximum concentration was reached at 20 min (2.3 μM), which correlated with the maximum antioxidant activity in the plasma of the rats. These results indicated a fast absorption of verbascoside in the gut barrier but a very low bioavailability, which may compromise to assign the observed effects to verbascoside. The low oral bioavailability of pure verbascoside was also corroborated in rats and the binding of this compound to plasma proteins was also shown, which could suggest a restricted distribution of this compound [66]. Nevertheless, there is evidence of the bioactivity of phenylpropanoids at very low concentrations in cell models (at the micromolar range), so low micromolar concentrations of verbascoside over a long-term in plasma could be responsible for some of the effects of LC.

The effect of the consumption of an oral acute dose of LC extract (1440 mg/kg) on the antioxidant response of blood cells was further studied in rats using high-resolution mass spectrometry in order to determine other potential metabolites in plasma [16]. In this study, verbascoside and isoverbasoside were identified as the most abundant metabolites (within the low micromolar range), suggesting that both compounds could be absorbed in their native forms. Five other metabolites, most probably derived from these two by deglycosylation (hydrolysis), methylation or glucuronidation [67], were also found in plasma, namely, hydroxytyrosol, caffeic acid, ferulic acid, ferulic acid glucuronide, and homoprotocatechuic acid, together with eight other phenolic compounds (some structural formulas shown in Fig. **3B**). Three flavone derivatives were also detected in plasma, namely, acacetin diacetate, luteolin diglucuronide and chrysoeriol diglucuronide. Acacetin diacetate could come from the conjugation of two acetate groups after the deglucuronidation of acacetin-7-diglucuronide, while luteolin and chrysoeriol diglucuronides could arise from the absorption of intact compounds present in the extract or from the absorption of free flavones in the gut and their successive glucuronidation (Fig. **3B**). Also, small amounts of gardoside, cistanoside F, theveside, eukovoside and martynoside were detected in plasma, suggesting that these compounds could be absorbed in their native forms. Therefore, the findings of this study indicated that the antioxidant response in blood cells may be due to the combined action of verbascoside, isoverbascoside and their metabolites, which could derive from the deglycosylation of phenylpropanoid glycosides in the gastrointestinal tract and the subsequent glucuronidation, sulfation or methylation of the aglycone forms.

The presence of verbascoside and isoverbascoside in the urine of healthy rats after the consumption of LC polyphenols has also been confirmed, which corroborates the absorption at the intestinal level of both compounds in their intact forms [68]. This study also revealed the possible deglycosylation or deglucuronidation of glycoside or diglucuronide derivatives present in the LC extract, respectively, and their subsequent glucuronidation or sulfation in the gut barrier. They concluded that the urinary and fecal excretion of LC in rats is low and that the influence of the gut microflora in the degradation of the polyphenolic compounds of LC deserves further attention.

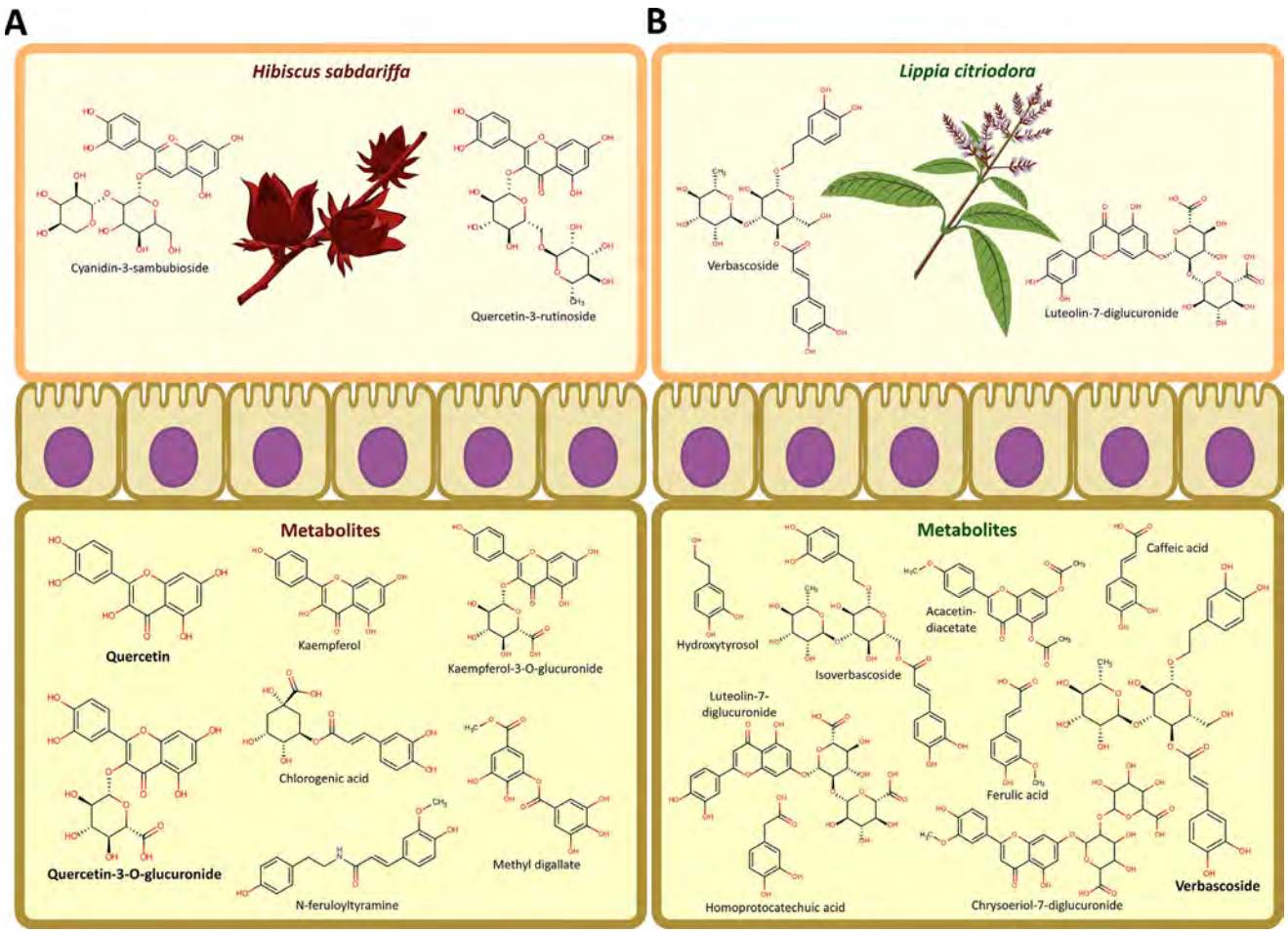

Fig. (3). Main polyphenolic compounds and metabolites from *H. sabdariffa* (**A**) and *L. citriodora* (**B**). Upper part shows a pictogram of each plant and the main polyphenolic compounds found in their respective extracts. The intestinal barrier is represented by the illustration of the enterocyte monolayer in the middle of the figure. Lower part shows the major metabolites derived from each plant found in plasma or tissues; the most significant ones are highlighted using bold letters.

No metabolites derived from LC consumption have been identified to date in plasma samples from human trials. Nevertheless, the effects of LC consumption on the oxidative damage and muscular injury related to intense physical activity has been studied in depth. Several single-blind, randomized and placebo-controlled trials have assessed the effects of LC extract consumption after exercise on several circulating parameters, antioxidant enzyme activities and expression and oxidative stress markers. The results evidenced the activation of glutathione reductase in erythrocytes and lymphocytes, lower levels of oxidative stress markers, such as malondialdehyde (MDA) and protein carbonyls in plasma, a modulation of the circulating lipid profile with an increase in high-density lipoprotein (HDL)-cholesterol, a modest decrease in acute inflammation and an increase in circulating urea after extract intake [69-73]. Furthermore, the protective effect of LC in muscle tissue was found through a 20% decrease in circulating myoglobin, while no interference was detected with an increase in glutathione-disulfide reductase gene expression due to cell adaptation to oxidative stress [71]. Although these effects are supposed to be due to the major compound of LC, verbascoside, further research on the potentially responsible metabolites is required to verify this hypothesis.

There is a lack of data about the toxicology and tolerability in the prolonged consumption of LC. In this sense, the acute oral toxicity was assessed in mice after an oral administration of 2000 mg/kg of LC extract; the extract did not show toxicity and could be classified as GHS Category 5 (Globally Harmonized System) or unclassified [55]. Moreover, several human intervention studies focused on sports nutrition have used dietary supplements based on LC extracts containing 10-20% verbascoside with a daily dosage varying from 500-1800 mg and a duration of the intervention from 3 to 9 weeks [69-71, 74, 75]. As a conclusion, no adverse effects were reported in any of these studies. Nevertheless, further studies on the toxicology of LC consumption are needed to establish safe doses of LC in humans.

To conclude, the absorption and permeability of LC polyphenols are also poorly characterized. The Caco-2 human colon cell line represents a useful model of human intestine in order to study the absorption and metabolism of polyphenols from LC. Information on the metabolites of LC reaching the blood circulation needs to be improved in order to try to explain their potential molecular targets and consequently explain the therapeutic effect of LC. Furthermore, little is known about the tissue distribution of these compounds, in which they could also be further metabolized. Therefore, further research to understand the complete absorption, metabolism and distribution mechanisms of LC polyphenols requires more attention.

1.3. *Rosmarinus officinalis*

1.3.1. Description and Composition

Rosmarinus officinalis L., commonly known as rosemary, is a shrub that belongs to the family Lamiaceae, which contains approximately 3500 species. It is a perennial, evergreen, aromatic plant that grows about one meter in height with whitish-blue flow-

ers and needle-like leaves. This herb blossoms twice a year (spring and autumn) and easily grows in all kinds of areas, especially in dry and sandy soils. Rosemary grows wild throughout the Mediterranean area and is currently cultivated worldwide due to its multiple uses as a culinary spice in food [76].

There is a wealth of information on the identification and quantification of the main components of rosemary-leaf extracts. The most representative classes of polyphenolic compounds present in this matrix are abietane-type diterpenoids, phenolic acids and flavonoids (flavones, flavanones and flavanols) (Fig. **2**). The main phenolic diterpenoid of rosemary is carnosic acid, and several derivatives of this compound can be found as well, namely, carnosol**,** rosmadial, rosmanol, 12-methoxycarnosic acid and its isomers epirosmanol and epiisorosmanol (Fig. **4A**). Triterpene acids are also abundant in rosemary-leaf extracts, such as ursolic and betulinic acids (Fig. **4A**). The most abundant phenolic acid is rosmarinic acid, followed by caffeic and ferulic acids, and several flavonoids, such as genkwanin, hispidulin, cirsimaritin, homoplantaginin, scutellarein, gallocatechin, apigenin, diosmetin, nepetrin, hesperidin, 6-hydroxyluteolin-7-glucoside and luteolin-3'-glucuronide, and phenylpropanoid derivatives, such as (9)-shogaol, are also detected in this plant [77-83] (structural formulas shown in Fig. **4A**).

The composition and quantity of compounds present in the different rosemary-leaf extracts depend on numerous factors, such as the soil type, climate, plant age and, more importantly, the extraction procedure. Basically, rosemary extracts can be classified into three main groups based on its chemical composition: essential oils, hydrophilic and hydrophobic extracts. The essential oil has been valued for its antibacterial, antioxidant and antiproliferative properties [81]. The major compounds found in the essential oil are 1,8-cineol, camphor, α-pinene, limonene, camphene and linalool. On the other hand, rosmarinic acid and other minor compounds, such as hydroxycinnamic acids and glycosylated flavonoids, are the major representative compounds in water-soluble extracts, while abietane diterpenes, such as carnosic acid, carnosol or rosmadial, and other hydrophobic compounds, such as methylated flavones genkwanin and cirsimaritin, and the flavone hesperetin are abundant in the non-water soluble extract. It is well known that the antioxidant activity of rosemary extracts is mainly due to phenolic abietane diterpenes and the phenolic rosmarinic acid. Nevertheless, this behavior depends on the hydrophobicity of the environment, where the hydrosoluble extract exhibits a high antioxidant activity in the absence of a membrane-based system, while the hydrophobic extract shows a higher antioxidant capacity in the presence of a membrane system [79].

1.3.2. Biological Activities

Rosemary, especially the leaf extract, is one of the most popular herbs, traditionally consumed as a culinary spice to adjust food flavor, and is widely used in the food and cosmetic industries as a natural antioxidant agent due to its inherently high antioxidant activity. Moreover, this plant has been used for centuries in folk medicine, and a wide variety of pharmacological activities have been attributed, such as hepatoprotective [84], antibacterial [85], anti-atherogenic [86], antidiabetic [87], antinociceptive [88-89], anti-inflammatory [89-91], anti-tumor [92, 93] and antioxidant [94, 95] activities. In addition, rosemary extracts and their isolated components have been shown to exert an antiproliferative effect on breast, liver, prostate, lung and colon cancer cells [96-100].

Most of the biological activities described for rosemary are associated with its phenolic content. In this sense, its strong antioxidant capacity is mainly due to phenolic diterpenes, such as carnosol, carnosic acid [101], rosmadial, rosmanol and epirosmanol, as well as the phenolic acid rosmarinic acid [79, 95, 102]. Although other phenolic compounds also contribute to the antioxidant activity of this aromatic plant, such as flavonoids like genkwanin and cirsimaritin. The mechanisms proposed for the antioxidant capacity of polyphenols are mainly related to the capacity of polyphenols to scavenge reactive oxygen species, such as peroxide ($^{\bullet-}O_2$), hydroxyl ($^{\bullet}OH$) or lipoperoxyl ($ROO^{\bullet}$) radicals. As mentioned above, the abietane diterpenes from rosemary have been shown to have a preference for a membrane environment, suggesting that the antioxidant effect against peroxidative damage that is induced by free radicals could be mediated by a membrane-related mechanism [79].

In addition to their potent antioxidant capacity, phenolic compounds from rosemary have been proposed to exert an important antiproliferative effect against several types of cancer cells. Researchers have focused their attention on the multiple mechanisms involved in this protective effect as well as the main responsible compounds [100]. For instance, it has been reported that two of the major compounds present in rosemary extract (carnosic acid and carnosol) inhibit the proliferation of colon cancer HT-29 cells by increasing oxidative stress, which results in the transcriptional activation of detoxifying genes by cells [99]. In the same cell model, rosmarinic acid exerted a preventive effect against the activation of the pro-inflammatory gene cyclooxygenase-2 (COX-2) [93], an inducible enzyme involved in metastatic mechanisms.

On the other hand, the literature on the therapeutic implications of rosemary in metabolic syndrome is also extensive since this aromatic plant possesses antidiabetic [87], anti-atherogenic [86] and hypocholesterolemic [103] activities, among others. These effects are basically related to the modulation of enzymes, transcriptional factors and the expression of key genes involved in several key metabolic pathways [104]. Definitively, rosemary represents another example of how medicinal herbs are composed of a complex mixture of phenolic compounds that exerts an important role in multifactorial diseases by interacting with a large number of metabolic targets.

1.3.3. Pharmacokinetics and Toxicology

In spite of a large number of studies focused on the potential therapeutic activities of rosemary extracts and the potential molecular mechanisms involved, little is known about the presence of these molecules and their metabolites *in vivo*. Therefore, research on the absorption, distribution, metabolism and elimination of rosemary components is needed to fully understand their activity *in vivo* and to establish a more effective and safe dosage in humans.

The pharmacokinetics of carnosic acid has been determined in the plasma, liver, intestinal content, urine and feces of rats receiving a single dose of the compound, intravenously or orally [105]. The results of the study revealed that carnosic acid has a slow absorption, but its elimination in the blood needs to be further clarified. The bioavailability was approximately 40% at 360 min after oral administration, likely due to the limited stability of carnosic acid in the stomach and the low uptake in the gut barrier. Moreover, only traces of carnosic acid were detected in the liver and intestinal contents, and there were no signs of enterohepatic recirculation. The analysis of the feces showed that part of carnosic acid is not metabolized; it is cleared by the liver into the intestine and mainly eliminated through the fecal route.

In an attempt to clear up the metabolism of some of the main diterpenes from rosemary, the bioavailability of the main compounds of a carnosic acid-enriched rosemary extract (0.5% w/w) has been studied in Zucker rats after oral administration of the extract for 15 days [106]. A total of 26 compounds were detected as early as 25 min after administration in the gut content, plasma and tissue samples, including carnosol, carnosic acid, rosmanol, epirosmanol, epiisorosmanol and rosmarinic acid. Most of the compounds and metabolites were identified in the liver and the gut lumen, with carnosic acid 12-methyl ether as the main derivative found in the liver, followed by 5,6,7,10-tetrahydro-7-hydroxyrosmariquinone, carnosic acid glucuronide, carnosic acid and epiisorosmanol (structural formulas in Fig. **4A**). On the other

hand, the main metabolites detected in the lumen of the small intestine were carnosic acid glucuronide, rosmanol glucuronide and carnosol glucuronide, suggesting that glucuronidation is the main form of conjugation, both within the intestinal epithelium and in the liver. The most abundant metabolites identified in plasma within the range of 150-300 μM were 5,6,7,10-tetrahydro-7-hydroxyrosmariquinone, which was probably derived from cell oxidative stress, and carnosic acid 12-methyl ether, due to the action of catechol-O-methyltransferases present in the intestine and the liver (Fig. **4A**). Further attention should be paid to these two latter compounds and their molecular targets since they may be relevant to explaining the biological activity of rosemary extract.

Recently, the permeability of 24 bioactive compounds derived from a rosemary extract enriched in diterpenes and triterpenes has been studied in the Caco-2 cell monolayer model, indicating that carnosic acid and epiisorosmanol showed the highest permeability values [107]. The flavonoids hispidulin, diosmetin, genkwanin and cirsimaritin exhibited significant permeation values, with cirsimaritin and genkwanin being the flavonoids with the highest permeations. Among the diterpenoids, carnosic acid, followed by epiisorosmanol and its isomers epirosmanol and rosmanol showed the highest permeability values, while triterpenoids were the class of compounds with the lowest permeability values (Fig. **4A**). The results of this study suggested that most bioactive compounds from rosemary extract are scarcely absorbed, and the major mechanism of absorption for most compounds is passive diffusion transport. Furthermore, the use of liposomes to vehiculize rosemary compounds does not improve their permeability.

Despite these studies, the lack of information about the absorption and pharmacokinetics of rosemary extract compounds is evidenced. Further research should be oriented to study the transportation mechanism at the gut barrier using cell models of human intestine. Moreover, special efforts should be made to identify the plasma metabolites derived from rosemary in human samples. Also, the study of tissue distribution and biotransformation of the plasma compounds or metabolites in animal models requires further attention. Putting all this information together may allow us to identify the specific molecular targets of rosemary metabolites.

The potential toxicity of the consumption of rosemary extracts is another issue that should be clarified. In this regard, only one study of acute oral toxicity (2000 mg/kg dose) in rats with two rosemary extracts, mainly enriched in diterpenes and containing a lower number of flavonoids, has been reported to date [108]. This study showed that the extracts were well-tolerated and had no adverse effects or mortality. Lastly, few human studies have been carried out to investigate the efficacy of rosemary extract on a prolonged basis. In a study performed in 90 subjects who daily consumed a mixture of a rosemary and citrus extracts for 3 months, no adverse effects were reported, and the polyphenol extract showed decreases in the UVB- and UVA-induced skin alterations and improvements in skin wrinkledness and elasticity [109]. Therefore, the toxicity and tolerability of rosemary require further research to set the maximum recommended dose for high effectiveness of the extract.

1.4. *Olea europaea*

1.4.1. Description and Composition

Olive tree (*Olea europaea*) is one of the most popular members of the family Oleaceae, which comprises approximately 30 genera of deciduous trees and shrubs [110]. The olive tree represents one of the oldest and most widespread tree species grown in the coastal areas of the eastern Mediterranean basin, southeastern Europe, western Asia and northern Africa. It is a short, thick tree that can reach up to 15 m in height with many branches. Its leaves are lanceolate and narrow and the olive fruit is small, ovoid and green and turns blackish-violet when ripe [111].

The olive fruit is widely consumed as either a ripe fruit or an unripe green fruit. Furthermore, olive oil constitutes the main food ingredient of the common "Mediterranean diet" and is the major source of dietary fat in the countries where olives are distributed [112, 113]. Likewise, several beneficial effects, such as a reduced risk of coronary artery disease [114, 115], neurodegenerative disease [116, 117] and certain types of cancer [118-120], have been widely attributed to the consumption of olive oil that is rich in phenolic compounds. Nevertheless, olive leaves were commonly discarded as byproducts of fruit harvesting [121] but have recently attracted more attention as nutraceuticals with health purposes due to their high content of phenolic compounds [122].

The chemical compositions of different parts of the olive tree have been extensively studied [122-129]. Fruits, seeds, leaves and oil of the olive tree are rich in phenols, flavonoids and secoiridoids. In particular, secoiridoids are the main phenolic compounds detected in olive leaf (OL) extracts, with oleuropein reported as the most representative compound. Other secoiridoids, such as oleuroside, hydroxyoleuropein, oleuropein diglucoside, oleoside, secologanoside, elenolic acid glucoside, 7-epiloganin or ligstroside, are also found in olive leaves. In addition, flavonoids represent another important group of phenolic compounds in the olive tree. Among them, luteolin, luteolin-7-O-glucoside, luteolin-7,4-O-diglucoside, luteolin-7-O-rutinoside, luteolin-4-O-glucoside, apigenin, apigenin-7-O-glucoside, apigenin-7-O-rutinoside, rutin and quercetin have been detected in OL extracts. Simple phenols were also identified in olive leaves, with hydroxytyrosol being one of the main components of OL extracts. In addition, cinnamic acid derivatives or phenylpropanoids (such as verbascoside and *p*-coumaric acid), other simple phenolic compounds (such as vanillin and *p*-hydroxybenzoic acid) and triterpene acids (such as oleanolic acid and ursolic acid) have also been identified in OL extracts [122, 127] (Fig. **2**).

1.4.2. Biological Activities

O. europaea has a large number of uses in folk medicine for the treatment of cardiovascular diseases, respiratory and urinary tract infections, diarrhea, stomach and intestinal diseases, asthma or rheumatism [111]; a wide range of beneficial health properties are attributed to their components as antidiabetic [130, 131], antihypertensive [132, 133], anti-inflammatory [134], antioxidant [131, 135, 136], antitumor [118, 137] and antimicrobial agents [136, 138]. Although previous literature on the phenolic compounds of the olive plant has focused on olive oil consumption, phenolic compounds in the olive tree are mostly concentrated in the olive leaves [121]. Furthermore, the leaves of *O. europaea* can be consumed as an herbal tea, and they have also been used as a traditional remedy in countries where it is cultivated. Several studies have shown antioxidant [128, 136], hypoglycemic [139], antihypertensive [140], antimicrobial [136, 141], tumoricidal [127], antiviral [142] and antiatherosclerotic [143] effects. Therefore, olive leaves may be considered a cheap and easily available natural source of phenolic compounds.

Certain researchers have studied the biological activities of isolated components from the olive plant. Oleuropein, which is the major phenolic compound present in OL extracts, represents a pharmacologically active molecule since several beneficial effects of this compound have been extensively reported, among them anti-inflammatory [144], anti-atherogenic [145], anticancer [146], antimicrobial [147] and antiviral [142] properties. In addition, this secoiridoid glycoside has skin photoprotective [148] and anti-aging [149] properties and is a potent antioxidant and radical scavenger [150]. In addition to its antioxidant activity, it is postulated that some of these effects could be related to the capacity of oleuropein to interact with biological membranes, consequently promoting changes in the membrane's physical properties and the function of membrane-related proteins [151].

1.4.3. Pharmacokinetics and Toxicology

In spite of the beneficial effects reported for the main components of *O. europaea*, such as oleuropein, the *in vivo* bioactivity depends on the absorption and metabolism of these compounds. *In vitro* gastric digestion of the breakdown of complex olive oil polyphenols [152] revealed that the relative amounts of hydroxytyrosol and tyrosol in the small intestine increased after gastric biotransformation of the complex secoiridoids derivatives of olive oil polyphenols. Likewise, these simple phenols crossed the human Caco-2 cell monolayer and the rat segments of the jejunum and ileum, while oleuropein was not absorbed. However, this secoiridoid glycoside was rapidly degraded by the colonic microflora, yielding hydroxytyrosol, which may then be absorbed. The findings of this study also indicated that hydroxytyrosol and tyrosol could be metabolized to O-methylated, glucuronidated and glutathionylated conjugates.

Hydroxytyrosol is the main derivative from oleuropein. While the secoiridoid glycoside is found in high amounts in unprocessed olive leaves and fruit, the higher concentrations of hydroxytyrosol may appear in the fruit and olive oil due to the chemical and enzymatic reactions that occur during the maturation of the fruit [153]. In addition, hydroxytyrosol has also been shown to be a strong antioxidant *in vitro* and in animal studies [154, 155]. The bioavailability of this simple phenol has been explored in humans (Fig. **4B**). Miro-Casas *et al*. quantified hydroxytyrosol and its main metabolite, 3-O-methylhydroxytyrosol, in plasma and urine after a dose of 25 mL of virgin olive oil in healthy humans and showed that approximately 98% of hydroxytyrosol was present in conjugated forms, mainly glucuronoconjugates [156]. It was suggested that the ingested hydroxytyrosol may be extensively first-pass metabolized in the intestine and liver and that the biological activity of this compound is most likely derived from its metabolites. The bioavailability of olive polyphenols in healthy volunteers after the consumption of twenty olives rich in hydroxytyrosol has also been examined [157]. From the fifteen phenolic compounds detected in olives, seven compounds significantly increased in plasma and urine after administration, namely, tyrosol, *p*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid, hydroxytyrosol, and three metabolites derived from hydroxytyrosol (homovanillic alcohol, homovanillic acid and 3,4-di-hydroxyphenylacetic acid) (structural formulas shown in Fig. **4B**). Moreover, other phenolic compounds were detected in plasma, mainly in their conjugated form as glucuronides. The results indicated that olive polyphenols are bioavailable, rapidly absorbed and metabolized, especially for the catechol-O-methyltransferase action of hydroxytyrosol in the liver and kidneys. In addition, a correlation between the increase on phenolic compounds after the ingestion of olives and an enhancement of antioxidant status in plasma was found.

The bioavailability of pure oleuropein in rats or in the same animal model supplemented with extra virgin olive oil (EVOO) has also been studied by HPLC-ESI-MS/MS of plasma samples after being consumed for 80 days [158]. The potential phenolic metabolites of oleuropein described in the literature are: hydroxytyrosol, 2-(3,4-dihydroxyphenyl)acetic acid, 4-(2-hydroxyethyl)-2-methoxyphenol or homovanillyl alcohol, 2-(4-hydroxy-3-methoxyphenyl) acetic acid or homovanillic acid, and elenolic acid [158] (Fig. **4B**). In the latter study, the metabolite homovanillic alcohol was found in plasma basal levels, whereas intact hydroxytyrosol was not detected, corroborating the biotransformation of this compound. The metabolites homovanillic acid and 3,4-di-hydroxyphenylacetic acid were detected but were not found in all the plasma samples of rats. This intraindividual variability on metabolite content was also reported in the plasma of volunteers after the ingestion of olives [157], which may suggest a genetic polymorphism of the enzymes involved in the metabolism of olive polyphenols or differential epigenetic regulation.

The above mentioned studies evidenced that EVOO phenolic compounds are absorbed by the small intestine upon oral administration and their levels are dose-dependently increased in plasma and urine [159]. Maximum concentrations of these compounds in urine have been detected within the first 4 h, with their free forms not exceeding 15%. The formation of hydroxytyrosol, tyrosol and their metabolites (especially as glucuronide conjugates) is also a key step in the biotransformation of olive polyphenols (Fig. **4B**). Oleuropein undergoes extensive non-enzymatic hydrolysis by the gastric environment [152] or decomposition by colon microflora [160], forming hydroxytyrosol, which enters the small intestine and is absorbed by passive diffusion or by the colon [158]. However, studies on the absorption of oleuropein are controversial since other studies have proposed that this secoiridoid glycoside can also be absorbed and subjected to phase II metabolism in humans [161].

The form of administration (oily or aqueous) or the administration route (intravenous or oral) significantly affects the bioavailability of EVOO phenolic compounds [162]. Nevertheless, most of the studies have been carried out with olive oil and fruit, while literature considering the absorption and metabolism of these compounds after consuming OL extract is scarce. Furthermore, given the unclear fate of oleuropein, it seems relevant to investigate the behavior of oleuropein from OL extract ingestion (rather than olive oil) since olive leaves are more concentrated in phenolic compounds, especially in oleuropein, and their bioavailability may differ from that of OL from EVOO.

Accordingly, the first study to assess the absorption and metabolism of oleuropein and hydroxytyrosol in human plasma after an ingestion of OL extract evidenced that the main olive phenolic metabolites found in plasma and urine were conjugated metabolites of hydroxytyrosol (mainly glucuronidated and sulfated), while homovanillic acid was detected in traces [121] (Fig. **4B**). Furthermore, the metabolites of hydroxytyrosol were rapidly detected in plasma after ingestion and the primary compounds were present in urine. Heterogeneous bioavailability and metabolism of oleuropein were also observed among volunteers in this study, suggesting a high dependence on several factors, such as the delivery method and gender. Nevertheless, whether the bioactivity of phenolic compounds from olive comes from hydroxytyrosol or its glucuronide metabolites, remains unclear. One study in rats showed that hydroxytyrosol metabolites, such as 3-O-glucuronide conjugate, were more potent than their precursor on radical scavenging activity [163], while another study carried out in humans reported that none of the glucuronides of hydroxytyrosol that were detected in plasma after an ingestion of olive oil contributed to antioxidant activity at real biological doses [164].

The bioavailability of phenolic compounds has also been studied in a human intervention study focused on the effect of the consumption of 250 mg of OL extract (>40% OL) on the menopausal status of women and its potential benefit in the prevention of osteoporosis [165]. Fifteen olive phenolic compounds were rapidly found in plasma and urine and were mainly phase II-derived metabolites; three were metabolites derived from hydroxytyrosol, four were oleuropein aglycon derivatives and two were homovanillic acid metabolites. Hydroxytyrosol glucuronide was the most abundant and tyrosol glucuronide, hydroxytyrosol-acetate glucuronide and luteolin glucuronide and its respective aglycone were found at trace levels. New metabolites derived from oleuropein were identified in urine, such as homovanillic alcohol sulfate, elenolic acid, and elenolic acid glucuronide. These findings confirmed the fast absorption of phenolic compounds from OL extract and the extensive biotransformation of hydroxytyrosol and oleuropein into metabolites, mainly as glucuronidated conjugates, as previously reported [121] (Fig. **4B**).

The toxicology of OL extract has been performed in Wistar rats fed several doses of the extract for 6 weeks [166]. The treated groups showed a significant increase in serum levels of alkaline

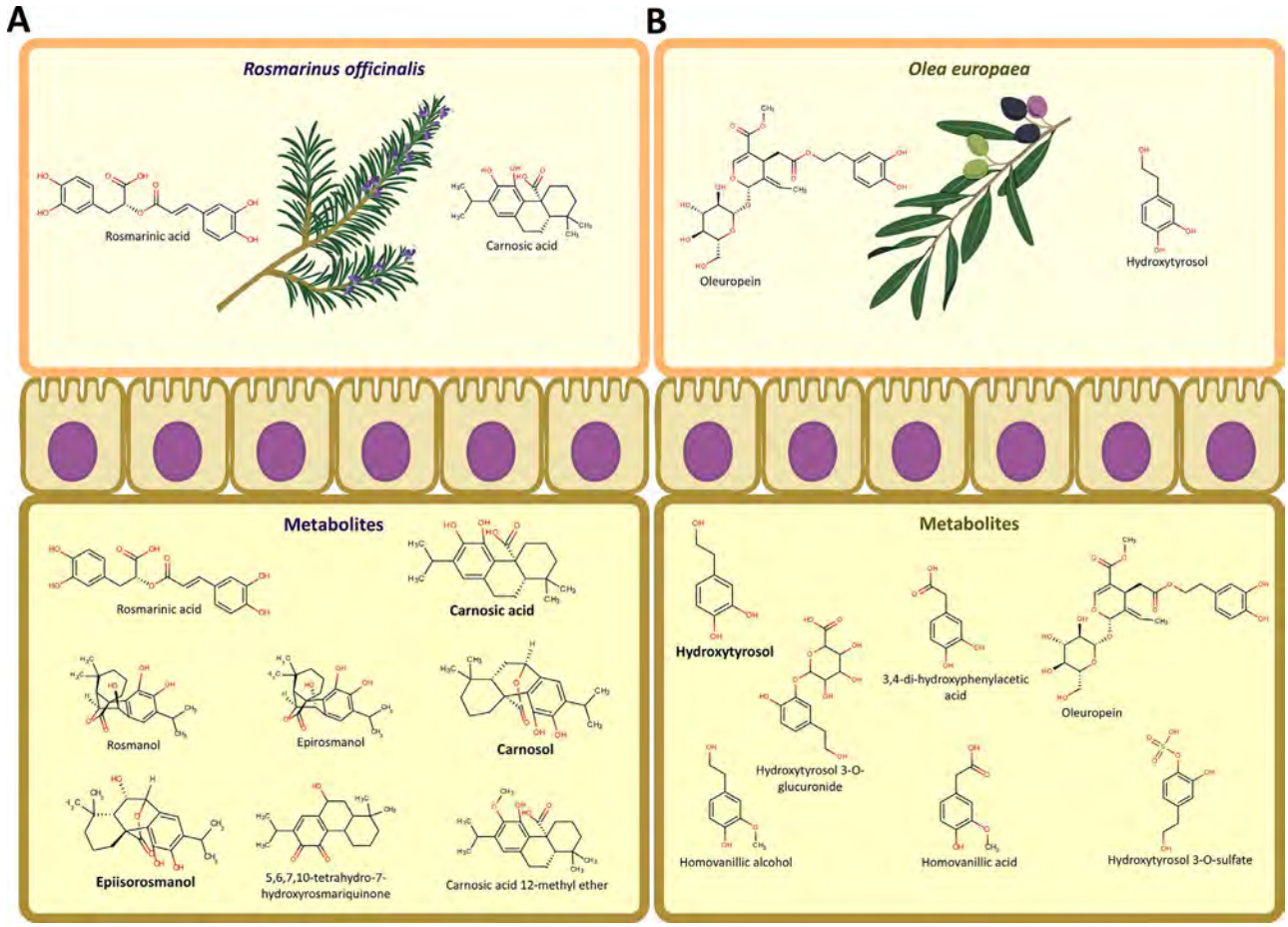

Fig. (4). Main polyphenolic compounds and metabolites from *R. officinalis* (**A**) and *O. europaea* (**B**). Upper part shows a pictogram of each plant and the main compounds found in their respective extracts. The intestinal barrier is represented by the illustration of the enterocyte monolayer in the middle of the figure. Lower part shows the major metabolites derived from each plant found in plasma or tissues; the most significant ones are highlighted using bold letters.

phosphatase and bilirubin and a significant decrease in serum triglyceride, glucose and cholesterol. Furthermore, the group with the higher dose showed decreases in red blood cells and hemoglobin and histological alterations in the liver and kidneys. The findings of this study proposed a careful use of OL extract, especially at higher doses for longer periods of times. Recently, a study on the efficacy and safety of a combination of two extracts (*Opuntia ficus-indica* and OL extracts) on gastroesophageal reflux showed that the consumption of 6 g/day of this formulation for two weeks did not exert any adverse effects and was well-tolerated [167]. In addition, the toxicological safety of OL extract was assessed in a preclinical study in which no evidence of mutagenicity or genotoxicity in the bacterial reverse mutation test, *in vitro* mammalian chromosomal aberration test or *in vivo* mouse micronucleus test was observed [168]. Moreover, the NOAEL derived from the 90-day study in rats was 1000 mg/kg per day.

To conclude, more studies are needed in order to investigate the accumulation of olive-leaf metabolites in tissues and their physiological significance *in vivo*. Furthermore, the pharmacokinetics of oleuropein from olive leaves requires further investigation to elucidate its absorption in the gut barrier, metabolism and various means of excretion, as well as its degradation into other phenolic compounds by the colonic microflora. The toxicological profile of the consumption of OL extract should be further studied in order to clarify a safe and well-tolerated dose for administration in humans, although the frequent consumption of olives and olive oil by humans, which contain many of the same components, has not shown any adverse events to date.

2. MOLECULAR DOCKING AS A TOOL FOR THE DISCOVERY OF POTENTIALLY BIOACTIVE METABOLITES

Biochemical pathways of energy metabolism have, as main objectives, the degradation of molecules to obtain energy (catabolism), the synthesis of simple molecules that polymerize, giving rise to macromolecules (anabolism) and the elimination of molecules that are toxic waste. These biochemical processes involve a large number of enzyme-catalyzed reactions that allow the transformation of numerous metabolites. The Kyoto Encyclopedia of Genes and Genomes contains annotations of 10,476 biochemical reactions and 17,931 metabolites and other small molecules [169]. With these numbers, it is easy to imagine the multiple possibilities of bioactive compounds to modulate the activity of enzymes involved in metabolic processes, either by acting directly on their catalytic or regulatory sites or by modifying their levels of expression. Human diseases, such as diabetes, obesity, neurodegeneration or cancer, lead to metabolic alterations through the modulation of certain key regulatory metabolic pathways of the cell [14, 28, 65, 118-120, 170]. In this context, the development of bioactive compounds that can counteract metabolic alterations has great therapeutic interest. Natural products have traditionally been a source of compounds for the development of drugs and having solved many of the technical problems associated with screening of these products in tests of high performance against specific molecular targets, there is a revived interest in them by pharmaceutical companies [171]. Natural products, such as polyphenols possess a high degree of stereochemistry and are usually substrates of various transport systems that can release them intracellularly, where they must interact with their

molecular target (metabolite-likeness property) [172]. In practice, it is economically impossible to test libraries of millions of compounds looking for bioactive molecules, and many compounds are not commercially available in the quantities suitable for *in vitro* experiments. For this reason, it is necessary to implement a guided search by computational methods to reduce the vast chemical space to a number of compounds that is experimentally approachable.

Metabolite identification is one of the key challenges in current mass spectrometry-based untargeted metabolomics studies. Identifying metabolites derived from plant polyphenols is a necessary task to establish candidate compounds that may interact with their cell targets and then can be correlated with the salutary effects of plant compounds in human health. Nevertheless, this may become a difficult task due to its high complexity in terms of protein targets and candidate compounds. These mass spectrometry-based metabolomics studies are generating an increasing number of metabolites whose data are incorporated into the main metabolomic databases: Human Metabolome DataBase (HMDB), Madison Metabolomics Consortium Database (MMCD), Metlin, and LIPID MAPS.

A simulated physiological approach for elucidating the molecular mechanisms of polyphenols uses computational techniques to search for protein-ligand interactions (Fig. **5**). Molecular docking techniques are widely used for the study of protein-ligand interactions. They enable the virtual screening of millions of compounds against known molecular targets with a reasonable economic cost. Docking experiments usually start with the crystallographic structure of a protein of medical interest and can predict bound conformations and the binding free energy of small molecules to the catalytic or allosteric binding sites [173]. Alternatively, when there are no high-resolution structures of a protein of interest, it is very useful to model its structure by homology to the resolved structures of other proteins with a minimum of 30% sequence identity [174]. After choosing the target protein and its possible binding sites for possible modulation of its activity by ligands, the next step is to have a chemical library with abundant structural variety in electronic format (Fig. **5**). The chemical structures of highly diverse structure compounds are available in different searchable databases, and these compounds can be used to perform virtual screening [175]. In some of these databases, we can also find valuable information on the chemical suppliers of these products, bearing in mind that, in later stages, we will need to have these compounds available for *in vitro* or *in vivo* testing to demonstrate their ability to modulate the activity of our protein of interest.

The computing time required to conduct molecular docking experiments is directly related to not only the size of the library of compounds to be tested but also to the precision of the virtual screening methods and the structural complexity of the compounds to be tested. Fast docking methods at atomic resolution that consider the receptor to be rigid (*i.e.*, protein) with flexible ligands require a few minutes (5-20 min) per ligand [176]. In contrast, molecular dynamics-based approaches require hundreds or thousands of hours per ligand [177]. We can also reduce the computation time if we perform a previous selection of the ligands. We must not forget that the final success of a modulator candidate is directly related to factors such as absorption, biodistribution, the rate at which it is metabolized, excretion, and toxicity (*i.e.*, ADMET profile) upon its administration [178]. Therefore, only compounds with an optimum ADMET profile will be evaluated *in silico* (Fig. **5**). We can consider different ADMET criteria for this selection: calculated logP value must not be greater than 5.0, calculated logS (logarithm base 10 of the solubility measured in mol/L) must be greater than -4.0, fragment-based druglikeness ≥ 0, drugscore ≥ 0.5, molecular weight ≤ 500, five or fewer hydrogen bond donor sites, and ten or fewer

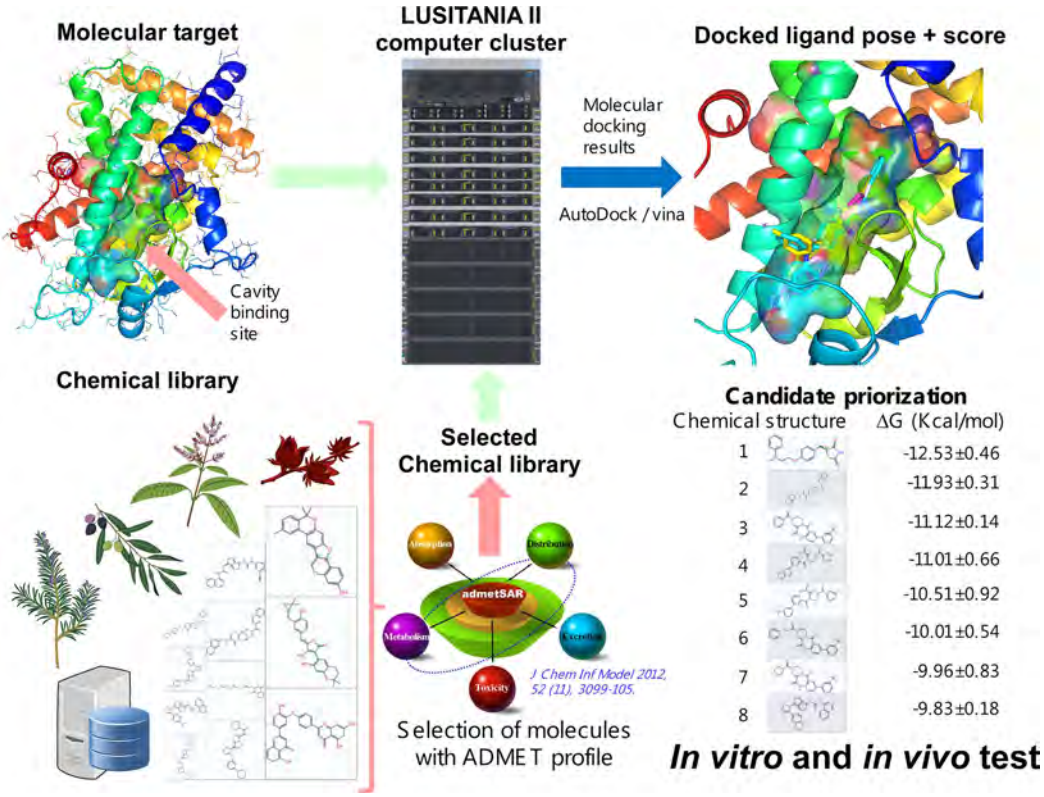

Fig. (5). Schematic representation of a virtual screening process of a phenolic compounds chemical library against the PPAR-gamma receptor structure using molecular docking techniques. On the one hand, a chemical library containing chemical structures to be screened must be built and asked to follow some ADMET criteria. On the other hand, molecular target high resolution structures must be obtained. Once both steps have been fulfilled, using computer clusters with a high computation capability, target structures are challenged with the chemical library to find the best docking results according ΔG binding values. Finally, all tested compounds are prioritized as a function of minor ΔG and proposed as candidates for subsequent *in vitro* and *in vivo* tests.

hydrogen bond acceptor sites [179, 180]. To facilitate the reader's understanding, we share the following data: a library of one million compounds, previously selected with ADMET criteria, can be tested *in silico* with molecular docking experiments, using AutoDock/vina [176] software, in one week. We are running similar calculations under a computing cluster with a Linux operating system that uses 20 computing nodes with 10 processors per node (LUSITANIA II cluster at the Research, Technological Innovation and Supercomputing Center of Extremadura-CenitS and COMPUTAEX). Obviously, the volume of data generated makes it necessary for the user to develop his own scripts to analyze this information. We use Python scripts to select the compounds with the lowest calculated free energy (ΔG, Kcal/mol) as possible modulators of different protein targets [179, 180].

We have applied these *in silico* screening techniques to select compounds from two sources, a library of olive polyphenols derived from OL extract and a library of plant-derived phenolic compounds and metabolites, as possible modulators of two proteins of pharmacological interest: the transcription factor receptor PPAR-gamma [179] and the AMP-activated protein kinase (AMPK) [181] (Fig. **5**). The cellular energy state is detected by various dynamic mechanisms that regulate the balance between catabolism and anabolism. AMPK is a cellular fuel sensitive kinase activated in deficient bioenergetic states that are caused by a lack of nutrients or hypoxia [182]. AMPK-phosphorylation of different proteins promotes inactivation of the energy-consuming pathways and activates the catabolism of fatty acids and other fuels. Therefore, this mechanism increases the available energy for the cell and decreases its content of reserves. In an attempt to identify the molecular targets of olive polyphenols derived from OL extract, our research group has recently reported the AMPK modulatory activity of olive phenolic compounds of an OL extract. In this paper, we demonstrated that OL extract (enriched in polyphenolic compounds) decreased the intracellular lipid accumulation through AMPK-dependent mechanisms in hypertrophic adipocytes [181]. A bioassay-guided approach was utilized to isolate the fractions from the extract that exhibited AMPK modulatory activity on the adipocyte cell model and to further identify the potential compounds responsible for such activity. Molecular docking experiments revealed that several polyphenols may be AMPK-gamma subunit modulators: secoiridoids, cinnamic acids, phenylethanoids and phenylpropanoids, flavonoids and lignans. Ongoing research is focused on corroborating the direct effect of these compounds and their metabolites on AMPK.

Thiazolidinediones (TZDs) are full agonists of the human PPARγ receptor, a nuclear soluble protein that, after binding to the agonist heterodimerize with the retinoic X receptor, recruits different transcriptional cofactors that bind to the promoter region of the fuel-related target genes and initiate their transcription. These synthetic drugs have been used in clinical practice to treat type 2 diabetes, and they effectively lower blood glucose levels and improve insulin sensitivity. However, their administration has been associated with severe side effects, and this makes this protein a target of interest in the search for new, safe modulators using *in silico* screening techniques. In our first paper [179], we performed molecular docking experiments with a big library of plant-derived phenolic compounds and metabolites to select 83 candidates with free energy variations ranging from -10.0±0.6 to -11.0±0.6 kcal/mol. Some of these compounds were tested *in vitro*, and the best candidates displayed encouraging bioactivities (manuscript in preparation).

CONCLUSION

Hibiscus sabdariffa, *Lippia citriodora*, *Rosmarinus officinalis* and *Olea europaea* are four medicinal plants with food uses that also represent a valuable source of the most representative plant bioactive polyphenols. There is abundant data about the biological and pharmacological effects of extracts derived from these plants as well as their isolated compounds, most of them by using *in vitro* or animal models, but human data are scarce. Several studies have reported the complex metabolism that its phenolic components undergo after ingestion in animal models and, in some cases, in humans. Available data provide information on the metabolites that reach the circulating plasma, but advanced research using targeted metabolomics should be utilized to elucidate the final intracellular metabolites that interact with the molecular targets and their associated biomarkers [183, 184].

In this review, we have compiled our own data as well as that of others on the wide biological effects and the metabolites of extracts derived from the abovementioned plants in cell and animal models, as well as in human trials. Most studies support that plant polyphenols exhibit rapid gut absorption, are highly metabolized through intestinal and hepatic cells or by colonic microflora and undergo mainly deglycosylations, glucuronidations, sulfations and methylations. The low bioavailability of the main phenolic compounds of these extracts has been reported, leading to plasma metabolite concentrations within the low micromolar range. Despite these low concentrations, the salutary effects of the consumption of these plants have been well documented in animal models and in humans. Biotransformation of these metabolites by target tissues deserves special attention in the future to find the final effectors of these metabolic effects and their protein targets. Molecular docking techniques provide a powerful method for virtually screening a large number of metabolites on selected protein targets in order to elucidate their potential mechanisms. The pleiotropic character of the polyphenols and its metabolites and the observed effect at multiple targets have led to propose that epigenetic regulation might be involved.

In summary, to fully elucidate the molecular mechanisms of plant polyphenols, targeted metabolomics in plasma and tissue samples, virtual screening on protein and membrane targets and cellular models using metabolites must be combined. This would also enable us to design more effective polyphenolic extracts or new drug candidates for pharmaceutical uses. Finally, most of the studies demonstrate that these plants are generally well-tolerated by humans. Nevertheless, further toxicological studies should be conducted on a chronic or subchronic basis to determine their effects when consumed in a concentrated form for medicinal purposes.

LIST OF ABBREVIATIONS

ACE	=	Angiotensin I-converting enzyme
AMPK	=	5'-adenosine monophosphate-activated protein kinase
DAD	=	Diode Array Detection
ESI	=	Electrospray
EVOO	=	Extra Virgin Olive Oil
GSH	=	Glutathione
HPLC	=	high-performance Liquid Chromatography
HS	=	*Hibiscus sabdariffa*
LC	=	*Lippia citriodora*
LDL	=	low-density Lipoprotein
LDLr	=	LDL Receptor
LPS	=	Lipopolysaccharide
MS	=	Mass Spectrometry
OL	=	Olive Leaf
ROS	=	Reactive Oxygen Species
TOF	=	Time-of-Flight

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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