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Proceeding Paper

Comparative Evaluation of the Total Antioxidant Capacities of Plant Polyphenols in Different Natural Sources †

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Abstract: Phenolic compounds during recent decades have been the object of study by the scientific community due to their high and diverse bioactive potential, with their antioxidant capacity being one of their most studied properties since, due to their structure, they have a high potential to act as effective compounds against oxidative stress. Due to their great structural diversity, these compounds have been classified into at least 10 different classes based on their basic chemical structures. The aim of this work was to carry out the characterization of the main phenolic content and the determination of the antioxidant capacity of four plant matrices. Firstly, *Lippia citriodora*, *Hibiscus sabdariffa*, *Olea europaea*, and *Silybum marianum* were chosen as natural sources due to their high content of phenolic compounds. After that, a qualitative characterization of the phenolic profiles of the selected plant extracts was performed using high-performance liquid chromatography coupled with mass spectrometry. Finally, the determination of the total content of polyphenols was carried out using the Folin–Ciocalteu method, and the antioxidant capacity using electron transfer methods (FRAP and TEAC) and hydrogen donation (ORAC). After the chromatographic analysis of the phenolic profile of each matrix, it was observed that there are different major phenolic compound families for each plant matrix. Regarding the antioxidant capacity, *L. citriodora* showed better results for the FRAP assay, while for the TEAC and ORAC assays the highest values were for *S. marianum*.

Keywords: phenolic compounds; antioxidants; HPLC–MS

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1. Introduction

Phenolic compounds present in plants are metabolites that have been shown to have a strong antioxidant capacity. Despite the fact that plants synthesize them mainly for their own defense against oxidative stress, these compounds retain the ability to act as ex-plant antioxidants and, therefore, they contribute greatly to the dietary and pharmaceutical properties of plant-based foods [1]. These compounds have a wide range of structures and functions; generally, they possess an aromatic ring with one or more hydroxyl substituents, and have been classified into at least 10 different classes based on their basic chemical structures [1]. However, there is no universal method for the measurement of antioxidant capacity, since these can measure different mechanisms of action, such as the transfer of electrons or the transfer of hydrogen atoms. In this sense, the selection of different assays to evaluate antioxidant activity would allow us to obtain a complete prediction of this bioactive potential [1,2]. The aim of this work was to identify the main families of phenolic compounds and to measure the antioxidant capacity of four selected plant matrices through different methods, in order to obtain a complete prediction of their antioxidant potential and the relationship of this bioactivity with the more abundant of these phenolic families.

2. Material and Methods

2.1. Chemicals and Reagents

All reagents used in this work were analytical grade and used without changes. The analytical procedures were performed using water purified by a Milli-Q system from Millipore (Bedford, MA, USA). LC–MS-grade methanol and acetic acid were purchased from Fisher Chemicals (Waltham, MA, USA) and Sigma-Aldrich (Steinheim, Germany), respectively.

To measure the antioxidant capacity and total phenolic content, the following reagents were provided from the indicated suppliers: AAPH (2,2′-azobis-2-methyl-propanimidamide dihydrochloride), ABTS [2,2´-azinobis (3-ethylbenzothiazoline-6-sulphonate)], ferric sulfate, Folin–Ciocalteu reagent, potassium persulfate, TPTZ (2,4,6-tripyridyl-S-triazine), and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) from Sigma-Aldrich (St. Louis, MO, USA); and gallic acid, sodium acetate, ferric chloride, hydrochloric acid, sodium acetate trihydrate, dehydrated sodium phosphate, and sodium carbonate from Panreac (Barcelona, Spain).

2.2. Sample Preparation

Extracts of *L. citriodora* leaves, *H. sabdariffa* calyces, *O. europaea* leaves, and *S. marianum* fruits were provided by Natac (Madrid, Spain). The dry extracts were stored in darkness and kept at room temperature until use. Prior to use, the dry extracts were reconstituted in the same extraction solvent mixture to a concentration of 5 mg/mL, and filtered with single-use syringe filters (0.20-µm pore size).

2.3. HPLC-ESI-TOF-MS Analysis

An RRLC 1200 system was used to analyze the extracts (Agilent Technologies, Palo Alto, CA, USA). This analytical platform was equipped with a vacuum degasser, an automated sampler, a binary solvent delivery system, and a UV–Vis detector. The RRLC system was coupled to a Bruker micrOTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) using an electrospray interface (ESI) (model G1607 from Agilent Technologies, Palo Alto, CA, USA). Phytochemical separation was accomplished using a 150-mm × 4.6-mm id, 1.8-µm particle diameter Zorbax Eclipse Plus C18 column (Agilent Technologies, Palo Alto, CA, USA). The eluents were water with 0.5% acetic acid (eluent A) and methanol (eluent B). Briefly, the multistep gradient used to separate the phytochemicals in *L. citriodora* was 0 min, 0% B; 15 min, 60% B; 33 min, 100% B; 38 min, 100% B; 38 min, 100% B; 46 min, 0% B; 55 min, 0% B min. For *H. sabdariffa* the gradient was 0 min, 0% B; 5 min, 25% B; 20 min, 40% B; 30 min, 60% B; 38 min, 100% B; 46 min, 0% B; 55 min, 0% B; for *O. europaea* 0 min, 0% B; 5 min, 30% B; 20 min, 60% B; 33 min, 100% B; 38 min, 100% B; 46 min, 0% B; 55 min, 0% B; while for *S. marianum* 0 min, 0% B; 7 min, 60% B; 25 min, 100% B; 30 min, 100% B; 36 min, 0% B; 45 min, 0% B. Finally, a conditioning cycle (10 min) was applied with initial conditions before each injection. The injection volume was 10 µL at room temperature. The flow rate was 0.3 mL/min. The effluent from the HPLC system was introduced into the mass spectrometer after reducing the flow rate with a "T"-type splitter, achieving a flow rate of less than 0.2 mL/min and ensuring correct ionization by ESI. All MS assays were developed in negative ion mode and considering a mass range from 50 to 1000 *m/z*. The identification was accomplished with the use of Data Analysis 4.0 software (Bruker Daltonics, Billerica, MA, USA), whose sophisticated CHNO algorithm supported good identification. Measurements were made in triplicate.

2.4. Total Phenolic Content by Folin–Ciocalteu

The total phenolic content was measured by the Folin–Ciocalteu method with some modifications [3]. Dry extracts were dissolved in ethanol:water (80:20) (different concentrations were tested). The absorbance measurements were carried out on a Synergy Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek Instruments Inc., Winooski, VT, USA) using 96-well polystyrene microplates. Phenol content was calculated based on

the calibration curves of gallic acid, and expressed as mg of gallic acid equivalents/g of dry extract. The experiments were performed in triplicate.

2.5. Antioxidant Activity Assays

The antioxidant activity of the dry extracts was evaluated using two methods based on single-electron transfer (SET)—the FRAP and TEAC assays—and the oxygen radical absorbance capacity (ORAC) method as a hydrogen atom transfer (HAT)-based method. To carry out these measurements, the same plate reader mentioned above was used.

2.5.1. Ferric Reducing Antioxidant Power

The FRAP assay was carried out following the method described by Benzie and Strain (1996) [4]. In this method, FRAP values were calculated by measuring absorbance at 593 nm using a microplate reader. A standard curve of $FeSO_4 \cdot 7H_2O$ was evaluated and, finally, the results were expressed in mmol of $FeSO_4$ equivalents/g of dry extract. The experiments were carried out in triplicate.

2.5.2. Trolox Equivalent Antioxidant Capacity

The TEAC test was performed as originally described by Miller et al. with some modifications according to Cádiz-Gurrea et al. [2,5]. TEAC values were calculated using Trolox as a standard and reading absorbance at 734 nm on a microplate reader. The results were expressed in mmol of Trolox equivalents/g of dry extract. The experiments were carried out in triplicate.

2.5.3. Oxygen Radical Absorbance Capacity

The ORAC method developed by Ou et al. was carried out with the modifications carried out by Cádiz-Gurrea et al. [2,6]. In this assay, where fluorescence is measured, Trolox was used as the reference compound, and a regression equation was used between its concentration and the net area of the fluorescence decay curve (area under the curve (AUC)) to calculate the final ORAC values of the expressed samples in mmol of Trolox equivalents/g of dry extract. The experiments were carried out in triplicate.

3. Results and Discussion

3.1. Characterization by HPLC-ESI-TOF-MS

In the identification of *L. citriodora* by HPLC-ESI-TOF-MS, it was observed that organic acids were present, such as gluconic acid, along with flavonoids such as chrysoeriol-diglucuronide, iridoids and secoiridoids such as loganic acid and shanziside, and phenylpropanoids as verbasoside and verbascoside—the latter being one of the compounds found at the highest concentrations. In the case of *H. sabdariffa*, after the analysis it was observed that organic acids such as hibiscus acid, phenolic acids such as caffeoylquinic acid and flavonoids such as quercetin and myricetin were present. In *O. europaea*, a greater diversity of groups present in its composition was found, including organic acids such as quinic acid, phenolic acids such as mycophenolic acid, simple phenols such as hydroxytyrosol, flavonoids such as epigallocatechin gallate and kaempferol, secoiridoids such as oleuropein, and phenylpropanoids such as acteoside. In relation to the *S. marianum* plant matrix, the following groups were identified: organic acids such as oxydobenzoic acid, diterpenes such as taxuspin, and flavonoids such as dihydroquercetin and silybin—the latter being one of the most abundant compounds in the plant matrix.

3.2. Total Phenolic Content and Antioxidant Activities

Table 1 shows the total phenolic content of the five plant extracts under study, as measured by the Folin–Ciocalteu method. As can be seen, the highest content is found in *S. marianum*, with a value of 536.95 ± 56.94 mg of gallic acid equivalents/g of dry extract—much higher than the rest of the plant extracts under study. This may be due to the fact that in the case of *S. marianum* the extract is from its fruit, while for *L. citriodora* and

O. europaea it is made with their leaves, and *H. sabdariffa* with its calyces. In this sense, since it is an extract obtained from the fruit, there may be a high sugar content, which has been seen to interfere with the Folin–Ciocalteu test [7], making the results much higher.

Table 1. In vitro total phenolic content and antioxidant capacity of *L. citriodora*, *H. sabdariffa*, *O. europaea* and *S. marianum* extracts, as measured by the TEAC, FRAP, and ORAC methods.

	Folin–Ciocalteu [a]	FRAP [b]	TEAC [c]	ORAC [d]
L. citriodora	293.96 ± 2.44	2.57 ± 0.09	1.13 ± 0.06	5.93
H. sabdariffa	51.09 ± 1.41	0.47 ± 0.05	0.24 ± 0.04	1.16
O. europaea	216.25 ± 28.70	1.67 ± 0.02	0.84 ± 0.05	6.01
S. marianum	536.95 ± 56.94	1.41 ± 0.28	1.34 ± 0.12	11.71

[a] Expressed in mg of gallic acid equivalents/g of dry extract. [b] Expressed in mmol of $FeSO_4$ equivalents/g of dry extract. [c] Expressed in mmol of Trolox equivalents/g of dry extract. [d] Expressed in mmol of Trolox equivalents/g of dry extract.

In the case of FRAP (Table 1), the plant matrix that reported the highest values was *L. citriodora*, with 2.57 ± 0.09 mmol of $FeSO_4$ equivalents/g of dry extract, while the plant matrix with the lowest results was *H. sabdariffa*, with 0.47 ± 0.05 mmol of $FeSO_4$ equivalents/g of dry extract. For the matrices *O. europaea* and *S. marianum*, the values were intermediate in comparison with the previous ones, being 1.67 ± 0.02 and 1.41 ± 0.28 mmol of $FeSO_4$ equivalents/g of dry extract, respectively. The high value obtained in this test for *L. citriodora* may be due to its high content of verbascoside—a characteristic and abundant phenylpropanoid in it—since the positive relationship between the amount of verbascoside in the matrix and the higher capacity in the FRAP test has been confirmed by various studies [1,8].

Despite the fact that both FRAP and TEAC (Table 1) are methods that determine the antioxidant capacity through electron transfer, the results obtained for the different matrices are not the same. In this sense, we observed that in the case of TEAC, the matrix with the highest result compared to the others was *S. marianum*, with 1.34 ± 0.12 mmol of Trolox equivalents/g of dry extract, followed by *L. citriodora*, with 1.13 ± 0.06 mmol of Trolox equivalents/g of dry extract which, in the case of FRAP, was the plant with the greatest ferric ion reduction capacity. This is consistent with the results obtained by Valentová et al., in which they observed that silybin—a flavonoid present in high concentrations in the fruit of *S. marianum*—had a higher antioxidant capacity in the TEAC test compared to the FRAP test, which indicates the better capacity of reduction of ABTS—which is the measure of TEAC—than reduction of ferric ions, the method on which the FRAP is based [9].

In relation to the ORAC assay (Table 1), which is based on hydrogen atom transfer, a trend similar to what occurs in the TEAC test is observed, since it is observed that it is the *S. marianum* matrix that has the highest value, with 11.71 mmol of Trolox equivalents/g of dry extract. However, in this case the values for *L. citriodora* and *O. europaea* are very similar, with 5.93 and 6.01 mmol of Trolox equivalents/g of dry extract, respectively. The results are consistent with those obtained by Viktorova et al. who observed that both the *S. marianum* extract and its major flavonoids in isolation had a high action capacity in the ORAC test, exerting a better hydrogen atom transfer capacity than the ABTS reduction capacity by electron transfer [10]. This may suggest that the group of flavonoids present in *S. marianum* has a better capacity to transfer hydrogen atoms than the characteristic phenylpropanoids in both *L. citriodora* and *O. europaea.*

It should be noted that for the *H. sabdariffa* plant matrix it was shown that both the total phenolic content and the antioxidant activity measured by the different methods used presented very low values compared to the rest of the plant matrices under study, which may be due to to the fact that the extraction of phenolic compounds was not carried out completely, indicating the importance of the selection of the conditions and the form of extraction of the compounds under study as the first step for a study of the biaoactivity of an extract.

4. Conclusions

A comprehensive characterization and biological antioxidant properties of selected sources have been obtained. After the chromatographic analysis of the phenolic profile of each matrix, it was observed that in *L. citriodora* the abundance of phenylpropanoids and iridoids stands out, in *H. sabdariffa* anthocyanins and flavonoids, in *O. europaea* secoiridoids and terpenes, and in *S. marianum* of flavonoids. Regarding antioxidant capacity, *L. citriodora* showed better results for the FRAP assay, while for the TEAC and ORAC assays the highest values were for *S. marianum*. In this sense, it is important to note that there is no universal method for measuring antioxidant capacity, so it is convenient to use a set of tests to evaluate antioxidant activity, since no single method can give a complete prediction of this bioactive potential.

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