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In-vitro Approach for the Determination of Antioxidant and Anti-inflammatory Activity of Wild Marjoram (*Thymus mastichina L.*)

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Abstract In the present study, it was investigated the antioxidant, anti-inflammatory properties of marjoram extracts and identified the major phenolic compounds which that may be responsible for these properties. Extracts of *T. mastichina* exhibited high antioxidant and anti-inflammatory activity. Methanol extracts showed higher antioxidant activity in comparison with ethanol and water extracts. The total phenolic content (195.71 ± 4.07 GAE/g dry extract) and the DPPH free radical scavenging activity (58.85 ± 0.52 g Trolox/100 g of dry weight extract) showed higher antioxidant activity than marjoram from the Portuguese region and an EC_{50} (0.028 mg/mL) superior to the synthetic antioxidant (BHT). *T. mastichina* was found to be a strong semicarbazide (SSAO) inhibitor with an EC_{50} one thousand times stronger than SSAO. Among polyphenols, flavonoids, mainly luteolin and quercetin, were the major constituents of the plant extracts and were present at levels of 6.22 and 5.46 µg kaempferol/mg DWE, respectively.

Keywords: *marjoram, polyphenols, antioxidant, anti-inflammatory*

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1. Introduction

Thymus mastichina L. also known as marjoram belongs to the genus *Thymus* which is one of the most important genera of the Lamiaceae family [1]. The species of *Thymus* genus are herbaceous perennial shrubs, well identified as aromatic and medicinal plants [2,3]. *Thymus mastichina* L. is endemic from the central Iberian Peninsula in Spain, except in East region, Cataluña and Aragón [1,4]. It's a specie widely distributed along the peninsula with an excellent adaptation to numerous ecological ecosystems [5]. *T. mastichina* was used traditionally in food preparations and was also used to make tea for stomach disease [6]. According to Blanco [5], above all, *T. mastichina* has been used since ancient times to aromatize, preserve, and facilitate the digestion of food.

T. mastichina is an aromatic and medicinal plant principally characterized by its high content in essential oils and phenolic compounds. The main components in the essential oil of *T. mastichina* are 1,8-cineole (30-68%) and linalol (3-48%), both are important compounds with therapeutic properties. These therapeutic properties include antiseptic, antispasmodic, anti-carcinogenic, digestive, diuretic and many other properties [7]. Phenolic compounds, which are presents in all parts of the plant such as seeds, leaves and roots, are one of the most important natural antioxidants. They are reported to exert a variety of valuable bioactivities including anti-inflammatory properties [8,9]. And, among phenolic compounds, flavonoids, which constitute the largest group of plant phenolics, are the most potent antioxidants. Flavonoids may counteract oxidative stress-induced endothelial dysfunction and platelet aggregation, which are the main causes of cardiovascular disease [10,11]. Furthermore, they play an important role in reducing the development of atherosclerotic plaque formation [12], the risk of neurodegenerative disease [11,13] and the risk of coronary mortality [14]. Besides, current research regardless cancer prevention has shown that flavonoids may interrupt various stages of the cancer process [15,16].

In the past, studies were carried out on various species of *Thymus* but little is known as regards *T. mastichina [5]*. Indeed, *T. mastichina* is scarcely studied despite of its crop growing potential, sustainability and profitability as source of antioxidant compounds that can give an add value to the products without additional costs for the

farmers. For this reason, the main objective was the compositional characterization of key bioactive compounds and evaluation of antioxidant and anti-inflammatory properties as indicator of potential ingredient use in the food industry.

2. Materials and Methods

2.1. Plant Material

Cultivated and wild marjoram (*T. mastichina* L.) was provided from two agriculture farms in the provincial city of Valladolid, Spain (Villabrágima en Ctra. Castromonte and San Pedro Latarce) where marjoram was cultivated for the first time from wild seeds, and also from a meadow as wild plant (Escuela de la Santa Espina). The cultivation of marjoram was initially performed in a nursery in order to ensure the growth of plants which were afterwards transplanted in fields.

Marjoram was harvested in June 2010 when the plant had a height of 40 cm. The plant was dried at a temperature of 19.5°C in a warehouse (80 m^2) with a natural ventilation system. The plants were kept in controlled conditions during 3 week in order to guarantee that the drying process was finished. Finally, the chopped plant was packed and stored in darkness until further use.

2.2. Determination of the Bioactive Properties

2.2.1. Samples and Reagents

Folin-Ciocalteu reagent, gallic acid (GA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox (anhydrous 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), rosmarinic acid, HPLC grade methanol, water and polytetrafluoroethylene filters (0.22 μm) were purchased from Sigma-Aldrich, Wicklow, Ireland. Luteolin-7-O-glucoside, luteolin, naringin, apigenin and apigenin-7-Oglucoside were purchased from Extrasynthese, Genay, France. The purity of standards and solvents were in the range of 95-99.8%. Only luteolin-7-Oglucoside had 90% purity.

2.2.2. Extraction of Phenolic Compounds

Samples of dried and ground plant were homogenized for 1 min at 24,000 rpm using an Ultra-Turrax T-25 Tissue homogenizer (Janke & Kunkel, IKA-Labortechnik, Saufen, Germany). Two grams of the ground sample was homogenized in 100 mL of solvent. Three different solvents were used for the extraction (water, ethanol and methanol). The sample suspension was shaken at 175 rpm overnight (19h) in a Max Q 4000 Incubator and Shaker (Thermo Fisher Scientific, Millcreek Road, Marietta, Ohio, USA) at 25°C. After that, the sample suspension was centrifuged for 5 min at 4,000 g (ROTINA 380R, HettichLab, Tuttlingen, Germany) and the supernatant was filtered through 0.22 μm polytetrafluoroethylene (PTFE) filters. The extracts were evaporated at 40°C in a rotary evaporator (Rotavapor RII, BÜCHI Laboratory, Flawil, Switzerland) and the extraction yield of each extract was estimated by weighing the material remaining after evaporation. Three replications of each extract were prepared and then stored at -20°C until subsequent analysis.

2.2.3. Determination of Total Phenols

The total phenolic content was determined using Folin-Ciocalteu Reagent (FCR) as described by Singleton et al. [17] and gallic acid was used as standard. In each replicate, 100 μL from the appropriately diluted sample extract, 100 μL methanol, 100 μL FCR and finally 700 μL sodium carbonate (Na_2CO_3) were added together and vortexed. The blend was incubated for 20 min in the dark at room temperature. After incubation the mixture was centrifuged at 13,000 rpm for 3 min (ROTINA 380R, HettichLab, Tuttlingen, Germany). The absorbance of the supernatant was measured at 735 nm by spectrophotometer. The TPC was expressed as mg of gallic acid /g dry weight extract (mg GAE/g DWE). The assay was performed in two batches which included three replications in each for both samples and standard.

2.2.4. The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

The capacity of the extracts to scavenge DPPH free radical was determined according to the method described by Sanchez-Moreno [18]. Briefly, 2,2-Diphenyl-1-picrylhydrazyl (Sigma-Aldrich, Wicklow, Ireland) was dissolved in methanol (0.238 mg/mL). 500 μL of the diluted samples, methanol as blank and Trolox dilutions as standards were added to 500 μL of DPPH solution. Solutions were incubated in the dark at room temperature for 30 min. The absorbance was measured at 515 nm by spectrophotometer (Hitachi Double Beam Spectrophotometer. model U-2900 UV-VIS, Japan). The radical scavenging activity was expressed as mg trolox/g dry weight extract (g Trolox/100g DWE) and also as EC50, which is the extract concentration providing 50% of radicals scavenging activity. All samples were replicated.

2.2.5. The Semicarbazide-Sensitive Amine Oxidase (SSAO) Inhibitory Assay

The SSAO inhibitory activity of *T. mastichina* was performed on water extracts. Standard curve was prepared with concentrations from 200 to 3.125 μM of SSAO. For the samples, 200 μl of the reaction solution was prepared using 50 μl of buffer as blank, standards and samples, which were preincubated with 50 μl of SSAO enzyme and then 100 μl of the cocktail reaction. The black flatbottom 96-well plate was incubated at 37°C for 2 hours. The fluorescence of resorufin was recorded at 530 nm excitation/ 590 nm emission) during 35 seconds using a Fluostar Omega (Offenburg, Germany). The results were expressed as μM of semicarbazide. The analysis was performed in triplicate.

2.3. Characterization of Phenolic Compounds

2.3.1. Preparation of the Extracts

Solid-phase extraction (SPE) was used for the clean-up of extracts prior to the LC-MS separation and quantification of phenolic compounds. C18 Sep-Pak cartridge (Mildford, USA) was pre-conditioned with 6 mL of methanol followed by 6 mL of water without allowing the cartridge to dry out. Then, the dissolved extracts (water, ethanol and methanol) were passed through the cartridge. The eluates were then transferred to vials and

evaporated to dryness with nitrogen. The residues obtained were dissolved in methanol. The distribution of the cleaned-up extracts was achieved with vortex agitation prior to the chromatographic analysis.

2.3.2. Liquid Chromatography-mass Spectrometry (LC-MS)

LC-MS analysis was performed on a Q-Tof premier mass spectrometer (Waters Corporation Micromass MS Technologies, Manchester, UK) coupled to Alliance 2695 HPLC system (Waters Corporation Milford, MA, USA). The Q-Tof Premier was equipped with a lockspray source where an internal reference compound (Leucine- Enkephalin) was introduced simultaneously with the analyte for accurate mass measurements.

Compounds were separated on an atlantis T3 C18 column (Waters Corporation Milford, USA, 100 mm x 2.1 mm; 3 µm particle size) using 0.5% aqueous formic acid (solvent A) and 0.5% formic acid in 50/50 v/v acetonitrile: methanol (solvent B). Column temperature was maintained at 40 °C. The gradient elution program applied was: 100% A for 15 min, 50% A: 50% B for 15 min and 80% A: 20% B for 50 min at a flow rate of 0.2 mL/min. Electrospray mass spectra data were recorded in the negative ionization mode for a mass range m/z 100 to m/z 1000. Capillary voltage and cone voltage were set at 3 kV and 30 V respectively. Collision induced dissociation or fragmentation (CID) of the analytes was achieved using 12-20 eV energy with argon as the collision gas. Identification of some of the phenolic compounds was carried out by comparing retention times and their masses with those of authentic standards.

And, for the compounds for which no standards were available a tentative of identification was based on accurate mass measurements of the pseudomolecular [MH]-ions in combination with collision-induced dissociation (CID) fragment ions.

Quantification of each compound was performed on the basis of the relative area of an internal standard, kaempferol. Results were expressed in µg kaempferol/mg DWE.

3. Statistical Analysis

Data were analyzed using the General Linear Models procedure using the SPSS package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA). Significant difference was declared at $P<0.05$ and values with statistically significant differences were compared using Duncan's Multiple Range test and designed by different letters.

The correlation analysis was performed using the Pearson's correlation coefficient (r). A partial correlation was also used to measure the degree of association between two variables (total phenolic content and DPPH free scavenging activity), with the effect of a set of controlling random variable removed (extraction solvent) in order to know the impact caused by this third variable on the relationship between the both primary variables.

4. Results and Discussion

4.1. Bioactive Properties

4.1.1. Extracts yield and Total Phenolic Content

The efficiencies of different solvents (water, ethanol and methanol) on the extraction of total polyphenols from *T. mastichina* L. were investigated. As shown in Table 1, the extraction yields varied significantly according to the extraction solvents used for extraction. In terms of total extraction yield, methanol was the most effective extractant solvent followed by ethanol and water (11.88, 8.51 and 7.91%, respectively).

Those results were in agreement with the results published by Hossain et al. [19] in which methanol was reported to be a highly efficient solvent for the extraction of phenolic antioxidant. According to Roby et al. [20], the polarity of solvents is correlated with the degree of extraction. Indeed, solvent with intermediate polarity are shown to have better chance in extracting a diverse range of phenolic compounds. .

The Folin–Ciocalteu phenol assay was used to evaluate the total phenolic content present in the samples [20,21] and the total phenolic content was expressed as gallic acid equivalent.

Total phenolic content of extracts varied significantly according to the solvent used for the extraction (Table 1). Methanol extracts had significantly higher phenol content than ethanol or water extracts (195.71 ± 4.07, 168.96 ± 4.48 and 152.30 ± 5.44 mg GAE/g dry extract, respectively ($P< 0.05$), Table 1). Those results were in agreement with the results reported by Barros et al. [22] for *T. mastichina*, in which methanol extracts showed the highest values in comparison with ethanol and water extracts (165.29 ± 1.11, 109.09 ± 0.46 and 59.93 ± 0.06 mg GAE/g dry extract, respectively). The total phenolic content in the present results was higher than values reported by Barros et al. [22], especially for water extracts. Those differences might be associated to the origin of the plants (Spanish vs Portuguese) since the edapho-climatological conditions were different in both areas.

Table 1. Extraction yield and total phenolic content of marjoram extracts

Sample	Extraction yield (%)[a]	Total phenols (mg GAE[b]/g dry extract)
Water extract	7.91[b]± 0.52	152.30 [c]± 5.44
Ethanol extract	8.51[b]± 0.27	168.96 [b] ± 4.48
Methanol extract	11.88[a] ± 0.77	195.71 [a] ± 4.07

[a] Extraction yield (%) = ((sample extract weight/sample weight) *100)
[b] GAE, Gallic acid equivalent
Values (mean ± SD, n=3) in the same column a different letter are significantly different ($P< 0.05$)

Other studies were as well carried out on *T. mastichina*, from the Spanish and the Portuguese regions. The results of the total phenolic content for the Spanish *T. mastichina* ranged between 2.90 and 9.15 mg GAE/g dry extract [4] and between 0.78 and 26.18 mg GAE/mL for the Portuguese *T. mastichina* [21]. These values were lower than those observed in the present study (Table 1). These differences might mainly be associated to the extraction

method used and also to the part of the plant on which the analyses were performed. Indeed, Delgado et al. [4] and Albano and Miguel [21], used for the extraction of phenolic content a procedure different to that used in the present study. Delgado et al. [4] performed a preliminary extraction with petroleum ether in order to eliminate chlorophyll and fats. And, Albano and Miguel [21] used two different procedures; the first one consisted on reextraction with methanol after hydrodistillation and the second one involved maceration with ethanol then suspension in distilled water and finally extraction with diethyl ether, ethyl acetate, and n-butanol. Moreover, in the present study, the analyses were performed on the whole plant whereas Delgado et al. [4] used the arterial parts (leaves and flowers) and Albano and Miguel [21] used the flowering aerial parts.

The present results were as well compared to species that belong to the *Thymus* genus. Roby et al. [20] reported that methanol was more efficient solvent in extracting phenols from the leaves of *Thymus vulgaris* L. than ethanol, diethyl ether and hexane (8.10; 7.30, 6.15 and 4.75 mg GAE/g of dry material, respectively).

Roby et al. [20] and Lagouri et al. [23] revealed that the highest TPC was observed on leaves of *Thymus vulgaris*, extracted with methanol, in comparison with ethanol, diethyl ether and hexane extracts and hexane and dichloromethane extracts, respectively (148.31; 11.97 and 7.35 mg caffeic acid/g, respectively) Rababah et al. [24] reported that the TPC determined in the methanol extracts of the *Thymus capitatus* leaves was higher than that of ethanol extracts, independently of the temperature used for the extraction (2419.9 vs 2323.6 mg GAE/100 g of dry material at 60°C and 1066.4 vs 219.8 mg GAE/100 g of dry material at 20°C, respectively).

Since there is no data as regards water extracts however different species from the same family showed similar results. Indeed, the values of the water extracts in the present study were similar to the values reported by Hinneburg et al. [25] for Basil (*Ocimum basilicum*) (mg GAE/g dry extract) and Dorman et al. [26] for savory (*Satureja cuneifolia* Ten.) (152.30 ± 5.44 vs 147 ± 1.60 and 151 mg GAE/g dry extract, respectively) although different methods of drying were used. In the present study air-dried extracts were used whereas in the study carried out by Hinneburg et al. [25] samples were freeze-dried.

Besides the solvent used for the extraction, the part of the plant on which the analysis was performed as described above, the specie can also be responsible to some extent for the variability of the total phenolic content. Furthermore, differences may also be associated to the wild origin of the plant.

4.1.2. Antioxidant Activity: Radical Scavenging Activity (DPPH Assay)

The DPPH free radical scavenging activity assay was used to measure the ability of the samples to reduce the free radical DPPH and this method has been extensively used for plants and spices [4,21,23]. DPPH radical scavenging activity was expressed as Trolox equivalents per 100 grams of dry extract or as the extract concentration providing 50% of radicals scavenging activity (EC_{50}).

The DPPH free radical scavenging activity was measured in marjoram extracts (methanol, ethanol and water extracts) and followed a concentration-dependent pattern (Figure 1). As expected, an increase of the extract concentration (0.015-0.06 mg/mL) improved the scavenging activity; exponentially for the water and the ethanol extracts (R^2 = 0.999; R^2 = 0.999, respectively) and in a polynomial fashion for the methanol extracts (R^2 = 1). Those results were in agreements with those reported by Delgado et al. [4], Lagouri et al. [23] and Rababah et al. [24]. However, the scavenging activity of methanol extracts increased significantly ($P<0.05$) above concentration 0.03 mg/mL since the optimum concentration at which it was suitable to compare the different solvent extracts was the central point which corresponded to 0.03 mg/mL. At this concentration, the DPPH free radical scavenging activity of methanol extracts was higher than that of water and ethanol extracts (58.85 ± 0.52, 35.12 ± 0.20 and 39.63 g Trolox/100 g of dry weight extract, respectively). The DPPH scavenging activity presented for the methanol extracts was in agreement with the results reported by Hossain et al. [19] for the synthetic antioxidant (BHT) (80.85 g Trolox/100gDW). The DPPH free radical scavenging activity expressed as EC_{50}, showed as well that methanolic extracts had higher antioxidant activity than ethanolic and water extracts (0.028, 0.044 and 0.052 mg/mL, respectively). Those results were in agreement with those reported by Barros et al. [22], in which the DPPH radical scavenging activity of the methanolic extracts of *T. mastichina* was higher than those extracted with ethanol and water (0.69, 0.94 and 2.57 mg/ml). In general, regardless of the solvent used, values of the DPPH free radical scavenging activity in the present study were higher than those reported by Barros et al. [22].

As for phenolic content, the discrepancy between the present results and those observed in other studies on *T. mastichina* [4,21] might be associated to the fact that different parts of the plants were used for the determination of the antioxidant activity and to the difference between the extraction procedures. Delgado et al. [4], in a study carried out on twenty Spanish *T. mastichina*, found that the DPPH scavenging activity of the methanolic extracts, expressed as EC_{25}, ranged between 0.90 and 3.45 mg/mL. Albano and Miguel [21] found, by using two different procedures and several solvents, as described for phenolic content that the DPPH scavenging activity of the Portuguese *T. mastichina*, ranged between 2.7 and 8.3 µg/mL.

In comparison with other species that belong to the Lamiaceae family, Rababah et al. [24] reported that the DPPH free radicals scavenging activity of the methanolic extracts of *T. mastichina*, expressed as EC_{50}, was higher than in oregano and thyme (0.119 ± 0.01 mg/mL). Moreover, the antioxidant activity of the methanolic extracts of *T. mastichina* was two times lower than that EC_{50} for the synthetic antioxidant (BHT) (0.019 mg/mL) [24].

A significant correlation (r=0.976) was observed between the content of total phenols (mg GAE/g dry weight extract) and the DPPH scavenging activity (equivalent Trolox) of marjoram extracts. As expected, the DPPH scavenging activity of marjoram followed the same pattern as the results observed for phenolic contents since extracts that contain high amount of polyphenols also exhibit high antioxidant activity [27] and vice versa. This

linear correlation between antioxidant capacity and total phenolic content of aromatic plants and spices was also reported by Rababah et al. [24] and Hossain et al. [29] who reported coefficients of correlation between 0.852 to 0.965.

In order to determine the effect of solvent as third variable, a partial correlation test was performed which showed the correlation between the primary variables (total phenolic content and DPPH scavenging activity) did not decrease (r= 0.996) in comparison to the Pearson's correlation (r=0.976) This means that the solvent used (methanol, ethanol and water) did not affect significantly the relationship between the total phenolic content and the DPPH scavenging activity. However, when the correlation included only the methanol and the water extracts, the effect of solvent became significant (r = -0.071). These results were in agreement with those mentioned before where a positive correlation was reported between antioxidant capacity and total phenolic content. The effect of other factors, such the solvent used, influencing significantly this correlative relationship has also been reported.

As it shown below (Table 1), *T. mastichina* was rich in flavonoids such as luteolin, quercetin and apigenin. The high antioxidant capacity of *T. mastichina* extracts might be associated with its high flavonoid contents. Several study found that luteolin, apigeninand quercetin has high antioxidant properties [30,31] with quercetin having highest radical scavenging activity among these flavonoids [32].

4.1.3. Anti-inflammatory Activity: SSAO Inhibitory Activity

The inhibition of SSAO activity represents a target for anti-inflammation because SSAO plays a key role in inflammation process through its ability to produce catalytic products such as hydrogen peroxide and other reactives aldehydes species.

The SSAO inhibitory activity of the water extracts of *T. mastichina* was compared with that of semicarbazide (positive control). The standard curve of semicarbazide was used to quantify the inhibitory activity of marjoram, which were 22.70, 28.13 and 57.27 µM of semicarbazide for 0.015, 0.3, and 0.9 mg/ml, respectively.

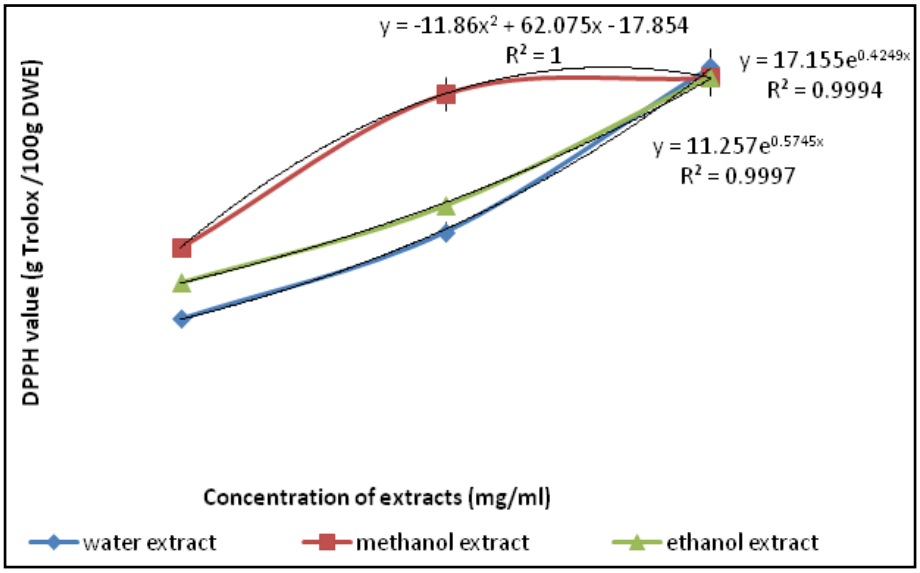

Figure 1. DPPH radical-scavenging effect of methanol, ethanol and water extracts of marjoram

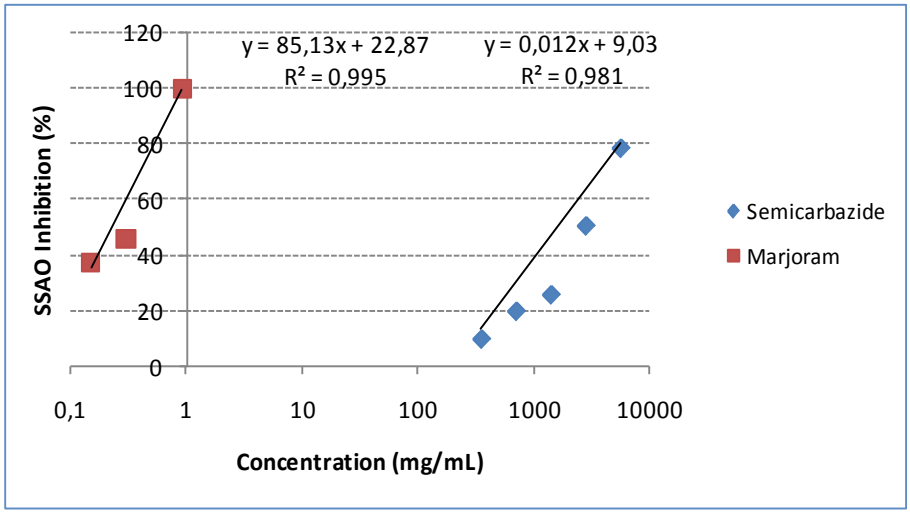

Figure 2. The inhibitory activities of marjoram (0.15, 0.3 and 0.9 mg/mL) and semicarbazide (3.125, 6.25, 12.5, 25 and 50 µM which corresponds to 348.5, 697.1, 1394.1, 2788.2 and 5576.5 mg/mL) on SSAO activities

T. mastichina was found to exhibit dose-dependent SSAO inhibitory activities. The extracts at concentrations of 0.15, 0.3 and 0.9 mg/mL showed inhibitions of 37.67%, 45.88% and 100%, respectively (Figure 2). The EC_{50} of *T. mastichina* was much lower than that of semicarbazide (0.39 mg/ml and 3.53 µM which corresponds to 393,63 mg/ml, respectively). *T. mastichina* was found to be a strong SSAO inhibitor, and its EC_{50} was 1/1009 lower than EC50 for semicarbazide, which means that *T. mastichina* was more than one thousand time stronger than semicarbazide. The high value of EC50 might be associated to the fact that the SSAO assay was performed on the water extracts of the whole plant (*T. mastichina*) and not on an isolated compound such as in the results found by Lin et al. [33], in a study performed on geranin isolated from *P. niruri*, in which geranin was reported as a strong SSAO inhibitor (about 1/5.2 that of semicarbazide).

Albano and Miguel [21] in a study performed on the flowering aerial parts of *T. mastichina* used extraction procedures different to that described in the present study and used the 5-lipoxygenase assay to measure the anti-inflammatory activity of *T. mastichina*. In the same and in comparison with other species that belong to the Lamiaceae familly (*Origanum vulgare, Salvia officinalis, Thymbra capitata, Thymus camphoratus* and *Thymus carnosus*) and for four extracts from a total of six, *T. mastichina* was found to be the strongest inhibitor of 5-Lipoxygenase. Consequently, regardless of the extraction procedures, the part of the plant on which the assay was performed and the method used for the determination of the anti-inflammatory activity *T. mastichina* was found to exert high anti-inflammatory activity.

4.2. Characterization of Phenolic Compounds

4.2.1. Context

Marjoram *T. mastichina* L. belongs to the Thymus species, which are well known as aromatic and medicinal plants [3], with antioxidant activities comparable to those of α-tocopherol and butylated hydroxytoluene [BHT] [34]. Their potent antioxidant properties have been mostly associated to the polyphenols present in the main composition [29,35]. Many aromatic herbs and spices have been studied and to some extent their phenolic chemistry is known but few information is available in relation to the phenolic composition of *T. mastichina* L.

The characterization of the polyphenols from *T. mastichina* L. was performed using the liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). Liquid chromatography coupled to mass spectrometry (LC/MS) was widely reported as an efficient, accurate and specific method for the analysis of polyphenols in many natural products [36]. Indeed, LC-ESI-MS/MS is recognized as a powerful analytical tool due to its high sensitivity, short run time, less use of toxic organic solvents compared to reversed phase HPLC coupled with diode-array detector and also for its ability to differentiate compounds with same nominal mass but different exact masses [28,29].

4.2.2. Mass Spectrometric Conditions for Polyphenols of *T. mastichina* L.

Polyphenols except polymeric tannins are usually small molecules and those are identified using mass spectral data in the mass range of m/z 100 to m/z 1000. The negative ionization mode is more suitable than the positive ionization mode for the acquisition of MS data from polyphenols, because they contain one or more hydroxyl and/or carboxylic acid groups [28,29]. Due to high phenolic content and DPPH scavenging activity the phenolic characterization of *T. mastichina* was performed only on the methanol extract. Several studies have also used methanol rather than other solvents to extract the phenolic components from different aromatic plants [20,28,29]. Figure 3 showed the total ion current (TIC) chromatogram of marjoram extracted with methanol. The identification of each peak is specified in Table 2.

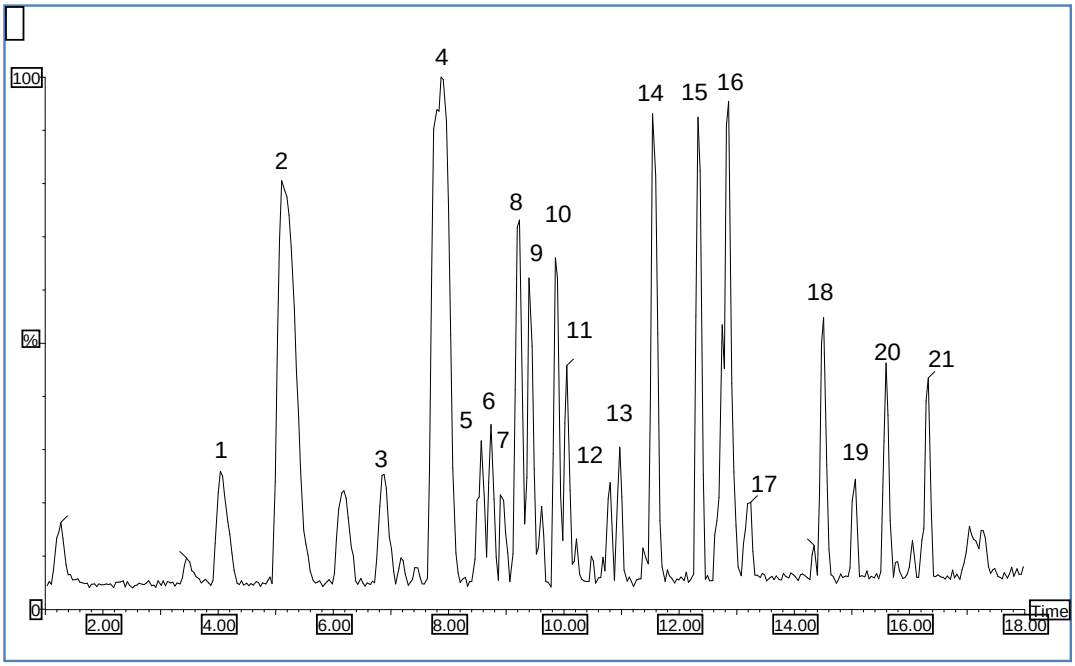

Figure 3. Phenolic analysis of marjoram extract by LC-MS

Table 2. Phenolic compounds identified in marjoram by LC-ESI-MS/MS

Peak	Polyphenols	Emperical formula	Obsd m/z [MH]-	Calcd m/z [MH]-	Major fragments	ug kaempferol/mg DWE	Retention time (min)
1	Dihydroquercetin-3-O-rhamnoside	$C_{21}H_{22}O_{11}$	449,1100	449,1084	287	1,42	3,51
2	Quercetin glucoside	$C_{21}H_{20}O_{12}$	463,0898	463,0877	301	5,46	4,33
3	Naringin glucoside	$C_{21}H_{22}O_{10}$	433,1150	433,1135	271	1,50	6,02
4	Luteolin-7-O-glucoside	$C_{21}H_{19}O_{11}$	447,0939	447,0927	285	6,22	7,30
5	Quercetin-3-O-arabinoside	$C_{20}H_{17}O_{11}$	433,0784	433,7710	301	1,63	8,13
6	Isorhamnetin-3-O-hexoside	$C_{22}H_{21}O_{12}$	477,1039	477,1033	315	1,18	8,29
7	Quercetin 2-hexoside	$C_{30}H_{25}O_{15}$	625,1190	625,1193	301	0,69	8,53
8	Eriodictyol	$C_{15}H_{11}O_6$	287,0559	287,0556	259	2,87	8,83
9	Apigenin-7-O-glucoside	$C_{21}H_{19}O_{10}$	431,0992	431,0978	431	1,59	9,06
10	Apigenin-7-O-glucuronide	$C_{21}H_{17}O_{11}$	445,0785	445,0771	269	0,65	9,28
11	Rosmarinic acid	$C_{18}H_{15}O_8$	359,0788	359,0767	161	1,94	9,52
12	Quercetin-hexoside-glucuronide	$C_{31}H_{27}O_{15}$	639,1339	639,1350	301	1,28	9,78
13	Luteolin/-hexoside-glucuronide	$C_{31}H_{27}O_{14}$	623,1389	623,1401	285	0,63	10,51
14	Luteolin	$C_{15}H_{10}O_6$	285,0406	285,0399	285	5,13	11,30
15	Naringin	$C_{15}H_{11}O_5$	271,0618	271,0606	151	3,03	12,09
16	Apigenin	$C_{15}H_{19}O_5$	269,0454	269,0450	269	3,76	12,58
17	Kaempferide	$C_{16}H_{11}O_6$	299,0569	299,0556	299	1,61	12,99
18	Unknown	$C_{18}H_{31}O_5$	327,2175	327,2171	291	2,17	14,31
19	Cirsimaritin	$C_{17}H_{13}O_6$	313,0724	313,0712			14,80
20	Unknown	$C_{18}H_{33}O_5$	329,2343	329,2328		1,77	15,39
21	Unknown	$C_{18}H_{15}O_7$	343,0835	343,0818		1,44	16,12

Eighteen different polyphenols were identified and quantified calculating the accurate mass. Most of the identified compounds belong to the flavonoids class (seventeen) and one compound is a hydroxycinnamic acid derivative (rosmarinic acid).The use of the hydrophobic C18 solid phase extraction cartridges for reducing intereferring compounds in mass spectrometric analysis might have promoted the presence of flavonoids at the expense of the phenolic acids. A number of highly hydrophilic phenolic acids might have been washed out with interfering sugars during the washing step of solid phase extraction.

Reference standards were used to identify 5 flavonoids; luteolin-7-O-glucoside, luteolin, naringin, apigenin and apigenin 7-O-glucoside. For the twelve remaining flavonoids, for which there were no 'in-house' standards, their identifications were based on accurate mass measurements and MS/MS experiments (Table 2). The twelve flavonoids identified were dihydroquercetin-O-rhamnoside, quercetin glucoside, naringin glucoside, quercetin-O-arabinoside, isorhamnetin–Ohexoside, quercetin- dihexoside, eriodictyol, apigenin 7-O-glucuronide, quercetin-hexoside-glucuronide, luteolin/-hexoside-glucuronide, kaempferide and cirsimaritin.

The fragmentation of the deprotonated molecular ion provided the aglycones as prominent fragments and the mass difference between the deprotonated molecular ion and the aglycone fragment determined the molecular mass of the glycoside substituent.

The majority of flavonoids reported were present as glycosyl or /and glucuronyl conjugates. Other studies also reported the presence of the glycosyl and glucuronyl conjugates [28,29], but not bounded to the same aglycone, as in this study. Among the glycosylated flavonoids, the glucoside conjugates were the most commonly observed. Delgado et al. [4]. Preira and Cardoso [37], observed that species of Thymus usually comprise distinct glycosidic forms of the flavonoids luteolin, apigenin and narginin. The MS/MS experiments revealed that the [M-H]- ions at m/z 463.09 eluting at 4.33 min, m/z 447.09 eluting at 5.16 min, m/z 433.11 eluting at 6.02 min and m/z 431.10 eluting at 9.06 min were quercetin-glucoside, luteolin-7-Oglucoside, narginin-glucoside and apigenin-7-O-glucoside, respectively. Moreover, isorhamnetin-O-hexoside and quercetin-dihexoside had similar fragmentation with the loss of one or two hexose moieties (162u), respectively. Glucoronide derivatives were also identified. Subsequent CID of apigenin-7-O-glucuronide showed the loss of a glucoronic acid (m/z 176.0) and produced the predominant fragment at m/z 269.0 corresponding to the aglycone apigenin. The apigenin-glucuronide has been described for the first time by Kaiser et al. [38] in marjoram (*Origanum majorana* L.).

Furthermore in the daughter scan mode (MS/MS), the spectra presented fragments at m/z 301.0 and at m/z 285.0 corresponding to the deprotonated molecules quercetin and luteolin, respectively, after the loss of both glucosyl and glucuronyl moieties.

Only one arabinoside derivative was detected in the *Thymus mastichina* L. extract. This compound eluting at 8.13 was identified as quercetin-O-arabinoside (Table 2). The CID of quercetin-O-arabinoside showed the loss of a pentose (m/z 132) and produced the predominant fragment at m/z 301.0 corresponding to deprotonated quercetin. Furthermore in the MS/MS experiment, cirsimaritin (m/z 313.1) lost two consecutive methyl groups, which resulted in two fragment ions at m/z 298.0 and at m/z 283.1. Hossain et al. [28,29] had previously reported similar results using LC-ESI-MS/MS on cirsimaritin in rosemary, oregano, sage, basil and thyme extracts.

The quantities of each compound were expressed in µg kaempferol/mg dry weight and, luteolin-7-O-glucoside was the most abundant phenolic compound (6.22 µg kaempferol/mg DWE) followed by quercetin glucoside (5.46 µg kaempferol/mg DWE) and luteolin (5.13 µg kaempferol/mg DWE). It has been reported by several authors that aglycones exhibit higher antioxidant activity than their glycosidic counterparts [39,40]. In addition,

apigenin, naringin and eriodictyol were also present in significant amounts (3.76, 3.03 and 2.87 µg kaempferol/mg DWE, respectively). Those results were similar to values reported by Wojdyło et al. [41] in which quercetin, luteolin and apigenin were the predominant flavonoids observed in 32 aromatic plants. Moreover, Delgado et al. [4] detected the presence of apigenin and luteolin in *T. mastichina* and those values were the highest in Salamanca and Soria provinces (0.15 to 0.91 mg/g and 0.35 to 1.85 mg/g, respectively). Additionally, Roby et al. [20] reported that apigenin was the principle flavonoids (more than 70% of the total flavonoids) in marjoram leaves.

Rosmarinic acid was the unique polyphenol from the hydroxycinnamic acid derivatives category identified with the LC-MS. It was recognized by comparing the retention time and the characteristic MS spectral data with that of the authentic standard (Table 2). Accurate mass measurements and fragmentation pattern during CID further confirmed their structural composition. The fragments produced, during CID analysis of the deprotonated ion at m/z 359.08 identified as rosmarinic acid, are caffeic acid at m/z 179.0 and the 2-hydroxy derivative of hydrocaffeic acid at m/z 197.0. Similar pattern of fragmentation of rosmarinic acid has been already observed by Shen et al. [42].

The rosmarinic acid in the current study was present at high levels (1.94 µg kaempferol/mg DWE) but not as the main constituent. This result was in agreement with values published by Kaiser et al. [43] in marjoram (*Origanum majorana* L.) extracts. Pseudomolecular ions at m/z 327.20 (observed exact mass 327,2171), m/z 320.20 (observed exact mass 329,2328) and m/z 343.10 (observed exact mass 343,0818) eluting at 14.31 min, 15.39 min and 16.12 min respectively could not be identified.

5. Conclusions

The whole plant, *Thymus mastichina*, from wild origin, showed a high antioxidant and anti-inflammatory activity, associated to its high content in phenolic compounds, especially flavonoids, mainly luteolin and quercetin.

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References

[1] Morales, V.M. 2005. Floraliteraria del Quijote alusiones al mundo vegetal en las obras completas de cervantes. Instituto de Estudios Albacetences “Don Juan Manuel”. 200.

[2] Amiri, H. (2012). Essential oils composition and antioxidant properties of three thymus species. Evidence-Based Complementary and Alternative Medicine.

[3] Jia, H.L., Ji, Q.L., Xing, S.L., Zhang, P.H., Zhu, G.L., & Wang, X.H. (2010). Chemical composition and antioxidant, antimicrobial activities of the essential oils of Thymus marschallianus Will. and Thymus proximus Serg. Journal of food science, 75(1), 59-65.

[4] Delgado, T., Marinero, P., Asensio-S-Manzanera, M. C., Asensio, C., Herrero, B., Pereira, J.A., & Ramalhosa, E. (2014). Antioxidant activity of twenty wild Spanish Thymus mastichina L. populations and its relation with their chemical composition. Food Science and Technology, 57(1), 412-418.

[5] Blanco, S.J. (2007). Folia Botanica Extremadurensis. 1.

[6] Bento, F., Galego, L., Gonçalves, V., Cavac, T., Almeida, V., & Margarida, C. (2006). Headspace solid-phase microextraction gas chromatography and gas chromatography-mass spectrometry analysis of the volatile compounds during packing of: Calamintha baetica, Thymus mastichina and Origanum vulgare. Traditional Food Processing and Techological Innovation in the Peripheral Regions.

[7] Sengupta, A & Bhattacharjee, S. (2009). Cardamom (Elettaria cardamomum) and its active constituent, 1,8-cineole. In: Molecular targets and therapeutic uses of spices (Modern Uses for Ancient Medecines), (eds, Aggarwal, B.B and Kunnumakkara, Singapore, London: WorldScientific Publishing.

[8] Nair, H.B., Sung, B., Yadav, V.R., Kannappan, R., Chaturvedi, M.M., & Aggarwal, B.B. (2010). Delivery of antiinflammatory nutraceuticals by nanoparticules for the prevention and treatment of cancer. Biochemical Pharmacology, 80(12), 1833-1843.

[9] Siriwardhana, N., Kalupahana, N.S., Cekanova, M., LeMieux, M., Greer, B., & Moustaid-Moussa, N. (2013). Modulation of adipose tissue inflammation by bioactive food compounds. Journal of Nutritional Biochemistry, 24, 613-623.

[10] Rahman, K. (2007). Studies on free radicals, antioxidants, and co-factors. Clinical Interventions in Aging, 2(2), 219-236.

[11] Dashwood, R.H. (2007). Frontiers in polyphenols and cancer prevention. J. Nutr, 137: 267S-269S.

[12] Pandey, K.B & rizvi, K.K. (2009). Plant polyphenols as dietary antioxidants in humanhealth and disease. Oxidative Medicine and Cellular Longevity, 2(5), 270-278.

[13] Vauzour, D., Rodriguez-Mateos, A., Corona, G., Oruna-Concha, M.J., & Spencer, J.P.E. (2010). Polyphenols and human health: Prevention of disease and mechanisms of action. Nutrients, 2(11), 1106-1131.

[14] Mink, P.J., Scrafford, C.G., Barraj, L.M., Harnack, L., Hong, C.P., Nettleton, J.A., & Jacobs, D.R. (2007). Flavonoid intake and cardiovascular disease mortality: A prospective study in postmenopausal women. Am J Clin Nutr, 85(3), 895-909.

[15] Mitjavila, M.T & Moreno, J.J. (2012). The effects of polyphenols on oxidative stress and the arachidonic acid cascade. Implications for the prevention/treatment of highprevalence diseases. Biochemical Pharmacology, 84(9), 1113-1122.

[16] Chahar, M.K., Sharma, N., Dobhal, M.P., & Joshi, Y.C. (2011). Flavonoids: A versatile source of anticancer drugs. Pharmacogn Rev, 5(9), 1-12.

[17] Singleton, V.L,, Orthofer, R., & Lamuela-Raventos, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Meth Enzymol, 299, 152-178.

[18] Sánchez-Moreno, C. (2002). Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems. Food Sci Tech Int, 8(3), 121-137.

[19] Hossain, M.B., Brunton, N.P., Barry-Ryan, C., Martin-Diana, A.B., & Wilkinson, M. (2008). Antioxidant activity of spice extracts and phenolics in comparison to synthetic antioxidants. Rasayan J. Chem, 1(4), 751-756.

[20] Roby, M.H.H, Sarhana, M.A., Selima, K.A-H., & Khalela, K.I. (2013). Evaluation of antioxidant activity, total phenols and phenolic compounds in thyme (Thymus vulgaris L.), sage (Salvia officinalis L.), and marjoram (Origanum majorana L.) extracts. Industrial Crops and Products, 43, 827-831.

[21] Albano, S.M & Miguel, M.G. (2011). Biological activities of extracts of plants grown in Portugal. Industrial Crops and Products, 33, 338-343.

[22] Barros, L., Heleno, S.A., Carvalho, A.M., & Ferreira, I.C.F.R. (2010). Lamiaceae often used in Portuguese folk medicine as a source of powerful antioxidants: Vitamins and phenolics. Food Science and Technology, 43, 544-550.

[23] Lagouri, V., Bantouna, A., & Stathopoulos, P. (2010). A comparison of the antioxidant activity and phenolic content of non polar and polar extracts obtained from four endemic Lamiaceae

species grown in Greece. Journal of Food Processing and Preservation, 34(5), 872-886.

[24] Rababah, T.M., Banat, F., Rababah, A., Ereifej, K., &Yang, W. (2010). Optimization of Extraction Conditions of Total Phenolics, Antioxidant Activities and Anthocyanin of Oregano, Thyme Terebinth, and Pomegranate. Journal of Food Science, 75(7), C626-C632.

[25] Hinneburg, I., Dorman, D., & Hiltunen, R. (2006). Antioxidant activities of extracts from selected culinary herbs and spices. Food Chemistry, 97(1), 122-129.

[26] Dorman, D.H.J., Bachmayer, O., Kosar, M., & Hiltunen, R. (2004). Antioxidant Properties of Aqueous Extracts from Selected Lamiaceae Species Grown in Turkey. J. Agric. Food Chem, 52(4), 762-770.

[27] Wong, S.P., Leong, L.P., & Koh, J.H.W. (2005). Antioxidant activities of aqueous extracts of selected plants. Food Chemistry, 99(4), 775-783.

[28] Hossain, M.B., Barry-Ryan, C., Martin-Diana, A.B., & Brunton, N.P. (2010a). Effect of drying method on the antioxidant capacity of six Lamiaceae herbs. Food Chemistry, 123(1), 85-91.

[29] Hossain, M. B., Brunton, N.P., Barry-Ryan, C., Martin-Diana, A.B and Wilkinson, M. (2010b). Characterization of phenolics composition in Lamiaceae spices by LC-ESIMS/ MS. J. Agric. Food Chem, 58 (19), 10576-10581.

[30] Materska, Małgorzata. (2008). Quercetin and its derivates: Chemical structure and bioactivity. Pol. J. Food Nutr. Sci, 58(4), 407-413.

[31] Özgen, U., Mavi, A., Terzi, Z., Kazaz, C., Asç1, A., Kaya, Y., & Seçen, H. (2011). Relationship between chemical structure and antioxidant activity of luteolin and its glycosides isolated from Thymus sipyleus subsp. sipyleus var. sipyleus. Rec. Nat. Prod, 5(1), 12-21.

[32] Majewska, M., Skrzycki, M., Podsiad, M., & Czeczot, H. (2011). Evaluation Pharmaceutica Drug Research, 68(4), 611-615.

[33] Lin, S.Y., Wang, C.C., Lu, Y.L., Wud, W.C., & Hou, W.C. (2008). Antioxidant, antisemicarbazide- sensitive amine oxidase, and anti-hypertensive activities of geraniin isolated from phyllanthus urinaria. Food and Chemical Toxicology, 46(7), 2485-2492.

[34] Brewer, M.S. (2011). Natural Antioxidants: Sources, Compounds, Mechanisms of Action, and Potential Applications. Comprehensive Reviews in Food Science and Food Safety, 10(4), 221-247.

[35] Sørensen, A-D.M., Petersen, L.K., de Diego, S., Nielsen, N.S., Lue, B-M., Yang, Z , Xu, X., & Jacobsen, C. (2012). The antioxidative effect of lipophilized rutin and dihydrocaffeic acid in fish oil enriched milk. Eur. J. Lipid Sci. Technol, 114(4), 434-445.

[36] Abad-García, B., Berrueta, L.A., Garmón-Lobato, S., Gallo, B and Vicente, F. (2009). A general analytical strategy for the characterization of phenolic compounds in fruit juices by high-performance liquid chromatography with diode array detection coupled to electrospray ionization and triple quadrupole mass spectrometry. Journal of Chromatography A, 1216, 5398-5415.

[37] Pereira, O. R., & Cardoso, S. M. (2013). Overview on Mentha and Thymus polyphenols.Current Analytical Chemistry, 9(3), 382-396.

[38] Kaiser, A., Carle, R., & Kammerer, D.R. (2013). Effects of blanching on polyphenol stability of innovative paste-like parsley (Petroselinum crispum Mill., Nym ex A. W Hill) and marjoram (Origanum majorana L.) products. Food Chemistry, 138(2-3), 1648-1656.

[39] Bravo, L. (1998). Polyphenols: Chemistry, dietary osurces, metabolism, and nutritional significance. Nutrition Reviews, 56(11).

[40] Rice-Evans, CA., Miller, NJ., & Paganga, G. (1997). Structure–antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med, 20(7), 933-956.

[41] Wojdyło, A., Oszmianski, J and Czemerys, R. 2007. Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chemistry, 105, 940-949.

[42] Shen, Q., Zhang, B., Xu, R., Wang, Y., Ding, X., & Li, P. (2010). Antioxidant activity in vitro of selenium contained protein from the se-enriched. Bifodobacterium animalis. Anaerobe, 16, 380-386.

[43] Kaiser, A., Carle, R & Kammerer, D.R. (2013). Effects of blanching on polyphenol stability of innovative paste-like parsley (Petroselinum crispum Mill., Nym ex A. W Hill) and marjoram (Origanum majorana L.) products. Food Chemistry, 138(2-3), 1648-1656.