THE PARTICLE SIZE INFLUENCE OF *MELISSA OFFICINALIS* L. POWDER ON *TEAC* AND *TPC* CORRELATED WITH THE *IN SILICO* STUDY OF ONE OF THE ANTIOXIDANTS: CAFFEIC ACID

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Abstract. *Melissa officinalis* L. (lemon balm) has been used since ancient times for its remarkable curative properties (anti-inflammatory, antidepressant, digestive, carminative, tonic or diuretic). These properties are due to the polyphenols that abound in the body of this plant species. In the present work, we aimed to determine the influence of particle size of macerated powders on the antioxidant activity and the extraction process of these polyphenols as well as the *in silico* study of one of the most abundant polyphenols of this plant species, namely caffeic acid. The results obtained indicate a significant Trolox equivalent antioxidant capacity (*TEAC*) of 2.31 μg/mL correlated with the highest amount of total polyphenols content (*TPC*) of 52.26 ±0.36 mg gallic acid equivalent per g of dry plant (mg *GAE*/g dry plant) for the macerates obtained with a pharmaceutical ethyl alcohol content of 50 % of the powder with average particle size (106–90 μm). The *in silico* analysis of caffeic acid revealed that natural compound exhibits good intestinal permeability and blood-brain barrier (*BBB*) permeability, but shows moderate bioactivity correlated with the probability of binding to a relatively small number of molecular targets.

Key words: lemon balm., particle size, antioxidants, *in silico* studies, caffeic acid.

INTRODUCTION

The plant species *Melissa officinalis* L., popularly known as lemon balm, is an aromatic herb belonging to the *Lamiaceae* family. This plant species is also a melliferous plant, a property suggested even by its scientific name, the word ”melissa” coming from the Greek *melitos* which means honey [21].

Widely used in Europe and Asia, the plant species *Melissa officinalis* L. has a large number of curative properties, such as cardiovascular, digestive, diuretic, anti-inflammatory, tonic, antidepressant and even antiviral [14, 21, 28, 38]. The

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remarkable curative properties are due to the natural compounds found in the body of this medicinal plant, compounds in the form of secondary metabolites. The main secondary metabolites abundant throughout the aerial part of lemon balm plants are phenolic compounds, especially hydroxycinnamic acids, such as rosmarinic acid (Fig. 1) and caffeic acid (Fig. 2) [10, 32].

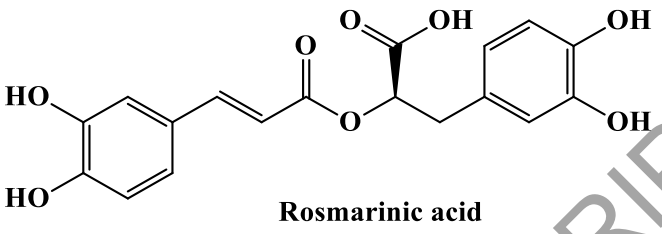


Fig. 1. Chemical structure of rosmarinic acid.

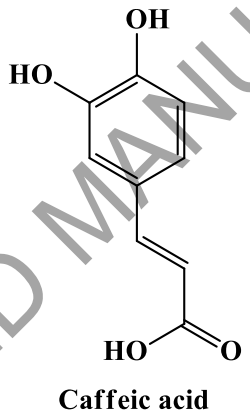


Fig. 2. Chemical structure of caffeic acid.

Recent *in silico* studies claim that these natural compounds are of great interest today because they have healing effects comparable to those of synthetic compounds [4, 5, 6, 7, 30, 46], but with much reduced or even absent side effects [3].

The antiviral activity of compounds from the plant species *Melissa officinalis* L. was reported by Prasanth *et al*. [35] who selected three phytocompounds from this species and using bioinformatics tools and Autodock, GROMACS, admetSAR and PASS analysis, observed that they exhibit high binding affinity with low binding energy to the main protease and spike protein and thus could be used as antivirals in SARS-CoV-2 infection.

The antiviral activity of rosmarinic acid has been reported by Priya *et al*. [36]. They collected the 3D structure of the Zika viral envelope protein from the Protein Data Bank [52] (PDB-5JHM) and studied its interaction with ligands of 25 phytocomplexes, analyses performed using SYBYL-X 1.3 software. The results

obtained from the molecular docking of each phytocompound with this viral protein showed that rosmarinic acid extracted from *Melissa officinalis* L. was among the top 5 compounds with the best total surflex-Dock score expressed as –logKd (6.2001) and with the best polar contact (8.0065), reflecting that this phenolic compound has activity with the host mechanism and responsible for protein folding, thus being able to be used as an antiviral.

Caffeic acid can also be used as a natural antiviral, as evidenced by Sathya and Gopalakrishnan [37] in their study. The results reveal that caffeic acid satisfies Lipinski's rule, namely, it has a molecular weight <500 Da the number of hydrogen bond donors <5, the number of hydrogen bond acceptor <10, CLogP <5, and also a good ADME nature, with hepatotoxicity and AMES mutagenicity equal to 0. The evaluation was performed using Accelerys Discovery Studio software.

The antiviral activity of caffeic acid was also studied by Güler *et al*. [17] who observed that this phenolic compound has the ability to inhibit the Covid-19 virus in terms of binding to the SARS-CoV-2 S1 protein. AutoDock 4.2 software was used for this study and a strong binding of the viral spike protein to the caffeic acid ligand was identified, the binding energy having a value of –5.31 kcal/mol.

However, the most important process in obtaining and identifying natural compounds from plants is the extraction technique [8]. This involves separating natural compounds, especially secondary metabolites, from inactive plant components.

The most commonly used extraction technique is maceration. This is the conventional extraction technique suitable for obtaining thermolabile compounds [33]. Maceration can be carried out from all parts of the plant and involves placing the plant material in a container over which solvent is poured until it is completely covered [9].

Periodic stirring of the contents is recommended for better miscibility of the two phases. At the end of the extraction process, the mycelium can be separated from the menstruum either by decantation, filtration or centrifugation [27].

In addition to selecting the appropriate extraction method for obtaining certain classes of natural compounds, the selection of the right type of solvent and the preparation of the plant material to be extracted play an essential role [1]. Thus, according to Tiwari *et al*. [44], the ideal solvent for extracting a significant amount of polyphenols is ethyl alcohol, which also has low toxicity compared to methanol. Moreover, ethyl alcohol can also be used as a solvent with water in various ratios [39].

Moreover, one of the most important parameters on which the production of certain quantities of natural compounds depends has been found to be the particle size of plant material being extracted. In this context, many studies show that the

finer the plant material is ground, the greater the extraction surface area, leading to an increase in the amount of natural compounds [12, 23, 26].

Thus, in the present study we aimed to identify the antioxidant activity of phenolic compounds from the aerial parts of *Melissa officinalis* L. species, depending on the particle size of the powders subjected to maceration. In addition, this study also aims to investigate the drug-likeness of one of the most abundant polyphenols of this plant species, namely caffeic acid, as well as the identification of possible molecular targets of this hydroxycinnamic acid.

MATERIALS AND METHODS

MATERIALS

Reagents

Distilled water, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin-Ciocâlteu reagent, Na_2CO_3 and gallic acid, were purchased from CARLO ERBA Reagents S.A.S.; 96.9 % pharmaceutical ethyl alcohol was purchased from SC.COMAN PRODUCT.S.A.

Plant material

The dried aerial parts of lemon balm (stems, leaves and flowers) were purchased from a local farmer and stored in paper bags until processing.

METHODS

Plant material primary processing

The dried plant material was carefully ground using a Retsch Grindomix laboratory mill, for 3 minutes in impulses, at 4000 rpm and for 20 seconds continuously at 10000 rpm.

The obtained powder was passed through a sieve with mesh sizes of 125 µm, 106 µm, 90 µm, 63 µm. The sieved material was used for the extraction process. The sieving process was performed using a Vibratory Sieve Shaker Retsch VS 1000, in pulses for 5 minutes, 1.8 mm amplitude.

Three types of powders were obtained from the sieving process: powders with particle sizes between 125 µm and 106 µm, powders with particle sizes between 106 µm and 90 µm, and powders with particle sizes between 90 µm and 63 µm.

Preparation of extract

2 g from each type of powder were immersed in 20 mL of solvent, for 7 days, protected from sunlight, 4 days with shaking at 30 rpm for 6 hours daily using the Biosan Rotator, and another 3 days without shaking.

Maceration was carried out in two types of solvent: solvent with 96.9 % pharmaceutical ethyl alcohol and distilled water in a volume ratio of 50:50 and solvent with 96.9 % pharmaceutical ethyl alcohol and distilled water in a volume ratio of 70:30.

The obtained macerates were centrifuged at 6000 rpm for 10 minutes, the supernatant was recovered and re-refuged at 6000 rpm for another 10 minutes using the Hettich EBA 200 laboratory centrifuge. Centrifuged extracts were filtered using the vacuum filtration assembly Rocker model VF6 and stored at −18 °C in glass vials until analysis.

Evaluation of the antioxidant capacity

As Shimamura *et al.* [40] described, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was used to measure the free radical scavenging capacity of the obtained extracts. This assay is widely used to assess antioxidant capacity through the phenomenon of DPPH free radical scavenging by antioxidants in the sample. For this purpose, an 80 μM DPPH solution is prepared in each type of solvent used for maceration. The reaction mixture consisted of extract and fresh DPPH solution in a volume ratio of 1:7. Samples were kept in the dark at room temperature for 30 minutes. For each sample, the absorbance was read at a wavelength of 517 nm after 5 minutes of stabilization under the UV influence of the Ocean Optics HR2000+ UV-VIS spectrophotometer. Based on the absorbance of each experimental variant, half maximal inhibitory concentration (IC_{50}) was calculated. The calibration curve for Trolox was plotted for 6 different concentrations of this reagent, and the DPPH radical scavenging activity of each sample was expressed as *TEAC* calculated by the formula:

$$TEAC = IC_{50}\ \text{Trolox}/IC_{50}\ \text{Probe}\ \ (\mu g/mL) \quad (1)$$

where IC_{50} Trolox is the half maximal inhibitory concentration of Trolox and IC_{50} Probe is the half maximal inhibitory concentration of the obtained extracts.

Estimation of total phenols by the Folin-Ciocâlteu test

A standard method was used to determine the total amount of polyphenols in the obtained extracts [49]. This method called the Folin-Ciocâlteu method involves the reduction of Folin-Ciocâlteu reagent by phenolic compounds with the formation of a blue complex.

Different volumes were taken from each experimental variant and diluted. From each dilution, 1 mL was taken over which 5 mL Folin-Ciocâlteu diluted solution was added, and after about 5 minutes another 4 mL of Na_2CO_3 7.5 % solution. Samples were kept in the dark at room temperature for 60 minutes and then analyzed on spectrophotometer (λ = 765 nm). The *TPC* amount was expressed as mg *GAE*/g of dry plant according to the gallic acid calibration curve. The calibration range of gallic acid was from 10 to 70 μg/mL. All experiments were performed in triplicate.

Statistical analysis

The results of the experimental analyses were expressed as mean values ± standard deviation (SD). For the analysis of differences between mean values One-way analysis of variance (ANOVA) was used. A probability of $p < 0.0001$ was regarded to be highly significant. The statistical analysis was performed using GraphPad Prism 9.0.0 software [48].

Calculation of caffeic acid drug-likeness properties

The drug-likeness property of a compound is given by ”Lipinski's rule of five” [37] which implies that a compound to behave as a drug in terms of pharmacodynamics and pharmacokinetics must have no more than five H-bond donors (HBDs), have no more than 10 hydrogen bond acceptors (HBA), have a molecular weight (*MW*) less than or equal to 500 Da and a LogP (cLogP) less than or equal to 5, and have low or even absent toxicity. In addition, the bioactivity score of this natural compound was identified. All these predictions were obtained using the software Molinspiration property engine v2018.10 [50] based on the natural compound canonical SMILES from the PubChem database [51].

Identification of possible molecular targets of caffeic acid

The online web tool SwissTargetPrediction [53] was used to predict the likely targets of this hydroxycinnamic acid. These are performed by reverse screening based on the similarity principle.

RESULTS AND DISCUSSIONS

INFLUENCE OF EXTRACTION SOLVENTS AND PLANT MATERIAL PARTICLE SIZES ON THE ANTIOXIDANT ACTIVITIES OF *MELISSA OFFICINALIS* L. AERIAL PARTS

According to Sousa *et al*. [43], when present in the body and in large quantities, reactive oxygen species (ROS) cause the body an oxidative stress situation that leads to the production of multiple cellular damages materialized as neurodegenerative or cardiac diseases or even to the production of various types of cancer.

From Fig. 3 it can be seen that the best ability of the lemon balm macerates to behave as hydrogen atom or electron donors for the conversion of the purple free radical DPPH$^{\bullet}$ to its reduced yellow form DPPH-H was for those with the 50:50 volume ratio solvent of pharmaceutical ethyl alcohol and distilled water *TEAC* values ranging from 1.47 μg/mL to 2.31 μg/mL. The results thus obtained are in correlation with those obtained by Dastmalchi *et al.* [13] who used a volume ratio of ethanol to water of 45:55.

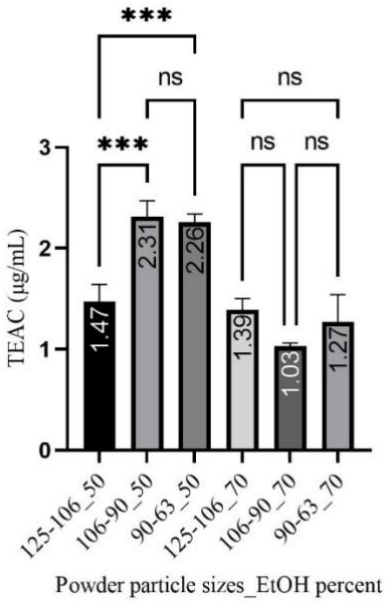


Fig. 3. Statistical difference of Trolox equivalent antioxidant capacity as a function of macerated powder particle sizes range and ratio of pharmaceutical ethanol used. Data are expressed as mean values ±SD of three independent experiments done in triplicate and *p* values were calculated by one-way ANOVA followed by Šídák's multiple comparisons test [48]. ***p = 0.0002; 0.0003; ns p = 0.9993; 0.0894; 0.9350; 0.4121.

In addition, it can also be seen that there is a statistically significant difference for the *TEAC* values between the macerate obtained from the powder with the largest particle size (125 μm–106 μm) with the other two types of macerates obtained with the same solvent with pharmaceutical ethanol and distilled water in 50:50 ratio.

For the extracts obtained with the solvent with alcohol and water in the ratio of 70:30, there are statistically insignificant differences regardless of the particle sizes of the macerated powders, in this case the *TEAC* values vary between 1.03 μg/mL and 1.39 μg/mL.

INFLUENCE OF EXTRACTION SOLVENTS AND PLANT MATERIAL PARTICLE SIZES ON THE TOTAL POLYPHENOLIC CONTENT OF *MELISSA OFFICINALIS* L. AERIAL PARTS

Phenolic compounds are the most common secondary metabolites in plant bodies. They have a number of significant health benefits including cardioprotective [19], antioxidant and neuroprotective [47], anti-diabetic [42], but also antiviral properties [24]. Thus, the recovery of large amounts of polyphenols from small amounts of plant material is currently of particular interest. For this the sample preparation process prior to extraction is particularly important. As can be observed from Table 1, the highest amount of total polyphenols extracted from the aerial parts of *Melissa officinalis* L. species was obtained for the macerate with a volume ratio of pharmaceutical ethyl alcohol and distilled water of 50:50 and for the plant powder with particle sizes ranging from 106 μm to 90 μm, respectively 52.26±0.36 mg *GAE*/g dried plant. Our results are in line with the results obtained by Kasparavičienė *et al*. [25] who observed an increase in the total amount of polyphenols from dry leaves of lemon balm when using the ratio of ethyl alcohol and water of 50:50 and 1:10 plant to solvent ratio.

Table 1

Variation in total polyphenol content as a function of macerated powder particle sizes range and ratio of pharmaceutical ethanol used

Probe code	Particle size range (μm)	Pharmaceutical ethanol:distilled water volume ratio	*TPC* (mg *GAE*/g dried plant) ±SD
125-106_50	125–106	50:50	46.05±0.05
106-90_50	106–90	50:50	52.26±0.36
90-63_50	90–63	50:50	47.79±0.70
125-106_70	125–106	70:30	43.05±0.20
106-90_70	106–90	70:30	37.59±0.09
90-63_70	90–63	70:30	38.21±0.31

Although it is considered that the highest *TPC* values are obtained from extracts of powders from different parts of lemon balm plants with the smallest particle sizes [18, 25], statistical analysis of the results obtained in this study (Fig. 4), reveals a highly significant difference ($p < 0.0001$) of the lemon balm powder macerate with average particle sizes between 106 μm and 90 μm (52.26±0.36 mg *GAE*/g dried plant) compared to both the lemon balm powder macerate with the smallest particle sizes between 90 μm and 63 μm (47.79±0.7 mg *GAE*/g dried plant) and those with the largest particle sizes between 125 μm and 106 μm (46.05±0.05 mg *GAE*/g dried plant).

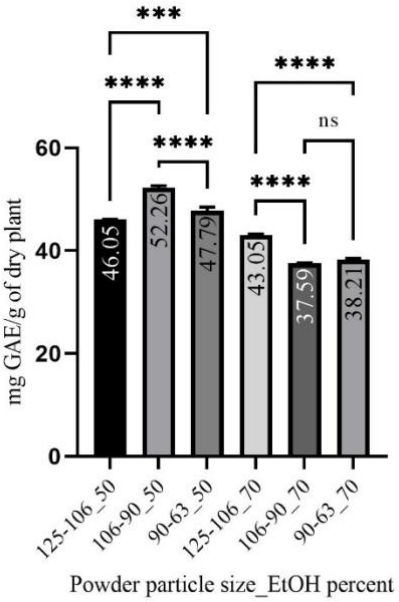


Fig. 4. Statistical difference of total polyphenol content as a function of macerated powder particle sizes range and ratio of pharmaceutical ethanol used. Data are expressed as mean values ±SD of three independent experiments done in triplicate and *p* values were calculated by one-way ANOVA followed by Šídák's multiple comparisons test [48]. ****$p < 0.0001$; ***$p = 0.0004$; ns $p = 0.2881$.

In addition, for the volume ratio of pharmaceutical ethyl alcohol and distilled water of 50:50, a statistically significant difference ($p = 0.007$) is also observed between the *TPC* value of the macerate from the powder with the largest particle size (125 μm – 106 μm) (46.05±0.05 mg *GAE*/g dried plant) and the macerate from the powder with particle size between 90 μm and 63 μm (47.79±0.7 mg *GAE*/g dried plant), in this case the smallest sizes of the macerated particles being favorable.

In the case of the macerate obtained with the solvent with the volume ratio of pharmaceutical ethanol and distilled water of 70:30, a highly significant difference ($p < 0.0001$) was recorded for the *TPC* value of the powder with particle sizes ranging from 125 μm to 106 μm (43.05±0.2 mg *GAE*/g dried plant), compared to both the *TPC* value of the powder with medium particle sizes from 106 μm to 90 μm (37.59±0.09 mg *GAE*/g dried plant) and the *TPC* value of the powder with the smallest particle sizes ranging from 90 μm to 63 μm (38.21±0.31 mg *GAE*/g dried plant). Moreover, it is observed that there is a statistically insignificant relationship ($p = 0.3371$) between the macerate obtained from the powder with particle sizes between 106 μm and 90 μm (37.59±0.09 mg *GAE*/g dried plant) and that obtained

from the powder with particle sizes between 90 μm and 63 μm (38.21±0.31 mg *GAE*/g dried plant).

Thus, although there are a number of studies in the literature claiming that there is an increase in the amount of total polyphenols as the particle size of extracted plant material is reduced [11, 22, 34] our study contradicts this claim.

The explanation for the reduction in the amount of total polyphenols as the particle size of the extracted powder decreases is that during the fine grinding of the plant material heat is released, which leads to oxidation of the polyphenols or even agglomeration of the powder particles, a process that affects their miscibility with the solvent [20, 41].

CORRELATION BETWEEN *TEAC* AND *TPC*

Figure 5 illustrates the correlation between antioxidant activity expressed in μg/mL *TEAC* and *TPC* expressed in mg *GAE*/g dried plant of macerates obtained from the aerial parts of *Melissa officinalis* L. plants.

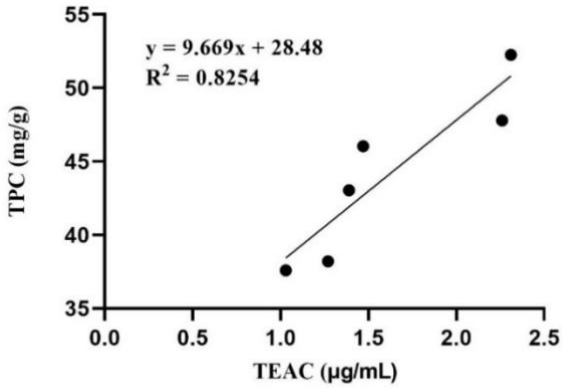


Fig. 5. Correlation between *TEAC* and *TPC* values (p = 0.0122).

It can be seen that there is a significant correlation between DPPH$^{\bullet}$ free radical scavenging activity and the amount of phenolic compounds present in the obtained macerates (R^2 = 0.8254). The results suggest that the antioxidant activity is due to the presence of phenolic compounds in the dried lemon balm plants, making the macerates of particular therapeutic importance [2, 45].

THE DRUG-LIKENESS PROPERTY OF CAFFEIC ACID

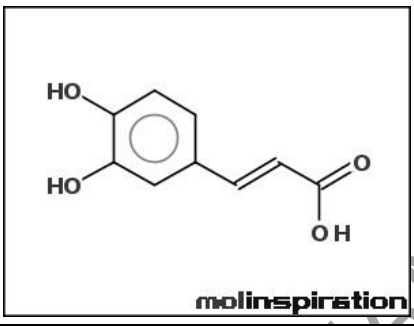
The structural properties of caffeic acid that claim its medicinal properties are presented in Table 2 [50]. The data obtained reveal that caffeic acid possesses a molecular mass of less than 500 Da (*MW* = 180.16 Da) together with an adequate

number of hydrogen bond acceptors (*nON* = 4) as well as an adequate number of hydrogen bond donors (*nOHNH* = 3), properties that infer a good permeability of this compound as a drug [29].

Moreover, the topological polar surface area value (*TPSA* = 77.75 Å^2) indicates a good intestinal absorption of this compound as well as a good *BBB* permeability [15].

Table 2

Structural properties of caffeic acid

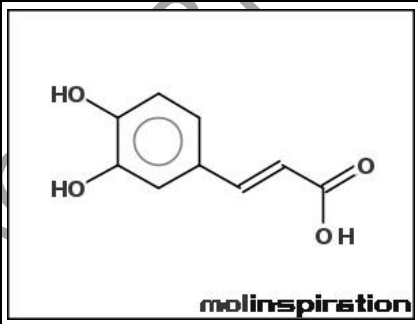


miLogP	0.94
TPSA	77.75 Å^2
natoms	13
MW	180.16
nON	4
nOHNH	3
nviolations	0
nrotb	2
volume	154.50 Å^3

Caffeic acid has also been studied in terms of bioactivity. According to Mishra *et al*. [31] the information illustrated in Table 3 [50] suggests that this hydrocinnamic acid shows moderate bioactivity (bioactivity score is between −0.5 and 0.00).

Table 3

Molinspiration bioactivity score of caffeic acid



G-Protein Coupled Receptors (*GPCR*) ligand	−0.48
Ion channel modulator	−0.23
Kinase inhibitor	−0.81
Nuclear receptor ligand	−0.10
Protease inhibitor	−0.79
Enzyme inhibitor	−0.09

THE POSSIBLE MOLECULAR TARGETS OF CAFFEIC ACID

The ability of natural compounds to interact with large numbers of lipids, proteins or enzymes is extremely important for identifying and understanding the molecular mechanisms of small molecules. Of particular importance for this is the identification of the specific molecular targets of these compounds [16].

Thus, as mentioned above, possible caffeic acid targets were identified using the SwissTargetPrediction tool [53]. For this, 100 molecular targets of caffeic acid were tested. However, values greater than 0 of the binding probability of the test compound to the specific target were identified only for the first 52 targets.

Figure 6 illustrates the top 18 targets whose probabilities of binding with caffeic acid had values between 0.150923844771 and 0.729303187844. These targets were represented by: CA2, ALOX5, CA7, CA1, CA6, MMP9, CA12, MMP1, MMP2, PTPN1, CA14, CA9, CA5B, CA5A, CA3, AKR1B1, ESR2, CA4.

Target	Common name	Uniprot ID	ChEMBL ID	Target Class	Probability*	Known actives (3D/2D)
Carbonic anhydrase II	CA2	P00918	CHEMBL205	Lyase	0.729303187844	33 / 13
Arachidonate 5-lipoxygenase	ALOX5	P09917	CHEMBL215	Oxidoreductase	0.729303187844	1 / 29
Carbonic anhydrase VII	CA7	P43166	CHEMBL2326	Lyase	0.729303187844	11 / 19
Carbonic anhydrase I	CA1	P00915	CHEMBL261	Lyase	0.729303187844	28 / 16
Carbonic anhydrase VI	CA6	P23280	CHEMBL3025	Lyase	0.729303187844	8 / 15
Matrix metalloproteinase 9	MMP9	P14780	CHEMBL321	Protease	0.729303187844	2 / 35
Carbonic anhydrase XII	CA12	O43570	CHEMBL3242	Lyase	0.729303187844	19 / 14
Matrix metalloproteinase 1	MMP1	P03956	CHEMBL332	Protease	0.729303187844	2 / 34
Matrix metalloproteinase 2	MMP2	P08253	CHEMBL333	Protease	0.729303187844	2 / 36
Protein-tyrosine phosphatase 1B	PTPN1	P18031	CHEMBL335	Phosphatase	0.729303187844	18 / 2
Carbonic anhydrase XIV	CA14	Q9ULX7	CHEMBL3510	Lyase	0.729303187844	10 / 18
Carbonic anhydrase IX	CA9	Q16790	CHEMBL3594	Lyase	0.729303187844	15 / 18
Carbonic anhydrase VB	CA5B	Q9Y2D0	CHEMBL3969	Lyase	0.729303187844	5 / 13
Carbonic anhydrase VA	CA5A	P35218	CHEMBL4789	Lyase	0.729303187844	6 / 14
Carbonic anhydrase III	CA3	P07451	CHEMBL2885	Lyase	0.212408695957	4 / 3
Aldose reductase	AKR1B1	P15121	CHEMBL1900	Enzyme	0.150923844771	11 / 35
Estrogen receptor beta	ESR2	Q92731	CHEMBL242	Nuclear receptor	0.150923844771	1 / 10
Carbonic anhydrase IV	CA4	P22748	CHEMBL3729	Lyase	0.150923844771	3 / 8

Fig. 6. SwissTargetPrediction [53] of caffeic acid top 18 targets.

CONCLUSIONS

Particle size directly influences the antioxidant activity of polyphenols from the macerates of *Melissa officinalis* L. aerial parts. The present study indicates that

the highest antioxidant activity correlated with the highest amount of polyphenols is shown by the macerates obtained with a pharmaceutical ethyl alcohol content of 50 % of the powder with average particle size (106–90 μm) (*TEAC* = 2.31 μg/mL) while for the macerates with a 70 % content in pharmaceutical ethyl alcohol, the highest *TEAC* (1.39 μg/mL) was obtained for the powder with particle size between 125 μm and 106 μm.

In silico analysis of caffeic acid revealed that it exhibits good intestinal permeability and *BBB* permeability, but shows moderate bioactivity correlated with the probability of binding to a relatively small number of molecular targets.

However, the present work represents the first stage of research on the therapeutic potential of natural compounds of the plant species *Melissa officinalis* L. Further *in vitro*, *in vivo* and *in silico* studies needs to be carried out to comprehensively evaluate the nutraceutical and therapeutic potential of *Melissa officinalis* L. extracts, as well as to assess the possible side effects of these extracts.

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