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Antioxidant capacity of *Melissa Officinalis* L. on Biological Systems

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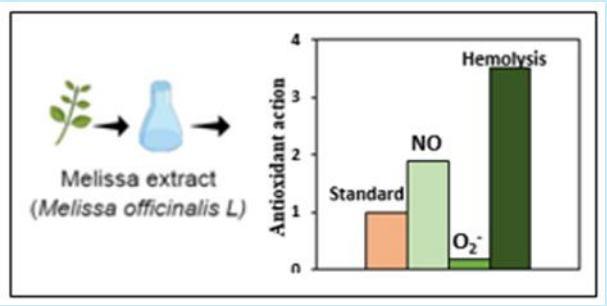
ABSTRACT: The aim of the present study was to evaluate *in vitro* antioxidant capacity of Melissa extract (ME) (*Melissa officinalis* L.) and its protective effect on peroxyl radical-induced oxidative damage in erythrocytes. ME used in the present study was obtained by rotary evaporation of the crude extract (ethanol:water/dried leaves). Total phenolic and flavonoids determined were 177 ± 13 mg GAE/g dried weight (dw) and 26 ± 3 mg QE/g dw, respectively. Total equivalent antioxidant activities, TEAC in mg TE/g dw, were 61 ± 6 and 512 ± 77 respectively for FRAP assay and DPPH• radical-scavenging. The ME acts as an antioxidant on NO and $O_2^{\bullet-}$, whereas ME exerted a higher antioxidant action on NO scavenging compared to the ascorbic acid (1.9 times). However, the antioxidant capacity of ME on $O_2^{\bullet-}$ was 5.6 times lower than ascorbic acid. The values of hemolysis inhibition from ME (IC_{50}, 2.0 ± 0.5 μg mL^{-1}) were higher than ascorbic acid (IC_{50}, 7 ± 2 μg mL^{-1}). Extract of Melissa was able to eliminate biological free radicals, suggesting a potential to prevent oxidative damage *in vivo*. In fact, the ME exerted protective action on cell membrane lysis *in situ*.



1. Introduction

Melissa officinalis L. (lemon balm) is a plant of the *Laminacea* family originated from the Mediterranean, East Asia, Southeast Siberia and North Africa but has adapted throughout the world[1]. Studies show the benefits of *M. officinalis* as antioxidant[2-4] which were used for the symptomatic treatment of gastrointestinal disturbances, adjuvant therapy for pain associated to functional dyspepsia, neurological diseases associated with oxidative stress[5]. The rosmarinic acid is the major compound present in the ethanol extract of *M. officinalis*[3, 6, 7]. Some properties of *M. officinalis* are related to the high levels of polyphenolic compounds as quercetin, caffeic acid and rosmarinic acid with antioxidant properties[3, 8].

Antioxidants are compounds with protective action on deleterious effect of reactive species such as reactive oxygen species (ROS) and reactive nitrogen species (RNS)[9]. Reactive species are atoms or molecules that have high oxidizing power and can be a radical with unpaired electron as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$) and nitric oxide ($NO^{\bullet}$ or NO) or non-radicalar forms as hydrogen peroxide (H_2O_2) or peroxynitrite anion ($ONOO^-$)[9, 10]. ROS can be formed in the cellular environment primarily as result of aerobic metabolism. Nitric oxide, a natural free radical, whose generation is related to normal physiological parameters; but when combined with ROS has potent pro oxidant effects[11].

Exacerbated reactive species in the cellular medium can be due to an unbalanced diet and xenobiotics exposure[12]. For food processing the oxygen level, the presence of transition metals and high temperatures are important to the reactive species generation[11, 12]. Macromolecules oxidation

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by reactive species can cause serious problems in biological systems by ultracellular damage or in food industry by off flavor, changes in nutritional properties and shelf life; so, the use of antioxidants is an important mechanism against reactive species. In recent years the interest for natural antioxidant has intensified for food manufactures and both human and animal diet[13, 14]. Different species of herbs have been investigated by the chemical composition, antioxidant activity and antimicrobial action, among them *M. officinalis*.

Antioxidant activity can be evaluated by radical scavenging using non-biological radicals such as $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) and $ABTS^{\bullet+}$ (2,2'-azino-bis-3-ethylbenzthiazoline)[15]. However, recently, radicals with biological characteristics as $O_2^{\bullet-}$ and NO have been employed to evaluate antioxidant activity[16]. Thermal decomposition of AAPH (2,2'-azobis-2-amidinopropane dihydrochloride) *in vitro* generates peroxyl radical that has been used to verify the antioxidant activity against oxidative erythrocytes damage induced by this radical[17]. Reactive species such as peroxyl radical and others as $O_2^{\bullet-}$ and NO are important for antioxidant assays by simulating a biological process.

The aim of this study was to evaluate the antioxidant activity of extract of *Melissa officinalis* L. on biological systems. For this purpose the aqueous *M. officinalis* extract obtained by ethanol:water (70:30) extraction was used to determinate: *i)* total phenolic by Folin-Ciocalteau method and total flavonoids by aluminum chloride reaction; ii) antioxidant activity on non-biological species as ferric ion reducing (FRAP assay) and $DPPH^{\bullet}$ scavenging; *iii)* antioxidant activity on free radical with biological characteristics, $O_2^{\bullet-}$ and NO scavenging and *iv)* antioxidant activity on hemolytic AAPH-induced.

2. Materials and methods

2.1 Plant and chemicals

Dried leaves of *M. officinalis* were acquired from Florien (Piracicaba, São Paulo, Brazil). All chemicals compounds were obtained from Sigma-Aldrich (St. Louis, EUA).

2.2 Obtainment of Extract

Dried leaves of *Melissa officinalis* were micronized using a micro mill Willye (TE-648, Tecnal) and stored in amber vial. Micronized sample (10 g) was subjected to lipids removal using n-hexane (300 mL) on Soxhlet extractor for 6 h[18]. Subsequently, the bioactive compounds from each sample was extracted with ethanol:water (70:30, v:v) by stirring (170 rpm) (Shaker TE-420, Tecnal), at 25 ºC for 24 h, in the dark. After extraction, the solution was centrifuged for 10 min at 4000 rpm (Centrifuge CT-500, Cientec), filtered Whatman no.1 (11 µM) and the solvent was evaporated under reduced pressure with the temperature not exceeding 50 °C (rotary evaporator SL-126, Solab). The concentrated extract was diluted in ultrapure water, sonicated 3 times for 20 s at 90 Hz (ultrasonic cell disrupter, UNIQUE) and centrifuged at 4000 rpm for 5 min. The supernatant was frozen and freeze-dried (Lyophilizer K-202, Liobras) and finally, the sample was resuspended in ultrapure water and used in all assays. To assay with cells the final extract concentration was adjusted to 10 mM phosphate buffer saline, pH 7.4, (PBS). Dry weight (dw) of aqueous extract was evaluated for results expression. The procedure described above was performed in triplicate (n=3).

2.3. Estimation of total phenolic and total flavonoids

Total phenolics were estimated using Folin-Ciocalteau reagent[19]. In this technique, the extract was pre-incubated with 10% (v/v) Folin-Ciocalteu reagent for 2 min and 7.5% (w/v) sodium carbonate was added. After 1 h at 25 ºC, absorbance at 760 nm was measured in a spectrophotometer (DU-800, Beckman Coulter®). Assays were performed in triplicate and results were expressed as mg of gallic acid equivalents (GAE)/g dw, using a standard curve of gallic acid (0.5 to 6.0 µg mL^{-1}).

Total flavonoids were carried in accordance with the colorimetric method[20]. Assay was performed in ethanol:water 70:30 (v:v) in the presence of 2% (w/v) aluminum chloride and extract. Absorbance at 415 nm was determinate, after 40 min of incubation at 25 ºC in the dark, using a spectrophotometer (DU-800, Beckman Coulter®). Assays were performed in triplicate and results were expressed in mg of quercetin equivalent (QE)/dw, using a standard curve of quercetin (0.6 to 9.6 µg mL^{-1}).

2.4. Ferric reducing antioxidant power (FRAP) assay

The reduction power of extract on ferric ion was described previously[21]. The reaction between extract and FRAP reagent produces a blue compound and absorbance was measured at 593

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nm (DU-800, Beckman Coulter®). FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 10 mM tripiridiltriazine in 40 mM HCl, and 20 mM $FeCl_3$ at ratio 10:1:1 (v:v:v). A standard curve was prepared with Trolox (0.63 to 3.78 µg mL^{-1}). Assays were performed in triplicate and results were expressed as mg trolox equivalente (TE)/g dw.

2.5. Radical DPPH• scavenging

Antioxidant activity of extract on DPPH• radical (2,2-diphenyl-1-picryl-hidrazil) was described above[22]. A methanol solution of DPPH• (65 mM) and extract at different concentrations (2.5 to 16.5 mg mL^{-1}) were used to determine the EC_{50} (concentration required to reduce 50% of DPPH• inhibition). A control assay was performed in the absence of extract to obtain the initial absorbance at zero time. After 3 h of incubation at 25 ºC, the absorbance at 515 nm was determined (DU-800, Beckman Coulter®). The antioxidant activity was assessed by reduction of absorbance value from DPPH• compared to the initial absorbance from control assay. The results were expressed as inhibition percentage of DPPH• in Equation 1. Assays were performed in triplicate and results were expressed as mean ± standard deviation mg TE/g dw, calculated from a standard Trolox curve with concentrations 0.32 to 6.3 mg mL^{-1}. EC_{50} values were expressed in mg mL^{-1} of extract.

$$DPPH^{\bullet} inhibition\ (\%) = [1 - (A_A / A_{C0})] \times 100 \qquad (1)$$

where: A_{C0} = absorbance at 515 nm from control assay at initial time; A_A = absorbance at 515 nm from assay in the presence of extract after 3 h of incubation.

2.6. Superoxide anion ($O_2^{\bullet-}$) and nitric oxide (NO) scavenging

The antioxidant activity on $O_2^{\bullet-}$ was determined according to the methodology proposed previously[8, 23, 24]. The assay used NADH (166 µM), NBT (43 µM), PMS (2.7 µM) and extract at different concentrations (20 to 600 µg mL^{-1}). All reagents were prepared in PBS. After incubation for 2 min at 25 °C, the absorbance was determined at 540 nm on microplate reader (Thermo Scientific Uniscience®). A control assay (without extract) was performed under the same conditions. Inhibition percentage of $O_2^{\bullet-}$ is described in Equation 2. Assays were performed in triplicate and results were expressed as mean ± standard deviation as inhibition percentage compared to control assay. Ascorbic acid, as standard antioxidant was used in similar conditions.

$$O_2^{\bullet-} inhibition\ (\%) = [1 - (A_A / A_{C0})] \times 100 \qquad (2)$$

where: A_{C0} = absorbance at 540 nm from control assay; A_A = absorbance at 540 nm from assay in the presence of extract.

For nitric oxide (NO) assay was used the proposed method[25], with modifications[8, 26]. Assay performed in PBS containing 10 mM sodium nitroprusside and extract (2.5 to 100 µg mL^{-1}) were incubated at 25 °C for 3 h. Control assay (without extract) was performed under the same conditions, when 100% nitrite formation was observed. Each 1 h, aliquot from each assay was transferred to 96-well plate containing sulfanilamide (1%, w/v). After 5 min, N-(1-Naphthyl)ethylenediamine dihydrochloride (0.1%, w/v) has been added, incubated for 5 min and absorbance at 540 nm evaluated using a microplate reader (Thermo Scientific Uniscience®). The concentration of sodium nitrite (µmol L^{-1}) in the assays was calculated from a standard curve of sodium nitrite (1.56 to 100 µmol L^{-1}). The NO inhibition percentage by the extract was determined after 2 h of incubation and was calculated relative to the control assay (Equation 3). Assays were performed in triplicate and results were expressed as mean ± standard deviation. Ascorbic acid, as standard antioxidant was used in similar conditions.

$$Inhibition\ NO\ (\%) = [1 - (A_A / A_{C0})] \times 100 \qquad (3)$$

where: A_{C0} = absorbance at 540 nm from control assay; A_A = absorbance at 540 nm from assay in the presence of extract.

2.7. Protective effect on hemolytic AAPH-induced in human erythrocytes

To evaluate the extract action on hemolysis induced by AAPH was employed the method described above[8, 27, 28]. Human erythrocytes, from venous blood, were obtained from healthy and nonsmokers people using vacutainer tubes containing ethylenediamine tetraacetic acid (EDTA). The cell suspension was prepared according to Gião *et al.*[17] and this suspension was kept at 4 °C and used within 4 h. The volunteers

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signed consent and informed, previously approved by the Local Committee of Ethics in Human Research (number: 493.382).

The erythrocytes suspensions (1%) were incubated for 30 min in the absence (control assay) as well in the presence of the extract (0.25 to 15 mg mL^{-1}) at 37 °C under agitation at 100 rpm (shaker TE - 420 Tecnal®). So, each assay received 5 mmol L^{-1}. AAPH and the incubation proceeded for 6 h. Simultaneous, assays in the absence of AAPH were performed, under same conditions, to evaluate spontaneous hemolysis; the effect of extract alone on cell lyses was also tested. The complete hemolysis (100%) was established by incubating erythrocytes (1%) in water at 37 °C for 10 min. Hemolysis was measured, at each 1 h, by hemoglobin absorbance in the extracellular medium at 540 nm using a microplate reader (Thermo Scientific Uniscience®). Assays were performed in duplicate and the results expressed as percentage of hemolysis according to Equation 4. The percentage of hemolysis inhibition related to the control assay was obtained according to Equation 5. Ascorbic acid, as standard antioxidant was used in similar conditions.

$$Hemolysis\ (\%) = (A_A / A_{100\%}) \times 100 \quad (4)$$

$$Hemolysis\ inhibition\ (\%) = [1 - (A_A / A_C)] \times 100 \quad (5)$$

where: A_A = absorbance at 540 nm from assay in the presence of extract; $A_{100\%}$ = absorbance at 540 nm from complete hemolytic assay (100%); A_C = absorbance at 540 nm from control assay.

2.8 Statistic analysis

The data were evaluated by analysis of variance (ANOVA), t-test and Tukey's test for comparison of means, with a significance level of 5%, using Minitab® software 16.2.2 (2010 Minitab Inc.).

3. Results and discussion

3.1 Total phenolic and flavonoid contents and antioxidant activity against ferric reducing power (FRAP) and free radical scavenging (DPPH•, NO and $O_2^{\bullet-}$)

The present study determined the antioxidant properties of *M. officinalis* and its action on reactive species. Total phenolic and flavonoids content on ME, evaluated by spectrophotometry, were 177 ± 13 mg GAE/g dw and 26 ± 3 mg QE/g dw, respectively. Results expressed by mean and standard deviation (n=3). *Lamiaceae* family, including the *M. officinalis* species, has been studied for polyphenolic presence and total antioxidant properties[2]. Results from Folin-Ciocalteau assays reflect the reducing power of the sample, including total polyphenols and other compounds such as: aromatic amines; ascorbic acid; sugars; organic acids and some inorganic compounds[12]. However, Folin-Ciocalteau method has been used to estimate total phenolic compounds. Folin-Ciocalteau values, in the present study, were similar the ethanolic (98%) *M. officinalis* extract (175 ± 11 mg GAE/g of dry extract) and slightly higher for the content of flavonoid (54 ± 4 mg catechin equivalent/g of dried extract)[4]. However, lower values using ethanolic (80%) *M. officinalis* extract by Folin-Ciocalteau assay (13.2 mg GAE/100 g dw) were found[6]. Another study showed the total polyphenols around 69.49–76.43 mg GAE/g dry plant and total flavonoids 7.0-10.0 mg QE/g dry plant from ethanolic (70%) *M. officinalis* extract, dependent of the harvesting period and hour[7].

Fernandes *et al.*[2] evaluated six families of aromatic herbs and the *Lamiaceae* family showed higher values of total phenolic by Folin-Ciocalteau assay, compared to other families. These authors found 42.86 mg GAE/g dw for ME using acetone:water:acetic acid (70:28:2) as extractor solvent. So, aqueous extracts of *M. officinalis* prepared by decoction were 1245 mg GAE/g dw and by infusion mode were 267 mg GAE/g dw[3]. Rababah *et al*. (2015)[29] studying methanolic extract from leaves of *M. officinalis* found 303.2 mg GAE/100 g fresh plant and 252.9 mg of cathequin equivalent/100 g fresh plant. Thus, several factors may affect the absolute values of total phenolics and flavonoids contents, including genotype, growing conditions, parts tested, time of taking sample and extraction methods. Recently, the content of total flavonoids by spectrophotometric method was evaluated in different plants extracts and *M. officinalis* specie showed high flavonoids content[30].

In the present study, the obtained total antioxidant activity of ME measured by FRAP assay was 61 ± 6 mg TE/g dw, result expressed by mean and standard deviation (n=3). A proportional decrease in the absorbance *versus* the sample concentration was observed (data not show). For DPPH• radical scavenging, 3 h were necessary to reach an absorbance plateau, thus ensured that the

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derivative zero of the absorbance by time (Figure 1A). Non-linear decay behavior of the absorbance *versus* time using some extract concentrations was observed, the stability achieved after 3 h of reaction. Dose-response on DPPH radical scavenging by extract after 3 h of reaction was studied (Figure 1B). High linear correlation coefficient (r^2 = 0.9935) was found for DPPH• scavenging and extract concentration (0.5-17.0 µg mL^{-1}). Similar behavior was presented by Trolox, a standard antioxidant, at concentration of 1.0-6.0 µg mL^{-1} (r^2 = 0.9826) (Figure 1B). IC_{50} values were 9 ± 1 µg mL^{-1} and 5.5 ± 0.2 µg mL^{-1} to extract and Trolox, respectively. These results suggest that the ME has antioxidant activity on non-biological radical DPPH•, but slightly less efficient than Trolox. Total antioxidant activity of ME measured by DPPH• scavenging was 512 ± 77 mg TE/g dw, as mean and standard deviation.

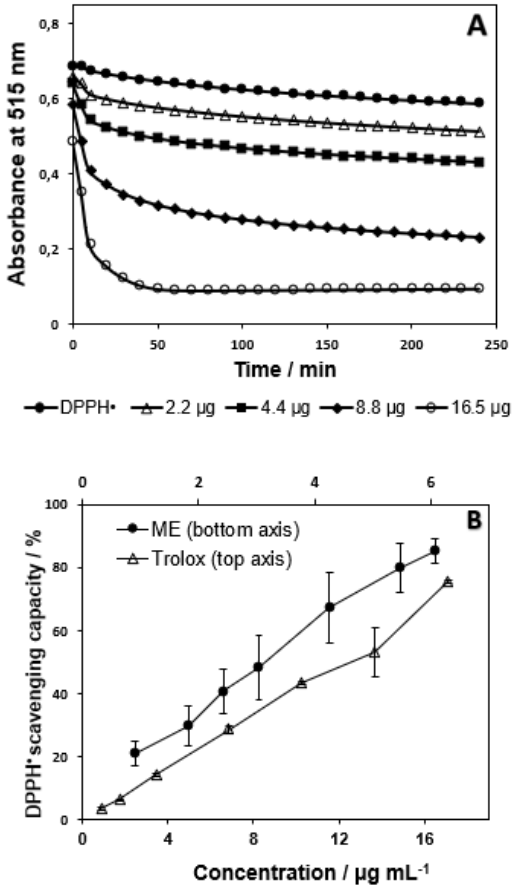

Figure 1. Antioxidant activity of ME on DPPH• scavenging. **(A)** Absorbance *versus* reaction time in absence of extract (only DPPH•) and presence of extract (2.2, 4.4, 8.8, 15.5 µg). **(B)** DPPH• scavenging by ME (0.5 at 17.0 µg mL^{-1}) and by Trolox (1.0 at 6.0 µg mL^{-1}), after 3 h of incubation mean ± standard deviation for assay made in triplicate from 3 extractions (n=3).

FRAP assay is based on the ability of different antioxidants, including phenolics, to reduce Fe^{3+} to Fe^{2+} in the presence of FRAP-reagent, forming a blue chromophore. Results obtained here indicating that ME had effective reducing power and showed similar characteristic when compared to the standard ascorbic acid. Total antioxidant activity of ME measured by FRAP assay was 61 ± 6 TE/g dw, in the present study, contrasting with the results found in previous study using another extractor solvent (464.8 µmol TE/g dw that correspond 117 mg TE/g dw)[2]. In the present study, FRAP assay corroborates the Folin-Ciocalteau values and can indicate high reducing power of the sample, suggesting great antioxidant potential from the ME. Reducing power can be associated with antioxidant activity of plant extract[31]. The reduction power indicates that the antioxidant compounds present in the sample are electron donors and can react with free radicals to stabilize and block chain reactions[1].

Antioxidant compounds can act on free radicals by scavenging mechanisms, which may be attributed to its hydrogen and/or electron donating; thus, they might prevent reactive radical species from reaching biomolecules such as lipoproteins, polyunsaturated fatty acids, DNA, amino acids, proteins and food systems[32]. It is well described that DPPH• radical is a good model for assessing antioxidant activity[22]. Here, *M. officinalis* showed a concentration-dependent DPPH• radical scavenging. The DPPH• radical assay is based on the reduction of DPPH• radical to a non-radical compound by antioxidant agents, e.g. *M. officinalis* extract. In the present study, the extract showed a potent action on the DPPH• scavenging which IC_{50} is similar to other studies of aqueous and methanol extract of *M. officinalis* (18.74 µg mL^{-1} and 13.74 µg mL^{-1}, respectively)[33].

Studies with aqueous ME exhibited IC_{50} to DPPH• scavenging from 1.53-1.62 µg mL^{-1} [7]. Results presented here show values 10× higher than antioxidant activity of ME against DPPH• radical scavenging found previously (5.57 g TE/100 g dw, that correspond 55.7 mg TE/g dw)[2]. There is a large variation in the absolute values for the DPPH• radical scavenging by ME. However, this found suggests that the ME can eliminate free radicals at physiological pH and can be of beneficial interest in preservation of biological systems, where free radical mediates some reactions including lipid oxidation. DPPH• radical-scavenging from ME ethanolic (80%) shows IC_{50}

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equal 48.76 µg mL^{-1}, which is less efficient than ascorbic acid (6.64 µg mL^{-1})[34]. Antioxidant activity of ME on DPPH• radical scavenging was 3.03-6.34 µmol Trolox/mL of water extract, depending on the extraction procedure as temperature and ultrasound bath[35].

To nitric oxide assay, the antioxidant activity of extract was measured by decrease of nitrite (NO_2^-) concentration over reaction time (Figure 2A). Melissa extract activity on NO radical scavenging was estimated by the decreasing of NO_2^- concentration. After 3 h of reaction, NO inhibition by extract in different concentrations (0.5-100 µg mL^{-1}) was studied (Figure 2B). However, a good linear correlation coefficient (r^2 = 0.9895) was found for NO radical scavenging and extract concentration (0.5-17.0 µg mL^{-1}). Similar characteristic was found by ascorbic acid evaluated in a concentration range of 0.5-200 µg mL^{-1} (Figure 2B); and good linear correlation coefficient (r^2 = 0.9901) between this radical scavenging and ascorbic acid concentration (0.5-25 µg mL^{-1}) was observed. So, IC_{50} value for the ME (35 ± 12 µg mL^{-1}) was better than ascorbic acid (68 ± 17 µg mL^{-1}).

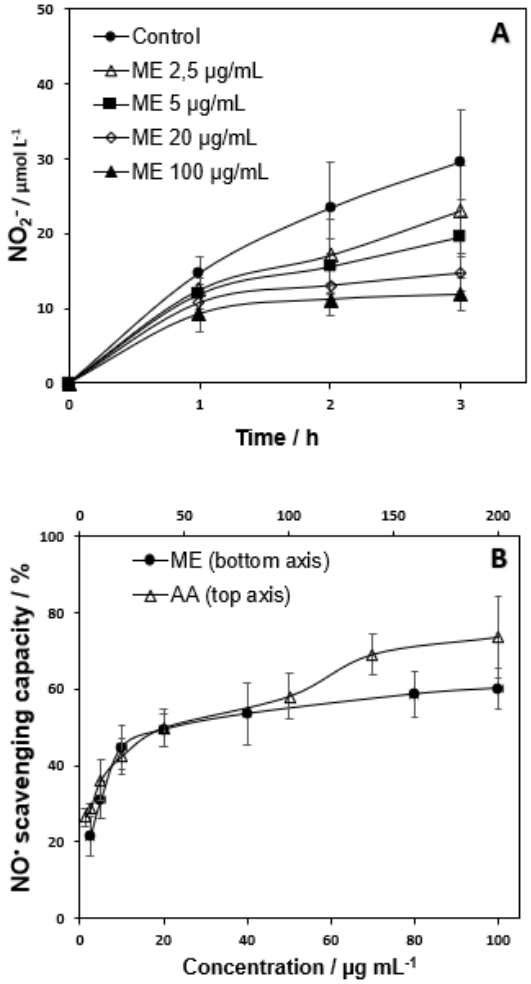

Figure 2. Antioxidant activity of ME on NO scavenging. **(A)** NO_2^- concentration *versus* reaction time in the absence of extract (control) and the presence of extract (2.5 at 100 µg mL^{-1}). **(B)** NO scavenging by ME (2.5 at 100 µg mL^{-1}) and by ascorbic acid (AA, 2.5 at 200 µg mL^{-1}), after 3 h of incubation. Mean ± standard deviation for assay made in triplicate from 3 extractions (n=3).

The NO radical-scavenging by antioxidants is performed by the quantification of nitrite ions (NO_2^-) generated by reaction between NO and molecular oxygen[25]. However, the antioxidant action of ME on NO can be deduced by the low concentration of NO_2^-. Other studies show that plant extracts were effective for NO scavenging, when IC_{50} values from 18 different plant species on NO inhibition were 51-604 µg mL^{-1} [10]. So, IC_{50} for the ME was 35 ± 12 µg/mL in present study.

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Normally, the reaction mechanism to NO radical scavenging by plants extracts involves phenolic compounds[36]. ME has shown various phenolic compounds such as rosmarinic acid and others[10], in fact, these compounds can be related to the NO radical scavenging. The NO is a natural compound produced *in vivo* by variety of cells and is an important bio-regulatory molecule with several physiological functions[11,.37]. However, under oxidative stress this reactive species combines with other reactive species to produce more toxic effects[11]. Therefore, the scavenging effect of ME was assessed against this radical. NO radical scavenging by extract has an interest in human health. It is well known that NO plays an important role in the prevention of various pathologies such as atherosclerosis, ischemia reperfusion, neurodegenerative diseases such as Alzheimer's and Parkinson's disease, cancer and diabetes[11, 37-39].

A relationship between the concentration of ME and $O_2^{\bullet-}$ inhibition was obtained in 0.5-600 μg mL^{-1} range to extract and 0.5-200 μg mL^{-1} to ascorbic acid (Figure 3). However, linear correlation between $O_2^{\bullet-}$ scavenging and ME extract at 0.5-80 μg mL^{-1} (r^2 = 0.9781) was obtained; and for ascorbic acid in the range 0.5-25 μg mL^{-1} (r^2 = 0.9879). Therefore, the effect of extract on $O_2^{\bullet-}$ scavenging was 5.6× lower than for ascorbic acid, observed, respectively, by IC_{50} values, 247 ± 43 μg mL^{-1} and 44 ± 18 μg mL^{-1}.

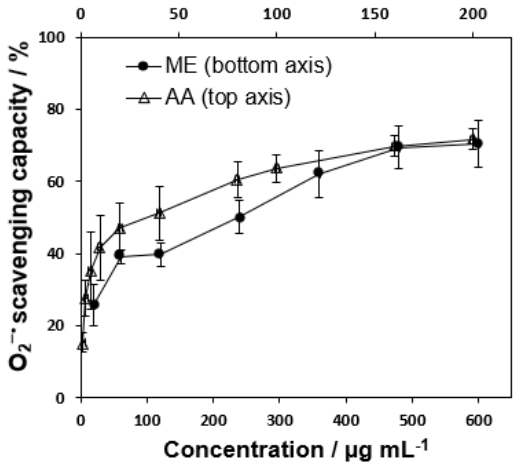

Figure 3. Antioxidant activity on $O_2^{\bullet-}$ scavenging by ME (20 at 600 μg mL^{-1}) and by ascorbic acid (AA, 10 at 200 μg mL^{-1}). Mean ± standard deviation for assay made in triplicate from 3 extractions (n=3).

The $O_2^{\bullet-}$ radical scavenging determination is based on the formazan chromophore formation by reaction between this radical and nitro blue tetrazolium, at physiological pH; antioxidant compound reacts with $O_2^{\bullet-}$ and chromophore formation is inhibited[24]. In the present study, the $O_2^{\bullet-}$ radical scavenging by ME was 5.6× lower than ascorbic acid. Other authors showed IC_{50} variation from 44 to 386 μg mL^{-1} for $O_2^{\bullet-}$ scavenging in aqueous ME from several cultivars[30]; our results are within the reported range (247 ± 43 μg mL^{-1}).

Recent studies reported that $O_2^{\bullet-}$ radical scavenging by plant aqueous extracts can be due to the presence of hydroxyl groups in phenolic compounds[38]. The $O_2^{\bullet-}$ is an important reactive oxygen species *in vivo* and can generate highly toxic species through reactions with other reactive species or by enzymatic reactions or metal catalyzed processes. The $O_2^{\bullet-}$ mediated oxidative stress and it is believed to be involved in pathogenesis disorders such as diabetes mellitus, Alzheimer's and Parkinson's diseases[39].

3.2. Anti-hemolytic action on AAPH-induced lysis

Results from action of ME on hemolysis inhibition are shown in Figure 4. The hemolysis percentage induced by AAPH has a sigmoidal characteristic over time (Figure 4A). When low hemolysis percentages up to 2 h and after 5 h of incubation 30% hemolysis was seen, compared to 100% hemolysis induced by water. However, MEs at 1-10 μg mL^{-1} were antihemolytic on AAPH-induced lysis following a dose-dependent characteristic. No hemolysis by the extract (10 μg mL^{-1}) alone, in the absence of AAPH, was detected. Figure 4B shows dose-dependence of antioxidant concentration and inhibition hemolysis percentage after 4 h of erythrocytes incubation in the presence of extract or ascorbic acid in a concentration range from 0.5 to15 μg mL^{-1}. Linear correlation between hemolysis inhibition and antioxidant concentration (0.5-8 μg mL^{-1}) showed r^2 = 0.9987 and r^2 = 0.7899 for Melissa extract and ascorbic acid, respectively. IC_{50} values indicated that the extract was more efficient in protecting red blood cell lysis, since IC_{50} for extract was 2.0 ± 0.5 μg mL^{-1} and for ascorbic acid 7 ± 2 μg mL^{-1}.

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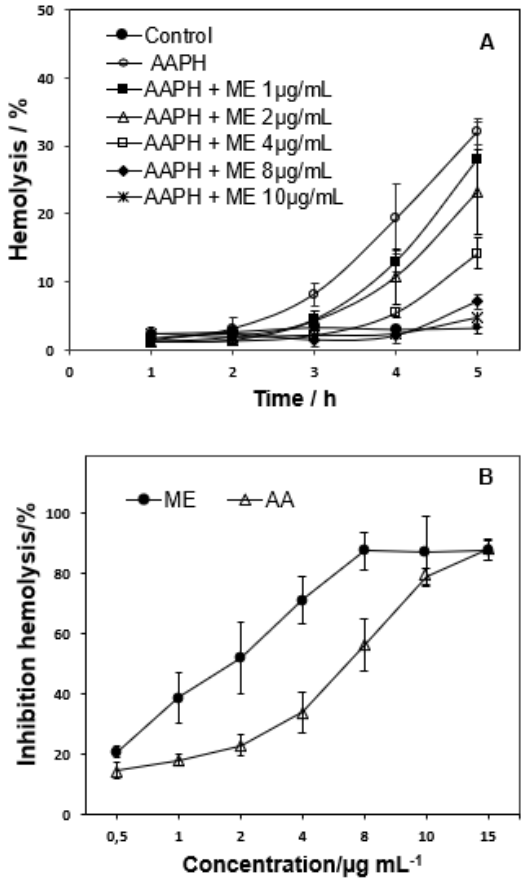

Figure 4. Antihemolytic action of ME. **(A)** Percentage of AAPH-induced hemolysis over time evaluated in absence and presence of ME (1 at 10 µg mL^{-1}), in relation with 100% hemolysis induced by water. A control test (only erythrocytes) in the absence of ME and AAPH was made (control). **(B)** Hemolysis inhibition by ME and ascorbic acid (AA, 0.5 at 15 µg mL^{-1}) after 4 h of incubation, in relation with control assay (absence of antioxidant). Mean ± standard deviation for assay made in triplicate from 3 extractions (n=3).

AAPH decomposition in the presence of oxygen generates peroxyl radical causing oxidative damage in cell membranes. Erythrocytes are very susceptible to oxidative damage by the high content of polyunsaturated fatty acids in their membranes and high cell concentrations of oxygen and hemoglobin[28]. Thus, peroxyl radical, generated by the decomposition of AAPH, attacks cell membranes of erythrocytes induced by lipid peroxidation and protein conformational changes, leading to hemolysis[8, 40]. Similarly, in the present study, it was observed effects of peroxyl radicals initiated by AAPH on human erythrocytes lyses. A lag phase in the progress curve of hemolysis during incubation with AAPH was observed, suggesting a complex mechanism in the oxidative damage process. Our findings indicated that the antihemolytic effect was more efficient by ME than by ascorbic acid. In addition, flavonoid compounds showed protective effect against hemolysis induced by AAPH in erythrocytes of rats[41]. The possible mechanisms of these protective effects of flavonoid-rich fractions may be through their radical scavenging, metal chelating and reducing power activities[41].

4. Conclusions

The antioxidant action found in this study clearly demonstrates that ME is able to eliminate synthetic and naturally occurring free radicals, suggesting its potential to prevent oxidative damage *in vivo* as well as in foodstuffs. In fact, the ME exerted protective action on erythrocytes membrane lysis.

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