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The protective effect of *Aloysia triphylla* aqueous extracts against brain lipid-peroxidation

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In a normal diet, the use of herbs may contribute significantly to the total intake of plant antioxidants and even be a better source of dietary antioxidants than many other food groups. Therefore, the aims of this study were to evaluate the protective effect of aqueous extracts of *Aloysia triphylla* (infusion and decoction) against lipid-peroxidation of brain homogenates and to determine changes in the prooxidant/antioxidant balance when the plant material is added. In order to elucidate a possible antioxidant mechanism *in vitro* evaluation of total antioxidant capacity, oxygen species scavenging ability and reducing power (RP) were studied. Tested extracts had shown a strong inhibition of lipid-peroxidation measured as thiobarbituric acid-reactive products of lipid-peroxidation (TBARS) and chemiluminescence. Furthermore, infusion and decoction exhibited free radical trapping ability, expressed by the capacity to scavenge superoxide and hydrogen peroxide. Additionally, both aqueous extracts presented antioxidant activity measured as total reactive antioxidant potential (TRAP), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid radical (ABTS) scavenging activity and RP. These results suggest that the lipid-peroxidation inhibition mechanism proposed is that the antioxidants present in *Aloysia triphylla* could act as strong scavengers of reactive oxygen species not only at the initiation of the lipid-peroxidation chain reaction, but also at the propagation step. Therefore, they could be used as prophylactic and therapeutic agents for those diseases where the occurrence of oxidative stress and lipid-peroxidation contributes to the progression of damage.

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1. Introduction

Medicinal plants are an important source of natural antioxidants. Many of them have been identified as free radical or active oxygen species scavengers. Within these substances polyphenolic compounds, phytochemicals with a wide distribution in nature, have this antioxidant activity.[1] In a normal diet, the use of herbs may contribute significantly to the total intake of plant antioxidants and even be a better source of dietary antioxidants than many other food groups. Therefore, much attention of preventive medicine research and the food industry is focused on natural antioxidants.[2]

Growing interest in natural antioxidants has developed due to a need for more effective, less toxic and cost effective antioxidants, and medicinal plants appear to have these desired advantages. Moreover, traditional medicine has generated a demand for therapeutic alternatives in Latin American, Caribbean and industrialized countries[3] because of the possibility that the negative impact of several agents on cells may be ameliorated or prevented by improving the dietary intake of natural herbs with antioxidant properties.

Within this context, the Verbenaceae family is composed of 100 kinds and approximately 2000 species of wide geographical distribution and it is characterized by including aromatic species mostly used in the traditional and popular medicine. Among the most important, *Aloysia triphylla* (L'Her), vulgarly known as "cedrón", grows spontaneously in South America and is cultivated in North Africa and Europe. It is commonly prepared as infusion or decoction and used in folk medicine in the treatment of asthma, cold, fever, colic, diarrhea and indigestion, insomnia and anxiety.[4,5] Although there are previous studies about the chemical characterization of this plant[6–8] there is only a little information about the antioxidant capacity, which also depends on the characteristics of the cultivation. Previous results have shown that other members of the Verbenaceae family presented antioxidant activity.[9] However, the radical scavenging ability of these species, including *Aloysia triphylla*, is an important issue to investigate.

Oxidative stress can be defined as an imbalance between the intracellular concentrations of reactive oxygen (ROS) and nitrogen species and the antioxidant defense systems. Evidence of oxidative stress and nitrative processes was found in several pathological disorders in terms of activity of antioxidant

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enzymes, levels of low-molecular weight antioxidants and markers in lipid-peroxidation. This situation is reflected by changes in the antioxidant defenses that could increase, as a protective response, or could be depleted owing to the radical species.[10,11] Cells are equipped with different kinds of defense mechanisms to maintain the redox homeostasis. When the mechanism of antioxidant protection becomes unbalanced in the human body, antioxidant supplements may be used to reduce oxidative stress damage.

It is very interesting to study the role of oxidative stress in several diseases and to evaluate the potential antioxidant protective effect of natural compounds on the affected tissues.

The aims of the present study were to evaluate the protective effect of aqueous extracts of *Aloysia triphylla* (infusion and decoction) against lipid-peroxidation of brain homogenates and to determine the changes in the prooxidant/antioxidant balance that occur in this biological system when the plant material is added. Additionally, *in vitro* evaluation of total antioxidant capacity, radical oxygen species scavenging ability and reducing power assay were studied for the purpose of elucidating a possible antioxidant mechanism of such a protection effect.

2. Results and discussion

Total polyphenolic and flavonoid contents are shown in Table 1. Decoction had a significantly higher amount of polyphenols than infusion ($p < 0.05$), whereas there was no significant difference between decoction and infusion in the flavonoid content.

In order to evaluate *in vitro* antioxidant activity TRAP, ABTS, DPPH and RP assays were performed (Table 2). Values were 9% higher in decoction than infusion for ABTS assay ($p < 0.05$). Moreover, in terms of DPPH assay decoction also displayed a good performance, showing 13% higher values than infusion ($p < 0.01$). In terms of TRAP assay, decoction presented values 23% higher than infusion ($p < 0.05$).

Reducing power ability of tested aqueous extracts steadily increased with increasing sample concentration. Using trolox as positive control, infusion and decoction showed almost similar activities (34.7 ± 0.4 μM trolox per g dry material and 31.4 ± 1.5 μM trolox per g dry material, respectively) and no significant difference between both was observed (Table 2). In all assays, calibration curves with well-known antioxidants (ascorbic acid or trolox) were developed in order to compare them to the aqueous extracts.

Table 2 Total antioxidant activity evaluated as TRAP, ABTS, DPPH and RP[a]

Antioxidant activity assay	Infusion	Decoction
TRAP (μmol trolox per g dry material)	74 ± 4	96 ± 5[b]
ABTS (μmol ascorbic acid per g dry material)	186 ± 10	205 ± 3[b]
DPPH (μmol ascorbic acid per g dry material)	135 ± 9	155 ± 2[c]
RP (μM trolox per g dry material)	34.7 ± 0.4	31.4 ± 1.5

[a] Values are expressed as mean ± standard error of mean. Results are mean values of three determinations. [b] $p < 0.05$. [c] $p < 0.01$.

Aloysia triphylla infusion and decoction exhibited scavenging activity for the superoxide anion radical in a concentration dependent manner, as shown in Table 3, with an IC_{50} of 162.6 ± 18.9 μg mL^{-1} and 170.8 ± 11.3 μg mL^{-1}, respectively. Trolox was used as a positive control. A trolox concentration of 6.7 μg mL^{-1} produced 45% of superoxide anion radical scavenging.

Furthermore, both aqueous extracts scavenged hydrogen peroxide in a concentration dependent manner (Table 3), with an IC_{50} of 136.6 ± 11.1 μg mL^{-1} for infusion and 140.9 ± 2.9 μg mL^{-1} for decoction. Quercetin was used as a positive control and exhibited an IC_{50} of 39.0 μg mL^{-1}.

Table 3 Reactive oxygen radical species scavenging ability[a]

Aqueous extract (μg mL^{-1})	Scavenging (%)	
	$O_2^{\cdot -}$	H_2O_2
Infusion		
10	5.9 ± 1.0	1.0 ± 0.1
100	37.3 ± 4.1	6.8 ± 0.1
250	58.5 ± 3.9	11.1 ± 0.2
Decoction		
10	5.6 ± 0.3	1.1 ± 0.1
100	37.1 ± 1.5	7.6 ± 0.5
250	59.4 ± 1.6	12.7 ± 0.9

[a] Values are expressed as mean ± standard error of mean. Scavenging ability is mean values of three determinations. There is no significant difference between infusion and decoction in both assays.

Table 1 Phytochemical analysis of aqueous extracts of *Aloysia triphylla*[a]

Phytochemical analysis	Infusion	Decoction
Total polyphenolic content (mg gallic acid per g of dry material)	39.33 ± 1.14	48.69 ± 5.27[b]
Total flavonoid content (mg quercetin per g of dry material)	9.74 ± 1.23	10.11 ± 2.18

[a] Values are expressed as mean ± standard error of mean. Results are mean values of three determinations. [b] $p < 0.05$.

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Table 4 Evaluation of antioxidant capacity on biological systems[a]

Aqueous extract ($\mu g\ mL^{-1}$)	Inhibition of lipid-peroxidation (%)	
	CL	TBARS
Infusion		
2.5	35.0 ± 10.3	2.0 ± 0.2
25	84.2 ± 2.3	19.8 ± 1.7
75	95.8 ± 3.1	59.3 ± 5.0
Decoction		
2.5	36.6 ± 1.3	2.7 ± 0.1[b]
25	88.1 ± 0.9	27.4 ± 1.5[b]
75	98.4 ± 0.5	82.2 ± 4.4[b]

[a] Values are expressed as mean ± standard error of mean. The results are mean values of three determinations. There is no significant difference between infusion and decoction in CL assay. [b] $p < 0.05$, CL control value = 61 253 ± 615 cpm mg^{-1} protein, and TBARS control value = 1.03 ± 0.05 nmol mg^{-1} protein.

Brain homogenates were used to determine the protection over lipid-peroxidation. Lipid-peroxidation was quantified by spontaneous brain chemiluminescence (CL) and TBARS. Autoxidation of brain was determined by the CL method in the presence or absence of different volumes of infusion or decoction of *Aloysia triphylla*. Addition of both aqueous extracts to the reaction medium exhibited a decrease in CL compared to the reaction medium without additives. This inhibition was concentration dependent, as shown in Table 4. Calculation of the concentration expressed in $\mu g\ mL^{-1}$ that inhibited 50% of CL (IC_{50}) showed that there was no significant difference between infusion and decoction (5.4 ± 1.9 $\mu g\ mL^{-1}$ for infusion and 4.3 ± 0.3 $\mu g\ mL^{-1}$ for decoction).

TBARS levels decreased with the addition of infusion or decoction in a concentration dependent manner in brain homogenates (Table 4). The concentration required to decrease 50% of TBARS levels in the absence of additives (IC_{50}) was calculated. Decoction produced a higher percentage of inhibition than infusion (IC_{50} = 45.5 ± 4.5 $\mu g\ mL^{-1}$ for decoction and IC_{50} = 63.5 ± 5.5 $\mu g\ mL^{-1}$ for infusion, $p < 0.05$). Quercetin was used as a positive control and exhibited an IC_{50} of 1.5 ± 0.1 $\mu g\ mL^{-1}$.

Oxidation is a central process in the energy management of all living organisms and is, therefore, kept under control by several cellular mechanisms. Oxidative stress is defined as an imbalance between the generation of reactive oxygen species and the endogenous antioxidant system. It is involved in several human diseases and conditions such as cancer, diabetes, inflammatory disorders, as well as aging processes.[12] In recent years, many reports have demonstrated that the oxidation of lipids was a crucial step in the pathogenesis of several human disease stages. Lipid-peroxidation is a process generated naturally in small amounts in the body, mainly by the effect of ROS that readily attack the polyunsaturated fatty acids of the membrane, initiating self-propagation chain reactions. This process, as well as the end-products of such reactions, is especially dangerous for the viability of cells and tissues. There is an efficient antioxidant defense system consisting of enzymatic (catalase, superoxide dismutase, and glutathione peroxidase) and non-enzymatic (vitamins A, C, E and glutathione) antioxidants. If this system fails or is overcome, lipid-peroxidation takes place. This process induces disturbance in structural alteration of integrity, fluidity, permeability and functional loss of biomembranes.[13] Since lipid-peroxidation is a self-propagating chain reaction, the initial oxidation of only a few lipid molecules can result in significant tissue damage.[14,15] ROS generated *in vivo* include hydroxyl radicals ($OH^{\bullet}$), superoxide anion radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), peroxyl radicals ($ROO^{\bullet}$) and singlet oxygen ($^1O_2$). The hydroxyl radical ($OH^{\bullet}$) is the most reactive species that can arise through the Fenton and Haber–Weiss reactions from H_2O_2 and $O_2^{\bullet -}$ in the presence of transition metals like iron or copper.[16–18]

In the present study, brain homogenate was chosen as a biological system for evaluation of lipid-peroxidation due to its high lipid content and its spontaneous autoxidation process that occurs at room temperature or after incubation at 37 °C.[19–21] Taking into account our results we suggest that both infusion and decoction of *Aloysia triphylla* strongly inhibited lipid-peroxidation of the brain homogenate. Although it has been established that there is a correlation between chemiluminescence and lipid-peroxidation,[22] spontaneous chemiluminescence truly provides a quantitative determination of steady state concentrations of excited molecules that give off photons in the process of returning to the ground state. Light emission from homogenates is related to the ROS concentration and gives an idea of prooxidant/antioxidant balance in the sample.[23] Therefore, aqueous extracts of *Aloysia triphylla* decreased the prooxidant steady state concentration in brain homogenates and it appears to have a positive effect on the prooxidant/antioxidant balance, improving antioxidant amounts. This is probably the main cause of lipid-peroxidation inhibition. We confirmed these results testing both aqueous extracts in other systems such as liver homogenates with the addition of *tert*-butyl hydroperoxide (*t*-BOOH) as an initiator of the lipid-peroxidation process.[24,25] The results of these experiments showed the same pattern as that obtained in brain homogenates, since decoction produced a higher percentage of inhibition of the lipid-peroxidation process than infusion, measured as TBARS (IC_{50} = 261 ± 2 $\mu g\ mL^{-1}$ for decoction and IC_{50} = 331 ± 10 $\mu g\ mL^{-1}$ for infusion, $p < 0.05$).

The protective effect of aqueous extracts of *Aloysia triphylla* against lipid-peroxidation could be explained by the following possible mechanism. The infusion and the decoction of *Aloysia triphylla* clearly exhibited $O_2^{\bullet -}$ and H_2O_2 scavenging capacity, compared to well-known antioxidants like trolox (hydrosoluble vitamin E analogue) or quercetin, respectively. The scavenging properties of this medicinal plant diminished the possible generation of $OH^{\bullet}$, and under this condition lipid-peroxidation initiation reaction could be prevented, as now $OH^{\bullet}$ is not available to start the oxidation process.

In addition to the efficiency in free radical scavenging, several factors influence the ability of antioxidants to inhibit lipid-peroxidation, such as antioxidant distribution between aqueous and lipid phases.[26] Thus, inhibition of lipid-oxidation

was probably expectable taking into account that both aqueous extracts exhibited high values in terms of DPPH assay, where the total charge of lipid-soluble antioxidants was evaluated. Vitamin E is the major chain-breaking antioxidant and it is considered as the first line of defense against lipid-peroxidation, protecting the cell membrane at the early stage of free radical attack.[27] Moreover, the charge of lipid-soluble antioxidants measured as DPPH is probably one of the main responsible reasons for the lipid-peroxidation inhibition. Previous reports of our group showed an inverse correlation between TRAP and extent of lipid-peroxidation, where a significant increase in TBARS levels is related to a decrease in TRAP values.[28,29] In this work, TRAP assay reflects the concentration of hydrosoluble antioxidants of low-molecular weight, since decoction values were higher than infusion and it was accompanied by a minor IC_{50} in TBARS assay. It is important to remark that decoction also showed higher values than infusion in DPPH and ABTS assays that contribute to the major inhibition capacity as well. Furthermore, it was found that the higher antioxidant activity of decoction in ABTS assay depends on the quantity of total polyphenols.[30] Consequently, it appears that decoction exhibits a better performance due to the vigorous extraction process of those compounds. Our study suggests that polyphenols present in aqueous extracts of *Aloysia triphylla* might be partially responsible for the antioxidant properties.

Since the antioxidant activity of a substance is usually correlated directly with its reducing capacity, the RP assay provides a reliable method to study the antioxidant activity of various compounds. This method that involves the reduction of Fe^{3+} to Fe^{2+} has been frequently used for rapid evaluation of the total antioxidant capacity of different plant extracts containing flavonoids with a good correlation.[31] In this study, both aqueous extracts had a similar flavonoid content. Therefore, it had been expected that both infusion and decoction showed an analogous performance in this assay. Nevertheless, compounds with reducing power are electron donors and reduce the oxidized intermediates of lipid-peroxidation processes, so they may act as primary and secondary antioxidants.

In recent years, antioxidants have gained a lot of importance because of their potential as prophylactic and therapeutic agents in many diseases. Traditionally, herbal medicines with antioxidant properties have been used for various purposes and epidemiological data also point out the widespread acceptance and use of these agents. Natural antioxidants, which are ubiquitous in fruits, vegetables and medicinal plants, have received great attention and have been studied extensively, since they are effective free radical scavengers and are assumed to be less toxic than synthetic antioxidants.[32] The present study is a step towards the exploration of natural antioxidants from the leaf aqueous extracts of *Aloysia triphylla* evaluating antioxidant capacity on biological systems, particularly, the protection against lipid-peroxidation. This process is important *in vivo* because it contributes to the development of several diseases such as atherosclerosis, diabetes, asthma, neurodegenerative diseases, kidney damage, preeclampsia, and cancer.[33] In addition, our work shows that both aqueous extracts are strongly reactive oxygen scavengers and have potential reducing power. The lipid-peroxidation inhibition mechanism proposed based on *in vitro* assays is that the antioxidants present in aqueous extracts of *Aloysia triphylla* could act as strong scavengers of ROS not only at the initiation of the lipid-peroxidation chain reaction, but also at the propagation steps. The capacity of trapping superoxide and hydrogen peroxide, chemical precursors of $OH^{\bullet}$, and the peroxyl scavenger ability of hydrosoluble and liposoluble antioxidants present in *Aloysia triphylla* support this mechanism. These compounds could act as electron donors that could reduce oxidized intermediates of chain reaction, enhancing the inhibition of lipid-peroxidation.

Further studies would be needed to complete the elucidation of the protective mechanism of the antioxidants present in this medicinal plant. However, these results suggest that both aqueous extracts could be used as prophylactic and therapeutic agents for those diseases where the occurrence of oxidative stress and lipid-peroxidation contributes to the progression of damage.

3. Experimental

3.1. Materials and methods

3.1.1. General experimental procedures. Trolox was purchased from Aldrich Chemicals (Milwaukee, WI, U.S.A.). 2,2′-Azo-bis(2-amidinopropane) was obtained from Acros Organics (New Jersey, U.S.A.) and sodium carbonate was obtained from Mallinckrodt (Hazelwood, MO, USA). All other chemicals were purchased from Sigma Chemicals (St Louis, MO, U.S.A.).

3.1.2. Biological material. *Aloysia triphylla* leaves were provided by PLATARIO S.A, from the Department of Calingasta, Cuyo, collected at Barreal, San Juan, Argentina (31° 40′ 730″ S, 69° 29′ 577″ W) in March 2012. The voucher specimens of the plant were kept in the Herbarium Museum of Pharmacobotany "Juan A. Dominguez", School of Pharmacy and Biochemistry of the University of Buenos Aires (BAF 9019).

3.1.3. Preparation of aqueous extracts. Decoction and infusion (extemporaneous aqueous preparations) were prepared by boiling air-dried aerial parts of the plants ground by mechanical milling in water (5%, w/v) (100 °C) for 20 minutes in the case of decoction, while in the case of infusion, by adding boiling water to the herb material and leaving it to stand for 20 minutes. The extracts were prepared according to the Argentine Pharmacopeia (Codex Medicamentario Argentino, 1979). Both preparations were filtered through filter paper and stored at −20 °C.

3.1.4. Determination of the total polyphenolic content. The total polyphenolic content was determined using the Folin–Ciocalteu procedure.[34] Aliquots (50 μL) of aqueous infusions and decoctions were transferred into test tubes and their volumes were made up to 0.5 mL with distilled water. Folin–Ciocalteu reagent, 0.25 mL, and 20% w/v sodium carbonate solution, 1.25 mL, were added and the tubes were vortexed. After 40 minutes, the absorbance was measured at 725 nm against a blank. A calibration curve was developed using different concentrations of gallic acid as standard solution (0, 6,

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12, 30, 60, and 90 μM). The amount of total polyphenols was expressed as mg of gallic acid per g of dry plant material.

3.1.5. Determination of the total flavonoid content. The amount of total flavonoid content was determined by the aluminium chloride method.[35] The reaction mixture (1.25 mL) comprised of 50 μL of aqueous extract, 450 μL of distilled water, 500 μL of aluminium chloride (1.2% w/v) and 250 μL of potassium acetate (120 mM) is incubated at room temperature for 30 minutes and absorbance was measured at 415 nm. A calibration curve was developed using different concentrations of quercetin as standard solution (0, 11, 22, 33, and 44 μM). The amount of total flavonoids was expressed as mg of quercetin per g of dry plant material.

3.1.6. Total reactive antioxidant potential (TRAP). TRAP was measured by chemiluminescence in a Luminoskan V 1.2-0 liquid scintillation counter. The reaction medium consisted of 20 mM 2,2-azo-bis(2-amidinopropane) (ABAP) and 40 μM luminol. The system is calibrated with different concentrations of trolox (0.25–0.50 μM), a vitamin E analogue. Aliquots of 10 μL of infusion 1/20 and 5 μL of decoction 1/20 were used, which means a total polyphenol amount of 0.98 μg of gallic acid for infusion and 0.64 μg of gallic acid for decoction. A comparison of the induction time after the addition of trolox and the infusion or decoction allows calculation of the total antioxidant capacity as the equivalent of trolox concentration necessary to produce the same induction time.[36,37]

3.1.7. Scavenging of 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid diammonium salt (ABTS) radical cations. The reaction mixture consisted of 0.36 mM 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid diammonium salt b (ABTS) and 18 mM ABAP in 100 mM phosphate buffer pH = 7.40. After 45 minutes of incubation at 45 °C, aliquots of the decoction and infusion were added to 3 mL of the solution. Aliquots of 10 μL of infusion and 5 μL of decoction were used, which means a total polyphenol amount of 19.66 μg of gallic acid for infusion and 12.17 μg of gallic acid for decoction. The absorbance was read at 734 nm at fixed time (3 or 4 minutes), under constant stirring. The calibration curve of ascorbic acid was developed (0, 4.75, 9.50, and 19.00 μM). The results were expressed as μmol ascorbic acid per g dry material.[38]

3.1.8. Scavenging of 2,2-diphenyl-2-picryl hydrazyl (DPPH) radical. The method consists of measuring the consumption of DPPH (stable radical) spectrophotometrically through the decrease in absorbance at 515 nm, measured at fixed time (10 minutes). Aliquots of the decoction or infusion were added to 3 mL of the solution prepared dissolving 2.5 mg of DPPH in 100 mL of methanol. Aliquots of 10 μL of infusion and 5 μL of decoction were used, which means a total polyphenol amount of 19.66 μg of gallic acid for infusion and 12.17 μg of gallic acid for decoction. The calibration curve of ascorbic acid was developed (0, 4.75, 9.50, and 19.00 μM). The results were expressed as μmol ascorbic acid per g dry material.[39]

3.1.9. Superoxide anion radical ($O_2^{\cdot -}$) scavenging assay. The superoxide anion radical was generated in the reaction mixture containing 0.5 mL of nitroblue tetrazolium (0.3 mM), 0.5 mL of NADH (0.936 mM), 1.0 mL of aqueous extract and 0.5 mL of Tris–HCl buffer (16 mM, pH = 8.00). The reaction was started by adding 0.5 mL of phenazine methosulfate solution (0.12 mM) to the mixture, incubated at 25 °C for 5 minutes and then the absorbance was measured at 560 nm against a blank sample. The concentration providing 50% scavenging ability was expressed in μg of dry plant material per mL. Trolox was used as a positive control.[40]

3.1.10. Hydrogen peroxide (H_2O_2) radical scavenging assay. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (50 mM, pH = 7.40). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Aqueous extracts were added to hydrogen peroxide and absorbance was measured at 230 nm after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The concentration providing 50% scavenging ability was expressed in μg of dry plant material per mL. Quercetin was used as a positive control.[41]

3.1.11. Reducing power (RP) assay. A volume of 25 μL of infusion 1/10 or decoction 1/10 was mixed with 175 μL of phosphate buffer (200 mM, pH = 6.60) and 100 μL of potassium ferricyanide (30 mM) and incubated at 50 °C for 20 minutes. Thereafter, 100 μL of trichloroacetic acid (TCA) (600 mM) was added to the reaction mixture and centrifuged for 10 min at 3000 rpm. The upper layer of solution (125 μL) was mixed with 125 μL distilled water and 1000 μL of ferric chloride (6 mM) and absorbance was measured at 700 nm. The calibration curve of trolox was developed (0, 6.25, 12.5, 25.0, and 37.5 μM). The results were expressed as μM trolox per g dry plant material.[42]

3.1.12. Spontaneous chemiluminescence (CL) of brain homogenates. Brains were obtained from female Swiss mice weighing 20–25 g. Mice were maintained in cages in a standardized environment and fed a laboratory diet and water *ad libitum*. Mice were killed by decapitation. Animal treatment was carried out in accordance with the guidelines of the 6344/96 regulation of the Argentinean National Drug, Food and Medical Technology Administration (ANMAT). The essentially blood-free brains were excised and placed in an ice-cold glass. The tissues were homogenized (1 g per 5 mL) in 30 mM potassium phosphate buffer pH 7.40 containing 120 mM KCl and centrifuged at 1500*g* for 10 minutes at 4 °C.

CL of brain homogenates was measured in a Packard liquid scintillation counter in the out-of-coincidence mode, at room temperature.

Spontaneous brain CL was measured in 250 μL of brain homogenates in the presence or absence of different aliquots of infusion 1/20 (0, 5, 20 and 40 μL) or decoction 1/20 (0, 2.5, 10 and 40 μL) in the reaction medium. A final volume of 2 mL was assessed with 100 mM phosphate buffer (pH = 7.40). This allows the calculation of 50% inhibitory concentration (IC_{50}) of chemiluminescence, expressed in μg of dry plant material per mL.[43]

3.1.13. Thiobarbituric acid-reactive products of lipid-peroxidation (TBARS). Brain homogenates were exposed to oxidation by incubation for 1 h at 37 °C. Incubation of 400 μL of brain homogenate was performed in the absence and presence of different aliquots of infusion 1/10 and decoction 1/10 (0, 5, 10 and 25 μL).

Samples of 400 μL of homogenates or buffer (blank) were diluted with 100 μL butylated hydroxytoluene (BHT) and 1 mL of

TCA to precipitate proteins. The precipitate was removed by centrifugation and the supernatant was incubated with 0.67% w/v thiobarbituric acid (TBA) for 1 h at 100 °C. The absorbance was measured at 535 nm.[44] This allows the calculation of 50% inhibitory concentration (IC_{50}) of lipid-peroxidation, expressed in μg of dry plant material per mL. Quercetin was used as a positive control.

3.1.14. Statistical analysis. Statistical calculations were performed (InStat statistical package for Windows; GraphPad, San Diego, CA). Data are expressed as mean ± standard error of mean. The statistical significance of the differences between the groups was calculated by the two-tailed unpaired Student's *t*-test.

4. Conclusions

In summary, this study suggests that the lipid-peroxidation inhibition mechanism proposed is that the antioxidants present in *Aloysia triphylla* could act as strong scavengers of reactive oxygen species not only at the initiation of the lipid-peroxidation chain reaction, but also at the propagation step. Therefore, they could be used as prophylactic and therapeutic agents for those diseases where the occurrence of oxidative stress and lipid-peroxidation contributes to the progression of damage.

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References

1 S. Chanda and R. Dave, *Afr. J. Microbiol. Res.*, 2009, **3**(13), 981–996.
2 E. Zamorano-Ponce, C. Morales, D. Ramos, C. Sepúlveda, S. Cares, P. Rivera, J. Fernández and M. A. Carballo, *Mutat. Res.*, 2006, **603**, 145–150.
3 G. Nigenda, G. Mora-Flores, B. Salvador Aldama-López and E. Orozco-Núñez, *Salud Publica Mex.*, 2001, **43**, 41–51.
4 M. E. Pascual, K. Slowing, E. Carretero, D. Sánchez Mata and A. J. Villar, *Ethnopharmacol.*, 2001, **76**, 201–221.
5 P. C. Santos-Gomes, M. Fernandes-Ferreira and M. A. S. Vicente, *J. Essent. Oil Res.*, 2005, **17**, 73–78.
6 H. Haraguchi, Y. Mochida, S. Sakai, H. Masuda, Y. Tamura, K. Mizutani, O. Tanaka and W. H. Chou, *Biosci., Biotechnol., Biochem.*, 1996, **60**, 945–948.
7 J. R. Soares, T. C. Dinis, A. P. Cunha and L. M. Almeida, *Free Radical Res.*, 1997, **26**, 469–478.
8 P. Valentão, E. Fernándes, F. Carvahlo, P. B. Andrade, R. M. Seabra and M. de Lourdes Basto, *Biol. Pharm. Bull.*, 2002, **25**, 1324–1327.
9 E. Portmann, M. M. Nigro, C. G. Reides, S. Llesuy, R. A. Ricco, M. L. Wagner, A. A. Gurni and M. A. Carballo, *Internet J. Toxicol.*, 2002, **31**, 192–202.
10 S. M. Ferreira, S. F. Lerner, R. Brunzini, P. A. Evelson and S. F. Llesuy, *Eye*, 2009, **23**, 1691–1697.
11 S. M. Ferreira, F. Lerner, C. G. Reides, R. Brunzini and S. F. Llesuy, *Acta. Ophthalmol.*, 2013, **91**, e64–70.
12 M. Z. Gul, L. M. Bhakshu, F. Ahmad, A. K. Kondapi, I. A. Qureshi and I. A. Ghazi, *BMC Complementary Altern. Med.*, 2001, **11:64**.
13 E. Niki, *FEBS Lett.*, 2012, **586**, 3767–3770.
14 G. Barrera, *ISRN Oncol.*, 2012, **137289**.
15 E. Niki, *Biofactors*, 2008, **34**, 171–180.
16 O. I. Aruoma, B. Halliwell, B. M. Hoey and J. Butler., *Free Radical Biol. Med.*, 1989, **6**(6), 593–597.
17 B. Br. Halliwell, *J. Exp. Pathol.*, 1989, **70**, 737–757.
18 B. Halliwell and J. M. C. Gutteridge, *Free radicals in biology and medicine*, Oxford University Press, Oxford, Third edn, 2009.
19 M. Cini, R. G. Fariello, A. Bioachetti and A. Moretti, *Neurochem. Res.*, 1994, **19**, 283.
20 E. L. Regalado, S. Tolle, J. A. Pino, P. Winterhalter, R. Menendez, A. R. Morales and J. L. Rodríguez, *J. Chromatogr. A*, 2011, **1218**, 7358–7364.
21 A. M. Lin, L. Y. Wu, K. C. Hung, H. J. Huang, Y. P. Lei, W. C. Lu and L. S. Hwang, *J. Agric. Food Chem.*, 2012, **60**, 9198–9194.
22 E. A. Lissi, T. Cáceres and L. A. Videla, *J. Free Radicals Biol. Med.*, 1986, **2**, 63–69.
23 S. Llesuy, S. Ferreira and P. Evelson, in *Handbook of Chemiluminescent Methods in Oxidative Stress Assessment*, ed. I. Popov and G. Lewin, Transworld Research Network, Kerala, India, 2008, ch. 3, pp. 29–40.
24 C. G. Fraga, V. S. Martino, G. E. Ferraro, J. D. Coussio and A. Boveris, *Biochem. Pharmacol.*, 1987, **36**, 717–720.
25 K. Yoshino, Y. Hara, M. Sano and I. Tomita, *Biol. Pharm. Bull.*, 1994, **17**, 146–149.
26 B. Halliwell, in *Handbook of antioxidants*, ed. E. Cadenas and P. Lester, Marcel Dekker Inc, New York, second edition, revised and expanded, 2002, ch. 1, pp. 1–45.
27 S. V. Landvik, A. T. Diplock, L. Packer, in *Handbook of antioxidants*, ed. E. Cadenas and P. Lester, Marcel Dekker Inc, New York, second edition, revised and expanded, 2002, ch. 4, pp. 75–97.
28 P. Evelson, C. P. Ordóñez, S. Llesuy and A. Boveris, *J. Photochem. Photobiol., B*, 1997, **38**, 215–219.
29 S. M. Ferreira, S. F. Lerner, R. Brunzini, C. G. Reides, P. A. Evelson and S. F. Llesuy, *Invest. Ophthalmol. Visual Sci.*, 2010, **51**, 4635–4640.
30 A. M. Campos, C. P. Sotomayor, E. Pino and E. Lissi, *Biol. Res.*, 2004, **37**, 287–292.
31 A. Luximon-Ramma, T. Bahorun, M. A. Soobrattee and O. I. Aruoma, *J. Agric. Food Chem.*, 2002, **50**, 5042–5047.
32 D. V. Ratnam, D. D. Ankola, V. Bhardwaj, D. K. Sahana and M. N. Kumar, *J. Controlled Release*, 2006, **113**, 189–207.
33 C. Mylonas and D. Kouretas, *In Vivo*, 1999, **13**, 295–309.
34 S. McDonald, P. D. Prenzler, M. Antolovich and K. Robards, *Food Chem.*, 2001, **73**, 73–84.

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35 C. C. Chang, M. H. Yang, H. M. Wen and J. C. Chern, *J. Food Drug Anal.*, 2002, **10**, 178–182.
36 P. Evelson, M. Travacio, M. Repetto, J. Escobar, S. Llesuy and E. A. Lissi, *Arch. Biochem. Biophys.*, 2001, **388**, 261–266.
37 E. Lissi, C. Pascual and M. D. Del Castillo, *Free Radical Res. Commun.*, 1992, **17**, 299–312.
38 A. Campos and E. Lissi, *Int. J. Chem. Kinet.*, 1997, **29**, 219–224.
39 C. Sánchez-Moreno, J. A. Larrauri and F. Saura-Calixto, *J. Sci. Food Agric.*, 1999, **79**, 1301–1304.
40 J. Robak and R. J. Gryglewski, *Biochem. Pharmacol.*, 1988, **37**, 837–841.
41 R. J. Ruch, S. J. Cheng and J. E. Klaunig, *Carcinogenesis*, 1989, **10**, 1003–1008.
42 E. A. Lissi, T. Cáceres and L. A. Videla, *J. Free Radicals Biol. Med.*, 1986, **2**, 63–69.
43 Y. Athukorala, K. N. Kim and Y. J. Jeon, *Food Chem. Toxicol.*, 2006, **44**, 1065–1074.
44 R. P. Bird and H. H. Draper, *Methods Enzymol.*, 1984, **105**, 299–305.

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