PHYTOCHEMICAL PROFILE, TOXICITY AND ANTIOXIDANT ACTIVITY OF *Aloysia gratissima* (Verbenaceae)

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Aloysia gratissima (Gill. et Hook) Tronc. (Verbenaceae) is native to South America with folk therapeutic applications for a wide range of diseases. The polyphenolic and carotenoid profile, toxicity, and antioxidant activity of aqueous extract of *Aloysia gratissima* were investigated. HPLC analyses showed high amounts of ferulic acid, *trans*-cinnamic acid and *p*-coumaric acid, and also *trans*-β-carotene and lutein which fluctuated throughout the seasons. Furthermore, the extract investigated not only exerted antioxidant activity but also inhibited lipid peroxidation. Toxicity was achieved only at the highest dose tested. Therefore, *A. gratissima* is a potential species for medicinal purposes.

Keywords: *Aloysia gratissima*; Verbenaceae; antioxidant activity.

INTRODUCTION

Aloysia gratissima (Gill. et Hook) Tronc. (Verbenaceae) is an aromatic native plant widely distributed in subtropical regions of South America, particularly Brazil, Paraguay, and Argentina. In Brazil, its folk name is "erva-santa", popularly named by local communities that use its aqueous extract to which they attribute several therapeutic benefits. In traditional medicine, it is thought to be a remedy to alleviate and help treat symptoms associated with headaches, bronchitis, and nervous system disorders[1] including depression[2] and also to treat digestive system disorders.[3]

However, there are no published studies investigating *Aloysia gratissima*'s toxicity while scientific articles about its chemical composition or antioxidant action are scarce, with those available focusing mainly on aqueous extract. Thus, while the chemical composition and some biological activities[4] of *Aloysia gratissima*'s essential oil have been investigated, information on the bioactive compounds belonging to the aqueous extract, to the best of our knowledge, has not yet been described. Furthermore, there is a lack of information about the seasonal phytochemical profile of *Aloysia gratissima*'s aqueous extract.

The methanolic extract of *A. gratissima* has shown the presence of kauranes, flavonoids and phenylethanoids[5] in addition to -bisabolol, triterpenes such as -amirin, the acids betulinic, oleanolic and ursolic, flavonoids such as genkwanin, 5-hydroxy-7,4'-dimethoxyapigenin, 5-hydroxy-7,3',4'-trimethoxyluteolin, and rutin, which exhibited antibacterial, antiedematogenic, and antioxidant properties.[6] Recently, Zeni *et al.*[7] demonstrated that *Aloysia gratissima*'s aqueous extract has both antidepressant-like and neuroprotective effects in mice.

Polyphenols and carotenoids have been studied as factors responsible for treating several cardiovascular and neurodegenerative diseases and cancer.[8] The aim of this study was to determine the polyphenolic and carotenoid composition of the plant throughout the seasons; to select the aqueous extract with the highest level of those compounds, and to investigate the toxicity and antioxidant activity of the aqueous extract containing the highest level of bioactive compounds analyzed.

EXPERIMENTAL

Plant material

The aerial parts of *Aloysia gratissima* (Gill et Hook) Tronc. (Verbenaceae) were collected from the surroundings of the Serra do Itajaí National Park (in Guabiruba county, 27°05'09" S, 48°58'52" W, 60 m altitude - Santa Catarina State, southern Brazil). The harvesting period occurred over the 4 seasons starting in March 2006 (summer), May 2006 (autumn), November 2006 (spring), and August 2007 (winter). The species was identified, taxonomically authenticated and a voucher specimen (N° 2658) deposited at the Regional University of Blumenau's herbarium, Santa Catarina, Brazil.

Sample preparation

The decoction of dried biomass samples from all seasons was extracted for 5 min in boiling water (2.5 g in 150 mL).

The aqueous extract of *A. gratissima* collected from the autumn season (AAE) was chosen, having shown the highest yield obtained, for further determination of antioxidant activity and toxicity. Subsequently, this sample was lyophilized and stored (-18 °C). At the time of the experiments, the lyophilized sample was dissolved in distilled water.

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Quantification of secondary metabolites

Total polyphenol content (TP)

TP was determined as described by Singleton and Rossi[9] by measuring absorbance (725 nm) after 1 h (room temperature, dark place). The quantification analysis was performed through a standard curve of gallic acid. The TP was expressed as gallic acid equivalent (GAE) in mg g^{-1} of dry weight (DW) of the material.

Total flavonoid content (TF)

Samples (0.3 g) were extracted with 10 mL of ethanol (room temperature, dark place) and analyzed as to their TF[10] by mixing samples with 2% AlCl$_3$ and 2.5 mL of ethanol for 1 h, and measuring absorbance at 420 nm. Quantification analysis used a standard curve of quercetin and TF was expressed as quercetin equivalent (QE) in mg g^{-1} of DW of the material.

Total carotenoid content (TC)

Dried samples (0.3 g) were extracted with hexane/acetone solution (1:1, v/v - 30 min, room temperature) containing 100 mg L^{-1} *tert*-butyl hydroxytoluene (BHT) and determined spectrophotometrically.[11] Calculations of the TC were based on the mean absorption coefficient ($A^{1\%}_{1cm}$ 2300, hexane - 450 nm) as previously described.[12] The TC was expressed as β-carotene equivalent (β-caroteneE) in mg g^{-1} of DW of the material.

HPLC analyses

Polyphenols

The dried samples were obtained (1 g, 5 min in 50 mL of boiling water) and then underwent liquid-liquid extraction with ethyl acetate. The determination of polyphenols was performed according to Morais *et al.*[13] The sample (10 µL) was briefly injected into a liquid chromatograph (Shimadzu LC-10A) equipped with a C$_{18}$ reverse-phase column (Shim-pack C$_{18}$, 250 × 4.6 mm ∅ column, 5 µm, 30º C), protected by a 5 µm C$_{18}$ reverse-phase guard column, and a UV-visible detector (280 nm). The samples were eluted in isocratic mode at a flow rate of 0.8 mL min^{-1}, using water:acetic acid:*n*-butanol (350:1:10, v/v/v). The duration of chromatographic analyses was 35 min and phenolic acid identification was performed by comparing retention times and by co-chromatography of standard compounds. The quantification analysis of phenolic acids was based on calibration curves built for each of the compounds identified in the samples. The curves showed good linearity and followed Beer's Law ($r^2 = 0.99$) as follows: gallic acid (y = 18929.62x, r^2 = 0.99), protocatechuic acid (y = 906.12x, r^2 = 0.99), chlorogenic acid (y = 19117.23x, r^2 = 0.99), *p*-hydroxybenzoic acid (y = 14412x, r^2 = 0.99), vanillic acid (y = 15294x, r^2 = 0.99), syringic acid (y = 24428x, r^2 = 0.99), caffeic acid (y = 29970x, r^2 = 0.99), *p*-coumaric acid/*trans*-cinnamic acid (y= 9.781x, r^2 = 0.99), and ferulic acid (y = 22388x, r^2 = 0.99).

Carotenoids

Prior to chromatographic analysis, 1 mL of the solvent extract used for total carotenoid determination was diluted in hexane (1:1, v/v) and added to KOH 10% in methanol (100 µL mL^{-1}) in order to completely saponify carotenoids. This solution was kept for 3 h (dark place, room temperature) and also washed 3 times with distilled-deionized water. The de-esterified extract was collected, concentrated under vacuum, and re-solubilized (hexane, 1 mL) for further chromatographic analysis.[14] A sample (10 µL) was injected into a HPLC device (Shimadzu LC-10A) equipped with a C$_{18}$ reverse-phase column (Vydac 218TP54; 250 × 4.6 mm ∅, 5 µm, 30 ºC), protected by a 5 µm C$_{18}$ reverse-phase guard column (Vydac 218GK54), and a UV-visible detector (450 nm). An isocratic mobile phase of CH$_3$OH:CH$_3$CN (90:10, v/v) at 1 mL min^{-1} with a total time of analyses of 15 min. Identification of the target compounds was performed using retention times, co-chromatography of standard compounds, and other reports on carotenoid composition analyzed by RP-HPLC-UV-visible detection under similar conditions.[15] For quantitative analysis, the calibration curve was obtained by plotting the peak area against different concentrations 0.5-45 µg/mL (y = 7040.96x, r^2 = 0.99) of lutein.

Animals

Adult (2-month-old) male mice *(Swiss albino)*, 5 individuals per group, were maintained at 25 °C on a 12:12 h light/dark cycle, with food and water *ad libitum*. The experiments followed the principles of laboratory animal care (NIH publication No. 85-23, revised 1985) and were approved by the local Ethics Committee for Animal Research (CEUA/UFSC).

Evaluation of acute toxicity

Acute toxicity

The animals received distilled water (control group) or AAE (100, 250, 500, 1000 or 2000 mg kg^{-1}) diluted in distilled water by the oral route (p.o.). After administration, the animals were observed as to any changes in behavior and autonomic activity according to Irwin.[16] This observation took place continuously during the first 24 h and once a day for the proceeding 14 days. Individual weight of animals was determined at the beginning and end of the experiment (14th day), when blood samples for biochemical studies and organs (liver, kidney, spleen, brain, and heart) for necropsy and morphological examinations were collected.

Blood analysis

Hematological analyses were performed including hemoglobin concentration, hematocrit, red blood cells, white blood cells, and platelets counts. The plasma was assayed for glucose, total cholesterol, and protein content, as well as for aspartate transaminase and alanine transaminase activities.

Tissue analysis

The organs were dehydrated with ethanol and submitted to diaphanisation with xylol, embedded in paraffin, sliced and subjected to hematoxylin-eosin staining. When observing lesions in the high dose group, the affected organs were also examined in the low dose group of AAE. Histopathological changes were graded on a severity scale ranging from 0 to 5 (0 = no lesion, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, and 5 =severe). This grading was determined with a scale that considered the average extent of the hepatic *acinus* affected (inflammatory infiltrate, degeneration and necrosis).

Determination of antioxidant capacity

Free radical-scavenger activity

The antioxidant capacity was determined spectrophotometrically (517 nm) by using the radical diphenylpicrylhydrazyl (DPPH).[17] The *A. gratissima* samples for all seasons (16 mg mL^{-1}) were assayed while the pure compounds, BHT and ferulic acid (0.5 mg mL^{-1}) were used as reference compounds. Results were expressed as % discoloration. The AAE was assayed at the concentration range 0.1-1000 mg mL^{-1} and results expressed as the Trolox equivalent antioxidant capacity (TEAC – µmol g^{-1}). The amount of antioxidants required to decrease the initial DPPH concentration by 50% (efficient concentration, EC$_{50}$) was calculated by linear regression.[18]

Evaluation of lipid peroxidation

The animals received distilled water only (control group) or AAE (30, 100, 300, and 1000 mg kg^{-1}, p.o.) diluted in distilled water. The AAE was administrated by gavage 1 h before the analysis. The whole brain (except cerebellum) was briefly dissected and homogenized in 5 volumes (1:5, w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. The homogenates were centrifuged (750 × *g*, 10 min, 4 ℃) to discard nuclei and cell debris. The suspension of mixed and preserved organelles called supernatant was separated and used for the analyses.

Non protein thiol groups (NPSH) were determined by the Ellman method[19] with minor modifications. One volume of homogenate was quickly treated with cooled trichloroacetic acid (20%) and centrifuged (10 x *g*, 2 min). The supernatant was maintained (5 min) with 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) in 1.0 M phosphate buffer, pH 8.0, and absorbances were measured at 412 nm.

Lipid peroxidation was assayed by the thiobarbituric acid reactive substances (TBARS) method.[20] One volume of trichloroacetic acid pre-treated samples was kept with one volume of 0.67% 2-thiobarbituric acid (TBA) prepared in 7.1% sodium sulfate at 100 °C for 30 min. The resulting pink colour was measured at 532 nm and compared to a standard curve of malondialdehyde (MDA). The protein content was quantified by the Bradford method.[21]

Statistical analysis

The results were expressed as mean ± standard deviation (SD) or standard error of the mean (SEM). Analysis of variance (ANOVA; Graph Pad version 3.09) followed by Tukey's test was applied to determine differences between the groups. A value of $P < 0.05$ was considered significant. For histological lesions, scores were statistically compared using the Kruskal-Wallis test, at a 5% significance level ($P < 0.01$).

Climatic data

Data for the geographic region were provided by CIRAM/EPAGRI (Florianópolis – Santa Catarina State) and used for calculations of correlations among phytochemicals and climatic data by using the Excel statistical package (v. 2003 - Table 1S, supplementary material).

RESULTS AND DISCUSSION

The highest yield obtained in the *A. gratissima* was found during autumn (25.71%), decreasing significantly in summer to 15.46% (spring, 20.08% and winter, 20.19%). Significant differences were found on analysis of TP, TF and TC in the samples throughout the seasons (Table 1S, supplementary material). TP and TC were found in higher amounts both in autumn (21.84 and 22.23 mg g^{-1}) and winter (18.93 and 12.94 mg g^{-1}), respectively. By contrast, TF showed higher values in spring and autumn (1.46 and 1.98 mg g^{-1}).

These data were obtained at the average temperature values of 31.7 to 24.5 °C (summer and autumn, respectively - Table 4S, supplementary material). Findings previously have shown that high temperatures and sunlight exposure increase carotenogenesis in fruits although these factors may also promote carotenoid photodegradation. In fact, the carotenoid levels in leafy vegetables cultivated in open fields are significantly lower in summer, suggesting that photodegradation prevails over carotenogenesis.[22] In many plant species, the flavonol content may be enhanced in response to increased irradiance levels.[23] In fact, during the particular year studied, the accumulation of polyphenols, carotenoids and flavonoids detected in AAE were

superior throughout the autumn season even though the temperature of 24.56 °C was not the lowest. In addition, the accumulated rainfall observed was lower (26.3 mm), as was the number of rainy days (6) during autumn, compared to the other seasons (Table 4S, supplementary material).

The HPLC analysis revealed that ferulic acid (11.57-86.40 mg 100 g^{-1}), *trans*-cinnamic acid (7.97-56.92 mg 100 g^{-1}), and *p*-coumaric acid (0.00-16.06 mg 100 g^{-1}), were the most abundant phenolic acids in the samples studied (Table 2S, supplementary material; Figure 1A). In addition, caffeic, chlorogenic, gallic, vanillic, and protocatechuic acids were also found in the samples, all of which are potent antioxidants.[24] Cinnamic acid derivatives are well studied secondary metabolites with useful biological activity, e.g., anti-atherosclerotic agent causing the inhibition of cellular cholesterol storage and transport, inhibition of LDL-oxidation, and HDL particle size rearrangement. Furthermore, *p*-hydroxybenzoic and syringic acids are antimicrobial and hepatoprotectant, respectively.[25]
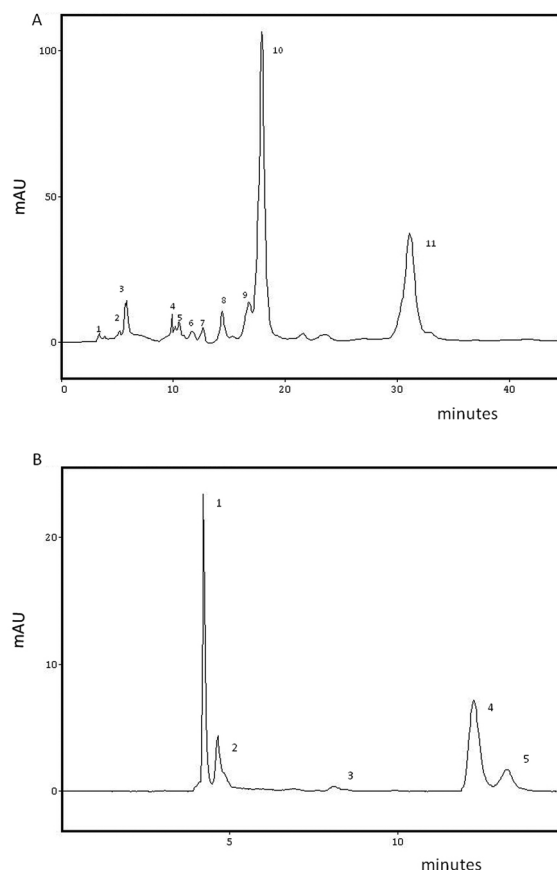

Figure 1. *(A) Polyphenolic HPLC profile of aqueous extract of A. gratissima of autumn (AAE, 1) unknown; (2) gallic acid; (3) protocatechuic acid; (4) chlorogenic acid; (5) p-hydroxybenzoic acid; (6) vanilic acid; (7) syringic acid; (8) caffeic acid; (9) p-coumaric acid; (10) ferulic acid; (11) t-cinnamic acid. Detection at 280 nm. (B) Carotenoidic HPLC profile (450 nm) of aqueous extract of A. gratissima of Autumn (AAE). (1) lutein; (2) zeaxanthin; (3) β-cryptoxanthin; (4) trans-β-carotene; (5) trans-α-carotene*

The aqueous extract of *A. gratissima* showed higher amounts of caffeic, ferulic and *t*-cinnamic acids in winter than in summer, whereas chlorogenic acid was more abundant in the summer season. Calculated correlations depicted chlorogenic acid as being positively influenced by maximum temperature ($r = 0.858$), caffeic ($r = 0.759$) and ferulic ($r = 0.720$) acids by ARH, gallic acid by AR ($r = 0.918$) as well as RD ($r = 0.839$). These results revealed the possibility

that the climatic factors could have had a significant influence on the phenolic acid accumulation in *A. gratissima* tissues. Seasonal changes in phenolic acid content have previously been detected, for example, in *Ginkgo* leaves,[26] indicating that biological effect studies should take into account the influence of seasonality on the metabolic profile of this species.

Lutein (0.30-1.30 mg g^{-1}) and *trans*-β-carotene (0.24-1.86 mg g^{-1}) were the major carotenoid compounds found, while zeaxanthin, β-cryptoxanthin, and *trans*-α-carotene were also detected in lesser amounts in the samples (Table 3S,supplementary material; Figure 1B). Lutein is a xanthophyll which, along with zeaxanthin (paired-pigments), produces a pigment in the macula of the human retina, having an important role in reducing the risk of age-related macular degeneration and cataracts.

Heinonen *et al.*[27] also observed minimum β-carotene and lutein content in tomatoes during summer, in accordance with the findings of the present study. Among the climatic factors studied, there seemed to be no influence on specific carotenoid production, except for ARH which showed an influence on α-carotene ($r = 0.836$) content. Further data are necessary to clarify whether climatic factors influence polyphenol and carotenoid production in this specific plant.

The acute toxicity evaluation of AAE did not show any changes in behavior, mortality, water and food ingestion or weight and revealed no pathological alterations indicating toxic effects (swelling, atrophy or hypertrophy). Moreover, AAE did not produce any detectable changes in the absolute or relative organ weights (data not shown). The hemogram and biochemical parameters evaluated also did not change (Table 5S, supplementary material). Increase or decrease in organ weight, cellular population or plasma constituents are parameters indicating organ toxicity.[28] Therefore, the data found in this study suggest an absence of AAE toxicity and safe use for the analyzed doses.

However, the morphological liver analysis (2000 mg kg^{-1} group) presented a slight increase in the severity score for centrilobular inflammatory infiltrate and degeneration, from Grade 1 to Grade 2 (Figure 2, Panel A-D), and there were no animals with higher severity grades (Figure 2, Panel E).

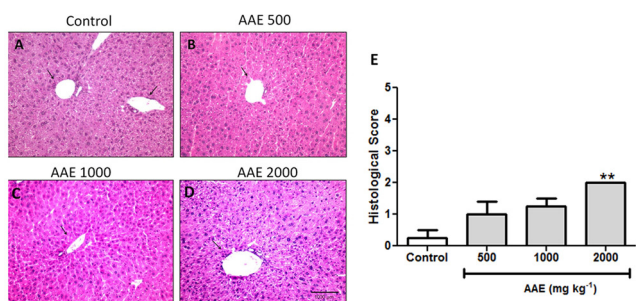Curiously, the antioxidant activity of the aqueous extract of *A.*

Figure 2. *Histopathology and quantification of histological score of livers collected after aqueous extract of A. gratissima of autumn (AAE) administration. Micrographies of liver sections with central veins indicated by arrows. (A) Control liver shows normal morphology. (B, C) grade 1 (minimal) "inflammatory infiltrated" from an animal treated with 500 or 1000 mg kg^{-1} is characterized by slim periportal/perilobular inflammatory infiltrated in the centrilobular region. (D) grade 2 (mild) "inflamatory infiltrated and degeneration" from an animal treated with 2000 mg kg^{-1} shows slight inflammatory infiltrated and centrilobular/periportal degeneration. (E) Treatment with 2000 mg kg^{-1} increased the mean severity score for centrilobular inflammatory infiltrated and degeneration from grade 1 to grade 2. Results are shown as mean ± SEM ** indicates statistically significant differences between control and treatment group (P < 0.01). 400x*

gratissima samples was similar throughout the seasons (Table 1S, supplementary material). The study results are in line with the findings by Horzic *et al.*[29] for chamomile and linden infusions. Furthermore, the AAE showed higher antioxidant activity than BHT, but lower than ferulic acid, its major compound. Ferulic acid is known to be a potent antioxidant,[30] suggesting it is the main compound responsible for the antioxidant activity of AAE.

It has been observed that the antioxidant activity of food extracts depends on both concentration and synergistic effects of phytonutrients.[31] In addition, assays for antioxidant activity measurement might be affected by other components involved in the cell's complex antioxidant systems (e.g., glutathione and enzymatic components).[32] Moreover, the polyphenolic profiles observed in this study have revealed that some phenolic acids which occurred at higher concentrations in one season decreased in another, showing some degree of balance across the seasons.

The overall relationship for the medicinal herb tested was positive and showed a highly significant linear correlation ($r^2 = 0.993$, $p < 0.05$), indicating that phenolic compounds contributed significantly to the AAE antioxidant capacity (Figure 3). In the Verbenaceae family, antioxidant activity has been detected in a large number of species[33] including *A. gratissima*.[6] The correlation between antioxidant activity and AAE ($r^2 = 0.9489$; $p < 0.05$) revealed an EC_{50} value of 1 mg mL^{-1}, similar to the values found for *Matricaria chamomilla* L. but higher than those for *Camellia sinensis*.[34]
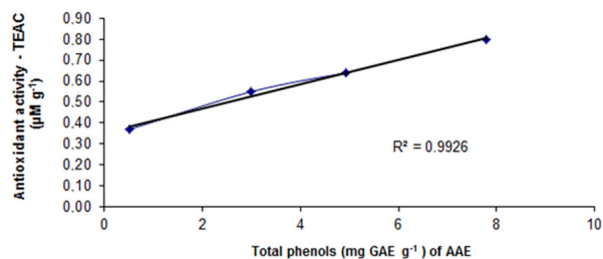
Figure 3. *Relationship between the antioxidant activity (TEAC, μmol g^{-1} DW) assayed by the DPPH test and the total phenolic content (mg GAE g[-1] DW) of aqueous extract of A. gratissima of autumn (AAE)*

In this study, AAE had a significant effect by lowering spontaneous brain lipid peroxidation (Figure 4A), mirroring studies conducted with *Vitex negundo* (Verbenaceae)[35] and *Hypericum perforatum*.[36] Of equal importance, the levels of NPSH were not modified by AAE administration (Figure 4B), leading to the speculation that *A. gratissima* antioxidant activity is directed to cell lipid environments, such as organelle membranes.

CONCLUSIONS

Considering the results showed in the present study, *Aloysia gratissima*'s aqueous extract emerges as an important source of polyphenolic and carotenoid compounds. To the best of our knowledge, this represents the first report of a phytochemical seasonal profile for *Aloysia gratissima* aqueous extract. This profile is possibly influenced by climatic factors that show an increase in bioactive compound production during the autumn season where such compounds appear to be involved in previously reported biological actions beneficial to health.

Moreover, the AAE also presented antioxidant activity *in vitro*, found to correlate with polyphenol content, and was capable of decreasing lipid peroxidation. Also, the acutely and orally administered extract has proven to be harmless, promoting no severe side-effects in mice confirming, at least in part, the folk knowledge on this plant.
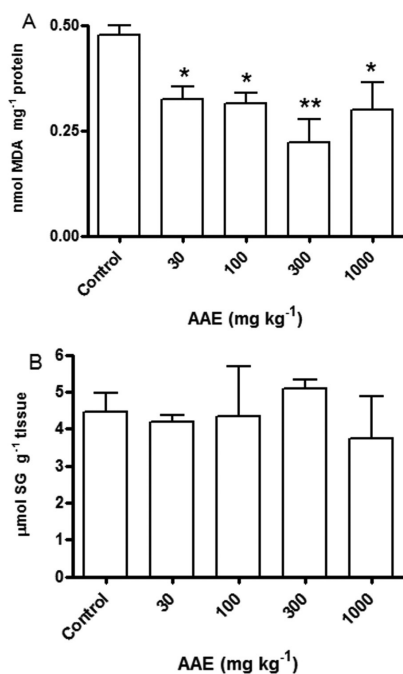
Figure 4. *Effect of aqueous extract of A. gratissima of autumn (AAE) treatment on levels of thiobarbituric acid reactive substances (TBARS) (A) and free thiol content (NPSH) (B) in mouse brain. * P < 0.05 and ** P < 0.01, statistically different from control group. Data were analyzed by ANOVA and Tukey's test, and are expressed as mean ± SD (n = 5 animals per group)*

These results for the chemical, toxicity and antioxidant activity evaluation of AAE should prompt further studies confirming this plant as a safe and effective therapeutic agent and candidate for testing of its biological effects in humans.

SUPPLEMENTARY MATERIAL

Available at http://quimicanova.sbq.org.br, in the form of a PDF file, with free access.

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