Original article

Psychopharmacological and antioxidant effects of hydroethanolic extract of Alpinia zerumbet leaves in mice

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1. Introduction

Epidemiological studies show that millions of people suffer from different types of psychiatric disorders throughout the world, and this number is increasing gradually, mainly in developing countries.\textsuperscript{1} Depression and anxiety are the major psychiatric disorders, which affect 10–15\% of the population.\textsuperscript{2} Despite heavy investments by the pharmaceutical industries in the research and development of new psychotropics, controlled trials have shown that there is little difference in the overall effectiveness of the drugs that are currently in use.\textsuperscript{3,4} Therefore, bioprospecting studies are increasingly directed at the rational pursuit of value-added bio-products, including new drugs. Thus, plants have emerged as potential alternatives for the treatment of psychiatric diseases.

\textit{Alpinia zerumbet} (Pers.) B.L. Burtt & R.M. Sm., Zingiberaceae, is traditionally used in Brazil to treat hypertension, inflammation, anxiety, and hysteria. However, investigations of antioxidant and central effects of \textit{A. zerumbet} extract are lacking.

\textbf{Background:} \textit{Alpinia zerumbet} (Pers.) B.L. Burtt & R.M. Sm., Zingiberaceae, is traditionally used in Brazil to treat hypertension, inflammation, anxiety, and hysteria. However, investigations of antioxidant and central effects of \textit{A. zerumbet} extract are lacking.

\textbf{Objective:} The aim of this study was to verify the effects of a hydroethanolic extract of \textit{A. zerumbet} (HEA) on tail suspension and light/dark tests to screen for possible antidepressant- and anxiolytic-like activities, respectively. We also evaluated the in \textit{vitro} antioxidant effects of HEA.

\textbf{Materials and methods:} Swiss male mice were orally treated with saline or HEA (200, 400 or 800 mg/kg) 60 min before testing. The in \textit{vitro} antioxidant activity of HEA was determined using the ferric-reducing antioxidant property method and assays involving free radical and reactive oxygen species scavenging.

\textbf{Results:} HEA (200, 400 and 800 mg/kg) significantly reduced the period of immobility in the tail suspension test, similarly to imipramine. In the tail suspension test, HEA (400 and 800 mg/kg but not 200 mg/kg) and diazepam significantly increased time spent in the light side. The antioxidant activity of HEA was remarkable, as it showed significant ferric-reduction power, 1,1-diphenyl-2-picrylhydrazyl radical and hydrogen peroxide scavenging activity, and protection against lipid peroxidation.

\textbf{Conclusion:} This study showed the antioxidant, antidepressant- and anxiolytic-like effects of HEA in mice. More studies and the identification of active components of the extract are necessary to further assess the therapeutic potential of this species in the treatment of psychiatric diseases.

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environmental conditions and were maintained in our own animal facility under controlled conditions.

The leaves of *A. zerumbet* were divided into twelve equal quadrants (50 cm² each). The essential oil was extracted using 70% (v/v) EtOH (17 L) by percolation. The extract was divided into two subfractions by evaporation under reduced pressure and further lyophilization.

Drugs and vehicles were injected intraperitoneally (i.p.). Diazepam (UniãoQuímica, Brazil) was purchased from common sources. All drugs were dissolved in saline (0.9% w/v NaCl).

Animals

Experiments were performed using 127 two-month-old male Swiss mice (25–30 g) obtained from the Bioterium Center of Unochapecó. Seven mice were housed per cage (30 x 19 x 13 cm) and were maintained in our own animal facility under controlled environmental conditions (22 ± 1 °C, 12 h LD cycle, free access to food (Nuvilab CR1) and water for at least two weeks before experiments. All procedures were performed in accordance with institutional policies related to the handling of experimental animals (approved by the URI Institutional Ethics Committee, process No. 091/PGA/11).

Plant material

The leaves of *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm., Zingiberaceae, were collected in Chapecó (SC) (26°58′36.06″S, 52°44′27.18″W) and were taxonomically identified by Osmar dos Santos Ribas (Curador do Herbário do Museu Botânico Municipal de Curitiba), with whom a voucher was deposited (MBM #306196).

Preparation of the extract

The leaves of *A. zerumbet* were dried at room temperature and powered (425 μm: 35 Tyler/Mesh). Powered leaves (500 g) were extracted using 70% (v/v) EtOH (17 L) by percolation, before evaporation under reduced pressure and further lyophilization (38.2% yield).

Drugs

Diazepam (UniãoQuímica, Brazil) was purchased from common commercial suppliers. Imipramine was obtained from Sigma–Aldrich (USA). All drugs were dissolved in saline (0.9% w/v NaCl). Drugs and vehicles were injected intraperitoneally (i.p.) or orally (p.o.) using a constant volume of 0.1 ml/10 g body weight.

Locomotion

Locomotion was measured using an open field apparatus that was divided into twelve equal quadrants (50 x 50 cm), facing away from the door and the following parameters: number of crossings, percentage in each quadrant, time spent in the center, time spent in the dark compartment, and the number of crossings between the light and dark compartments. The test was performed in a quiet darkened room. The mice were kept in this room for at least 1 h before the test. Groups (n = 9–11) were treated orally with the vehicle (0.9% w/v saline), diazepam (1.0 mg/kg) or HEA (200, 400 or 800 mg/kg) 60 min before testing.

Determination of the total phenolic content

Quanti
de Ciaolteu in an alkaline medium, was used to determine the total phenolic content according to the Folin–Ciocalteu method. 17,18 Briefly, 100 μL of extract HEA solution was added to 1 ml of 10-fold-diluted Folin–Ciocalteu reagent. After agitation, the mixture was kept at room temperature for 5 min. Next, 1 ml of 10% (w/v) Na2CO3 was added, and the mixture was incubated in the dark for 90 min. The absorbance of the blue product, which indicated the capacity of phenolic compounds to reduce Folin–Ciocalteu in an alkaline medium, was measured at 725 nm against a blank sample. The total phenolic content was expressed as gallic acid equivalents, i.e. mg GAE/g dry weight (d.w.), and the values were calculated as the means of triplicate experiments.

Determination of the total flavonoid content

In spectrophotometric quantification of flavonoid compounds HEA (5 ml) was added to 2.5 ml of a mixture containing 5 mM AlCl3 and 48 mM sodium acetate. After 30 min of incubation, the absorbance was measured at 425 nm and compared with a blank. A standard curve was prepared using quercetin and the results were expressed as quercetin equivalents, i.e. mg QE/g dry weight (d.w.), and calculated as the means of triplicate analyses.
reaction mixture. After incubating at 37°C for 30 min, the absorbance of the Fe²⁺-TPTZ complex was recorded at 593 nm. Controls containing the extract without TPTZ were also evaluated to determine the possible absorbance of natural compounds in the extract at the wavelength used to measure the absorbance of Fe²⁺-TPTZ. The absorbance of the extract at 593 nm in the absence of TPTZ was subtracted from that obtained using the extract plus the TPTZ mixture. The increase in the absorbance due to the Fe²⁺-TPTZ complex formation induced by HEA was compared to that induced by ascorbic acid (standard), and the results were expressed as the mean absorbance of triplicate experiments (n = 3).

2.11. 1,1-Diphenyl-2-picrylhydrazyl radical scavenging assay

A stable solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used to determine the total antioxidant capacity of HEA. DPPH solution (0.24 mg/ml) was diluted with methanol until an absorbance of 1.10 ± 0.02 at 517 nm was obtained. The extract was diluted with methanol to produce different concentrations (5–250 μg/ml) and 50 μl of each dilution was mixed with 1.95 ml of methanolic DPPH solution. The antiradical power of the different concentrations of extract and standard were determined by measuring the decrease in the DPPH absorbance after 24 h in the dark versus a blank. The addition of the antioxidant resulted in a relative decrease in the absorbance that was proportional to the antioxidant activity of the extract. The same procedure was followed for ascorbic acid to compare the antiradical capacity of HEA to that of ascorbic acid in vitro. This analysis was performed in triplicate (n = 3), and the results were expressed as mean % inhibition of the DPPH radical, which was calculated as follows: % inhibition = ([Abscontrol − Abssample]/Abscontrol) × 100. The concentration of HEA that could scavenge half of the DPPH radical (IC₅₀) was calculated by nonlinear regression analysis using GraphPad Prism version 4.0 (La Jolla CA, USA).

2.12. Evaluation of the hydrogen peroxide scavenging capacity

The ability of HEA to scavenge hydrogen peroxide (H₂O₂) was assessed by measuring the decrease in the H₂O₂ absorbance at 240 nm in a medium used to assess catalase (CAT) antiradical activity that contained 50 mM phosphate buffer (pH 7.0) and 17 mM H₂O₂. The H₂O₂ scavenging ability of ascorbic acid was also evaluated to compare its H₂O₂ scavenging ability with that of HEA. The results were calculated as the mean of triplicate experiments (n = 3) and expressed as the CAT-like activity (k/mg d.w.).

2.13. Evaluation of the superoxide anion radical scavenging capacity

The ability of HEA to inhibit the auto-oxidation of epinephrine to adrenochrome, which is mediated by superoxide anions (O₂⁻), was evaluated at 480 nm. The reaction assay contained 50 mM glycine buffer (pH 10.2) and 1 mM epinephrine at 30°C. This assay was performed in triplicate (n = 3) and the O₂⁻ scavenging ability of ascorbic acid solution (20 μM) was evaluated for comparison.

2.14. Protection against glutathione oxidation

The capacity of HEA to prevent GSH oxidation was assessed in the absence or presence of H₂O₂ by measuring the disappearance of the reduced sulphydryl groups of GSH. The reduced sulphydryl groups were quantified, 30 min after GSH addition to a reaction mixture containing 200 mM potassium phosphate buffer (pH 6.4), HEA (5–250 μg/ml), and H₂O₂ (0 or 0.5 mM) at 39°C. Controls containing extract lacking GSH were run to verify the possible absorbance of the extract at the wavelength used to assess GSH oxidation (412 nm). A low absorbance was detected in the extract, which was subtracted from that obtained with the extract plus GSH. The same procedure was adopted for ascorbic acid for comparison. This assay was assessed in triplicate (n = 3), and the results were expressed as the % of remaining sulphydryl groups of GSH.

2.15. Protection against lipid peroxidation

Lipid peroxidation was performed using brain homogenates from mice. Animals were decapitated; the forebrain was immediately dissected and then homogenized in 50 mM Tris–HCl (pH 7.5; 1:9, w/v). The homogenate was centrifuged for 20 min at 2000 × g to yield a low-speed supernatant that was used for determination of lipid peroxidation. This supernatant (350 μl) was pre-incubated at 37°C for 1 h in the presence or absence of 50 μM FeCl₂, 1 mM H₂O₂ and HEA (0–250 μg/dl) in a final volume of 500 μl, which was made up using 50 mM Tris–HCl. After pre-incubation, the level of thiobarbituric acid-reactive substances (TBARS) was determined.

A decrease in the formation of the pink chromogen in tubes pre-incubated with HEA was considered to be an indicator of the inhibition of lipid peroxidation, which was calculated as stated above (DPPH radical scavenging assay). The IC₅₀ value, which represents the concentration of HEA that was necessary to inhibit 50% of lipid peroxidation, was determined by non-linear regression analysis using GraphPad Prism version 4.0 (La Jolla CA, USA).

2.16. Statistical analysis

The results (cumulative spontaneous locomotion counts, time(s) of immobility and in vitro antioxidant activity) were expressed as the mean ± S.E.M. Comparisons between groups were performed using ANOVA followed by Tukey’s test, using SPSS 20.0. Differences between data were analyzed using the Student’s t-test to determine the in vitro antioxidant activity (different concentrations of HEA × different concentrations of ascorbic acid). For the lipid peroxidation assay, comparisons using different concentrations of HEA were analyzed by one-way ANOVA followed by Tukey’s test using SPSS 20.0. P < 0.05 was considered significant.

3. Results

Fig. 1 shows the effects of HEA on the tail suspension test. HEA significantly reduced immobility time in the TST (F₁,₄₇ = 15.0, p < 0.0001). Imipramine was not more active than HEA in this test. Spontaneous locomotion of groups treated with HEA did not differ from controls (F₁,₂₄ = 0.54, data not shown).

Fig. 2 shows the effects of HEA on light/dark test. HEA (200, 400 and 800 mg/kg) and diazepam (10 mg/kg) significantly increased the latency for the first crossing from the light to the dark compartment (F₄,₄₅ = 7.3, p < 0.0001). The number of crossings between compartments was significantly increased by
HEA (400 and 800 mg/kg) and diazepam (1.0 mg/kg) in the light/dark test \( (F_{(4,45)} = 8.7, p < 0.0001, \text{Fig. 2B}) \). Like diazepam (1.0 mg/kg), HEA (400 and 800 mg/kg) significantly increased the time spent in the light side of the apparatus \( (F_{(4,45)} = 6.8, p < 0.0002, \text{Fig. 2C}) \).

The total phenolic content expressed as gallic equivalent (GAE) was found to be around 80.29 \( \mu \text{g} \text{GAE/g d.w.} \), while flavonoid content was recorded in quercetol equivalents (QE) and was around 4.85 \( \mu \text{g} \text{QE/g d.w.} \).

Fig. 3 shows the results of the FRAP assay. One-way ANOVA showed that HEA and ascorbic acid had significant reducing power at a concentration of \( >5 \mu \text{g/ml} \). However, the ferric-reducing power of ascorbic acid was significantly higher than that of HEA for all concentrations evaluated, according to the Student’s \( t \)-test.

Fig. 4 shows DPPH radical scavenging antioxidant activity. The DPPH radical scavenging ability of HEA was lower than that of ascorbic acid, but it was high in the evaluated concentration range. For 5 and 10 \( \mu \text{g/ml} \), the inhibition of DPPH by HEA was 27% and 46%, respectively, and it produced >80% of inhibition for all concentrations between 20 and 250 \( \mu \text{g/ml} \). The IC\(_{50}\) value for DPPH radical scavenging activity of HEA was 9.86 \( \mu \text{g/ml} \) (confidence interval = 5.71–17.02), whereas for ascorbic acid it was 4.94 \( \mu \text{g/ml} \) (confidence interval = 3.87–6.32).

HEA also had \( \text{H}_2\text{O}_2 \) scavenging activity (Fig. 5). However, this property was less evident compared with its reducing potential and DPPH radical scavenging activity (Figs. 3 and 4, respectively), i.e., it was evident at concentrations of 50–150 \( \mu \text{g/ml} \) HEA (Fig. 5) but not at higher concentrations (data not shown). HEA failed to prevent the \( \text{H}_2\text{O}_2 \)-induced oxidation of the thiol groups of GSH and it also had no superoxide radical \( \text{O}_2^- \) scavenging activity (data not shown). Ascorbic acid is not shown in Fig. 5 because it also did not present protective effect against \( \text{GSH} \) oxidation.

Fig. 6 shows the protective effect of HEA during the Fenton reaction-induced lipid peroxidation of homogenates of mice brains. The extract inhibited lipid peroxidation at all concentrations, where the inhibition rate was 46–79% at concentrations of 5–250 \( \mu \text{g/ml} \), while the IC\(_{50}\) value for HEA was 15.7 \( \mu \text{g/ml} \) (confidence interval = 9.3–26.4).

4. Discussion

Preclinical evaluation is essential for studies involving new potential drugs. In this context, this study evaluated the effects of \textit{A. zerumbet} on the central nervous system in animal models. Our results showed that HEA (200, 400 and 800 mg/kg) had an antidepressant-like activity in TST. Furthermore, this effect was comparable to the tricyclic antidepressant imipramine. In addition to this antidepressant-like activity, HEA (400 and 800 mg/kg) had an anxiolytic-like profile in the LD test that was comparable to diazepam.

Phenolic compounds are found mainly in fruits, herbs, and vegetables, and their antioxidant properties are recognized in addition to their many other biological effects.\(^{26,27}\) Flavonoids, such as flavonols and flavanes, are a major class of phenolic compounds found in plants.\(^{27}\) In this study, the total phenolic and flavonoid contents were quantified in HEA, which could be related to the antioxidant properties of this extract.

The antioxidant activity of a substance is correlated to its reducing properties, so FRAP is a reliable method for assessing the
antioxidant activity of several compounds, including polyphenols.\textsuperscript{20} The FRAP assay measures the ability of an antioxidant substance to reduce the 2,4,6-tripyrindyl-s-triazine-Fe(III) complex to 2,4,6-tripyrindyl-s-triazine-Fe(II) complex by donating an electron.\textsuperscript{20} HEA had a remarkable reducing activity with the 2,4,6-tripyrindyl-s-triazine-Fe(III) complex, which indicated the presence of compounds that could donate electrons. The reducing activity could also be attributed to compounds other than phenolics in the present study, however the activity of leaf and seed extracts of \textit{Abelmoschus moschatus} Medik., was strongly suggested to be attributable to polyphenol compounds.\textsuperscript{28} Indeed, phenolic compounds are widely recognized as important reducing agents.\textsuperscript{29}

The reducing power of HEA was supported by the DPPH radical scavenging assay, which also evaluated the ability of antioxidants to transfer a single electron. DPPH scavenging was detected at all concentrations (5–250 μg/ml) evaluated. These results strongly suggest that the DPPH radical scavenging capacity of HEA was related to its reducing properties in the FRAP assay. The DPPH scavenging capacity of the extract was lower than that of ascorbic acid at all concentrations evaluated. However, the antioxidant potential of HEA was similarly high because ascorbic acid inhibited 90–92% of DPPH oxidation at 10 μg/ml, whereas HEA inhibited 81–87% at concentrations of 20–250 μg/ml.

HEA was also capable of removing H2O2, which is an ROS that is produced continuously during cellular oxidative metabolism.\textsuperscript{30} H2O2 scavenging occurred at 50–150 μg/ml of HEA and it was lower than its reducing power and DPPH scavenging activity, which were observed from 5 μg/ml. The H2O2 scavenging capacity indicates indirect protection against −OH radical formation, which is produced by Fenton-type reactions involving H2O2 and transition metals.\textsuperscript{20}

Extracts of other plants have H2O2 scavenging capacity that may be attributed to phenolic compounds, which can transfer electrons to H2O2 and reduce it to water.\textsuperscript{28,31,32} However, the weak H2O2 scavenging ability of HEA appeared to be insufficient to inhibit the H2O2-induced oxidation of GSH, because none of the extract concentrations tested produced this effect. The lack of H2O2 scavenging with ascorbic acid may be explained by H2O2-induced ascorbic acid oxidation in the reaction mixture used in these assays, as previously demonstrated.\textsuperscript{33}

The inhibition of lipid peroxidation is considered to be an important index of antioxidant activity because membrane lipid peroxidation is an endpoint of biological damage that occurs in several diseases, including neurodegenerative disorders.\textsuperscript{34} However, antioxidants may protect from lipid peroxidation by scavenging...
free radicals, thereby preventing diseases.\textsuperscript{28,35} Therefore, the inhibition of lipid peroxidation is recognized as an important index of antioxidant activity. Interestingly, the present study showed that HEA inhibited lipid peroxidation and provided a good degree of protection against oxidative damage to lipids. This protective effect may be related to its capacity for scavenging the OH radical, which is produced by the Fenton reaction between iron and H\textsubscript{2}O\textsubscript{2}.\textsuperscript{36} When present in excess, the OH radical may initiate a lipid peroxidation chain that terminates in brain injury.\textsuperscript{37}

Oxidative damage to lipids has recently been detected in anxiety- and depression-like rat models\textsuperscript{38} and in patients with major depressive disorders.\textsuperscript{39} The inhibition of lipid peroxidation by HEA and its ability to scavenge free radicals might be a link between antioxidant activity and its central effects in the present study using mice. Further in vivo studies should be performed to corroborate this hypothesis.

In summary, this study demonstrated the in vitro antioxidant, antidepressive, and anxiolytic effects in mice of HEA. The in vitro antioxidant activity may be attributable to its radical scavenging ability and protection against lipid peroxidation. The central nervous system activities of HEA might be in part due to antioxidants and flavonoids detected in the plant.

Conflicts of interest

All authors have none to declare.

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