Involvement of TRPV1 receptors on protective effect of *Acmella ciliata* extract and spilanthol in streptozotocin-induced sporadic Alzheimer's disease model

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ABSTRACT

*Acmella ciliata* (AC) is a plant widely used in the cuisine of the North and Northeast of Brazil and this study aims to investigate the effect of AC extract on memory impairment and oxidative stress dysfunction in the brain after intracerebral (i.c.v.) administration of 0.5 mg/kg streptozotocin (STZ) in mice. After i.c.v. injections, animals received oral treatment with ethanolic extract (50-150 mg/kg), hexane fraction (100 mg/kg) and galantamine (3.0 mg/kg) for 15 days. They were subjected to memory tests, inhibitory-avoidance test (IAT) and new object recognition (NOR) test. Spilanthol (10 mg/kg), an alkaloid isolated from the hexane fraction was also tested acutely. Posteriorly, biochemical analysis were performed with mice's brains to evaluate the activity of catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione-S-transferase (GST) enzymes and to estimate the concentration of malondialdehyde (MDA). We found that treatments prevented STZ-induced memory loss, as assessed by IAT and NOR. Biochemical analysis revealed that STZ significantly increased levels of MDA and depleted GSH in mice's brain. The extract decreased oxidation, as evidenced by a significant decrease in MDA and lead to an increase in levels of antioxidant markers in mice treated with STZ. The positive effects of plant extract and fraction on memory deficits induced by STZ seem to be related to spilanthol and its activity on TRPV1 receptors, since pretreatment with capsazapine promotes the reversal of these effects. The results together demonstrate the beneficial effects of *A. ciliata* and spilanthol in preventing memory impairment and dysfunction caused by STZ in mice. Therefore, there is a potential for this plant and spilanthol in the treatment of neurodegenerative diseases.

Key words: Jambu, neuroprotection, alkamides, neurodegenerative disease, memory.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease related to aging, characterized by slow and progressive loss of neurons in different regions of the central nervous system (CNS) and consequent deficits in memory and cognition. The main cause for this disease is still unknown, but it occurs in the patients’ brains, with accumulation of beta-amyloid peptides (Aβ) and hyperphosphorylation of the tau protein, which are respectively responsible for the formation of the two histopathological findings of AD, the senile plaques and the neurofibrillary tangles (Kozlov et al., 2017; Schmidt et al., 2017; Gao et al., 2018; Lane et al., 2018; Swarbrick et al., 2019). AD is the most common type of dementia, which accounts for 60-80% of cases. The World Alzheimer’s Report indicates a prevalence of 46.8 million people affected by AD worldwide. As population ages, this number is projected to triple by 2050 unless...
effective interventions are developed and implemented. Urgent efforts are required for an early detection of this disease (Breijye and Karaman, 2020). Most AD cases are sporadic (sAD), which involves several different etiopathogenic mechanisms, including environmental, genetic, and metabolic factors (Kozlov et al., 2017; Tellechea et al., 2018; Lei et al., 2021). For the research of pharmacological targets for AD many animal models are used (Drummond and Wisniewski, 2017; Denner et al., 2020). The intracerebroventricular (i.c.v.) streptozotocin (STZ) is a well-established animal model that has been widely used for investigating this pathology (Grieb, 2016; Jayant et al., 2016; Drummond and Wisniewski, 2017; Halawany et al., 2017; Ravelli et al., 2017; Berté et al. 2018; Salem et al., 2020). STZ is a glucosamine-nitrosourea compound biosynthesized by Streptomyces achromogenes, which is commonly used in the systemic induction of diabetes due to its ability to damage the pancreatic β cells and to induce insulin resistance.

Furthermore, its administration also decreases cerebral glucose uptake and produces effects related to AD, such as reduced cognition and increased cerebral aggregated Aβ fragments, tau protein, and Aβ deposits (Grieb, 2016; Drummond and Wisniewski, 2017). Transient receptor potential cation channel subfamily V member 1 (TRPV1), formerly known as vanilloid receptor 1 (VR1), is a nonselective cation channel with high Ca²⁺ permeability, that is expressed in primary sensory neurons as well as in the brain. It is activated by heat (>42°C) and phytochemicals such as the alkamide capsaicin (Čroutilin et al., 2010; Nomura et al., 2013; Premkumar, 2014). TRPV1 has clinical relevance to neurodegenerative disorders as its activation can decrease neuroinflammation, production of cytokines (Jayant et al., 2016), cellular injury, and oxidative stress, prevents hyperphosphorylation of AD-associated tau protein (Xu et al., 2017), also participates in cognition, ischemic damage and neuroprotection (Gupta et al., 2014). Pharmacological activation of TRPV1 rescues Aβ-tolerant microglial dysfunction, the AKT/mTOR pathway activity, and metabolic impairments and restores the immune responses including phagocytic activity and autophagy function. Amyloid pathology and memory impairment are accelerated in microglia-specific TRPV1-knockout APP/PS1 mice. Metabolic boosting with TRPV1 agonist may decrease amyloid pathology and reverse memory deficits in AD mice model (Lu et al., 2021). Thus, results reported in the literature demonstrate that TRPV1 is an important target regulating metabolic reprogramming for microglial functions in AD treatment.

_Acmella ciliata_, one of the plants popularly known as Jambu in Brazil, is an edible herb used in typical northern Brazil cuisine and folk medicine. Recent study revealed that in comparison with conventional vegetables, raw Jambu presented higher levels of protein (24.01%) (Neves et al., 2019). Jambu is famous for its sensorial effects, such as anesthetic, numbing, tingling and cooling feeling, due to the well-known phytochemical alkamide spilanthalh [{(2E,6,8E)-N-isobutyl-6,8-decadienamide} (Spelman et al., 2011; Silveira et al., 2016). A patent review on spilanthal indicated that it is widely used in oral care compositions, personal products, cleaning products, foodstuffs, and beverages, due to spilanthal’s sensory properties (Silveira et al., 2018). Alkamides represent a class of natural compounds that are highly active in the CNS as they produce antinociceptive (Rios et al., 2007; Gertsch et al., 2008; Déciga-Campos et al., 2010; Ong et al., 2011; Das et al., 2014), immunomodulatory (Gertsch, 2008), anticholinesterases and antioxidant effects (Tu et al., 2016) pointing out its anti-Alzheimer potential. Previous studies suggest that the analgesic and antinociceptive effects could be related to TRPV1 receptors modulation (Nomura et al., 2013; de la Rosa-Lugo et al., 2017). As the phytochemical analysis of the plant under study showed the presence of alkamides, that have anticholinesterase and antioxidant effects, in addition to having effects on TRPV1 receptors which are related to AD, we investigated in this work the anti-AD potential in animals with streptozotocin-induced AD, not only for the extract but also for the hexane fraction and the spilanthal compound.

**MATERIALS AND METHODS**

**Plant extraction and spilanthal isolation**

*A. ciliata* aerial parts were collected at the garden of the Associação dos Funcionários Fiscais do Estado de Santa Catarina (AFFESC, Florianópolis, Santa Catarina, Brazil) in October 2013. The ethanol extract, hexane fraction and spilanthal were obtained as previously described (Silveira et al., 2016). Phytochemically, from the hexane fraction of _A. ciliata_ ethanol extract (HFAC), 10 alkamides were identified: spilanthal (2E,7Z)-6,9-endoperoxyn-N-isobutyl-2,7-decadienamide, (2E-4E-6Z-8E)-N-isobuty-12,4,6,8-dodecatrienamide, (2E-6Z-8E)-N-2-methylbutyl-2,6,8-decatrienamide,2,3-epoxy-N-phenylethylamide-6,8-nonadiinamide, (2Z)-N-phenylethyl-6,8-nonadiinamide, (2E)-N-isobutyl-2,4-undecaden-8,10-dinamide, (2E)-N-isobutyl-2-undeca-8,10-dinamide, (2E,7Z)-N-isobutyl-2,7-tridecadien-10,12-diaminamide and (7Z)-N-isobutyl-7-trideca-10,12-diaminamide. HR-MS spectrum and chromatogram of spilanthal can be found as supplementary material.

**Drugs, reagents and doses**

The following substances were used: STZ, capsazepine (TRPV1 channel antagonist) and galantamine (drug commonly used in the treatment of AD) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The reagents (ethanol and hexane) used to obtain the spilanthal were
commercial grade, purchased from VETEC (RJ, BR). Ketamine (Vetbrands, FL, USA), xylazine (Agener União, SP, BR), lidocaine with epinephrine 2% (Cristália, SP, BR). All drugs were dissolved in saline (except STZ which was dissolved in artificial cerebrospinal fluid prepared as described by Jayant et al. (2016) and were infused at room temperature). The dose of galantamine used in this study as positive control in memory experiments was determined from a preliminary study based on the doses used by Kita et al. (2013). The other doses used were determined based on pilot experiments.

**Animals and ethical statement**

Male Swiss albino mice (25 - 30 g), approximately 90 days old, were used for the study. Animals were obtained from the Universidade do Vale do Itajaí (UNIVALI). They were kept at 22 ± 2°C with free access to food and water, under a 12:12 h natural light (sunlight) and dark cycle, except during pharmacological assays. This study followed the guidelines established by the Research Ethics Committee of UNIVALI, and the Brazilian Law on Animal Experimentation and was approved by the Research Ethics Committee of UNIVALI (CEUA/UNIVALI), protocol 11/18.

**Experimental groups**

Mice were randomly divided into nine groups, each containing 8 – 10 mice:

Group 1 (n = 10) regards the sham-operated animals that did not receive STZ nor oral treatment; however, received the same amount of the vehicle used to solubilize STZ (artificial liquor), 2 μL; i.c.v. This group is important to show that i.c.v. does not cause hippocampal lesion interfering with behavioral and biochemical assays. Group 2 (n = 9) is the negative control, injected only with STZ (2 μL of 2.5 mg/mL; i.c.v.) and treated with a vehicle per os (p.o.) route in which extract and spilanthol were solubilized (DMSO 2% and distilled water) treatment for 15 days. Group 3 (n = 10) represents the positive control, was injected with STZ (2 μL of 2.5 mg/mL; i.c.v.), and followed by injection of galantamine (3.0 mg/mL i.p.) treatment for 15 days. Groups 4 to 6 (n = 10) are the animals that were injected with STZ (2 μL of 2.5 mg/mL; i.c.v.) and treated for 15 days with ethanolic extract (50 mg/kg, 100 mg/kg e 150 mg/kg, p.o., respectively), dissolved in dimethyl sulfoxide (DMSO) 2% and distilled water. Group 7 (n = 10) are the animals that were injected with STZ (2 μL of 2.5 mg/mL; i.c.v.) and treated for 15 days with hexane fraction of A. ciliata (100 mg/kg; p.o). Group 8 (n = 10) was injected with STZ (2 μL of 2.5 mg/mL; i.c.v.) and treated with spilanthol (10 mg/kg; p.o) one time only (acute treatment). Group 9 (n = 10) are the animals that were injected with STZ (2 μL of 2.5 mg/mL; i.c.v.) and treated with spilanthol (10 mg/kg; p.o) and capsazepine (40 mg/kg) one time only (acute treatment). Neurobehavioral tests were carried out within 24 h after the 15th day of treatment (Figure 1). Groups 8 and 9 had no daily treatment, but pharmacological tests with these groups occurred only 15 days after induction of AD by STZ.

**STZ dosing regimen**

STZ dosing regimen was conducted by the freehand i.c.v. procedure previously reported (Pinton et al., 2010) with slightly modifications. Briefly, mice were anesthetized with xylazine/ketamine, then they were submitted to a minor surgery to remove the cutaneous tissue aiming at the exposure of the skull. After cleaning and asepsis of the cranial region, the animals still under anesthesia received STZ (2 μL of 2.5 mg/mL solution; i.c.v.) through a hypodermic needle attached to a cannula, which was linked to a 5 μL Hamilton syringe. After 48 h, each mouse received a second injection of STZ (same way as the first one). Drug treatment started after the second dose of STZ and lasted

![Figure 1: Timeline (in days) of experiments. OFT- Open field test, NOR- Novel object recognition test, IAT- Inhibitory avoidance test.](image-url)
for more than 15 days, including the days of the behavioral tests. Treatment was done orally (p.o.) or intraperitoneally (i.p.) (depending on the treatment) and experiments were carried out 1 h or 30 min after them.

Behavioral tests

**Novel objects recognition (NOR) test**

The NOR test is used to evaluate long-term memory also cognition. It is based on the principle that in a familiar environment, laboratory animals show an instinctive affinity for novelty or preference for exploring a new object rather than a familiarity (Ennaceur, 2010). To this end, animals from Group 1 to 8 were evaluated. The test consisted of three different phases (habituation phase, training phase, and test phase) conducted on three successive days and followed the protocol previously described (Myskiw et al., 2008) with slight modifications (Cazarin et al., 2021). Briefly, in the first phase, mice were exposed to an open field apparatus consisted of a wooden box of 40 x 60 x 50 cm dimensions and were left for 15 min in the absence of stimulus objects to adapt to the apparatus. On the next day animals were placed on the apparatus for 10 min in the presence of two identical in shape, color, and size objects put in opposite corners of the wooden box for familiarization. On day three, mice were tested by placing them again in the open field apparatus but now in the presence of a familiar and a novel object (different in shape, color and size from the other) and were left to explore the objects for 10 min. The time taken by each mouse to explore the two objects during the acquisition and retention phases of the test were recorded manually and separately with two stop watches (by a trained observer). Its Recognition Index (RI) was calculated for the retention trial as $RI = B/A1+B$ where $B = $ novel object and $A1 = $ familiar object.

**Effect of treatments on the locomotor activity of animals (Exploratory behavior) – Open Field Test (OFT)**

Locomotor activity was assessed using the same open field apparatus used in the NOR test. In this test, during animals' habituation phase, their exploratory behavior was evaluated during the first six minutes. Mice were placed individually in the center of the open field and crossing movements were recorded during this time.

**Inhibitory avoidance test (IAT)**

This experiment is used to measure long-term memory. In the present study, all nine groups of animals were submitted to an IAT as previously performed (Izquierdo and Dias, 1983) with some modifications (Cazarin et al., 2021). Mice were trained and tested in an apparatus consisted of a 50 cm long, 25 cm wide, 25 cm high plywood box with a glass frontal wall and floor made of steel plywood stainless steel parallel with caliper of 0.1 cm and to 1 cm of distance of each other, with a 2.5 cm high, 7.0 cm wide, 2.5 long platform. A 15 W lamp lit up the apparatus, while the room remains dark. On the training trial, animals were gently placed and held on the platform facing the rear left corner. Their latency to step down (placing the four paws on the grid) was timed, and immediately after a 0.4 mA foot shock was delivered for 2 s. The test session was conducted 24 h later. The procedure was identical to training session with omission of the foot shock. Their latency to step down was also measured up to a limit of 180 seconds.

**Biochemical analysis**

**Preparation of brain homogenate**

Twenty-four hours after the last behavioral test, the animals were euthanized and their brains were dissected into cortex and hippocampus. These structures were homogenized in potassium phosphate buffer solution 200 mM (pH 6.5) at a dilution ratio and rinsed with 0.9% NaCl solution for cleaning and weighed equal to 1.3 (w/v) that immediately was used to quantify the levels of reduced glutathione (GSH). The remain homogenate was centrifuged at 9000 × g for twenty minutes at 4°C to obtain the supernatant which were used to determine the activity of superoxide dismutase (SOD), catalase (CAT), Glutathione-S-transferase (GST), Glutathione reductase (GR) and Glutathione peroxidase (GPx), as described below. The protein concentrations were determined by the Bradford method (Bio-Rad, 159 Hercules, CA, USA).

**Assessment of brain antioxidant markers**

**Catalase (CAT) activity**

CAT activity was determined by a previous protocol (Maehly and Chance, 1954) with slight modifications. CAT reaction solution consists of 625 μL of 50 mM of potassium phosphate buffer (pH 5), 100 μL of 5.9 mM H₂O₂ and 35 μL enzyme extract. Change in the absorbance of the reaction solution was noted after 1 min at 240 nm. Absorbance changes of 0.01 as units/min denotes one unit of catalase activity.

**Superoxide dismutase (SOD) activity**

In this experiment, Kakkar et al. (1984) method was utilized. Buffers were exploited for the assessment of SOD
activity. Centrifugation of tissue homogenate was done at 1500 × g for 10 min and then at 10,000 × g for 15 min. Supernatant was collected and 150 μL of it was added to the aliquot containing 600 μL of 0.052 mM sodium pyrophosphate buffer (pH 7.0) and 186 mM of phenazine methosulphate (50 μL). 100 μL of 780 μM NADH was added to initiate enzymatic reaction. After 1 min, glacial acetic acid was added following the previous protocol. Following addition of 1.0 mL 1.65 mL containing 0.58 mL sodium phosphate buffer together with 100 μL of reduced glutathione (1 mM) and 12.5 μL of 1-chloro-2,4-dinitrobenzene (CDNB) 1 mM. Changes in absorbance were recorded at 340 nm and enzymes activity was calculated as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6 × 10^3 M^-1 cm^-1.

**Glutathione-S-transferase (GST) assay**

Scheme of Habig et al. (1974) protocol was strictly followed for the estimation of GST potency. 150 μL aliquot of tissue homogenate was added to 720 μL of sodium phosphate buffer together with 100 μL of reduced glutathione (1 mM) and 12.5 μL of 1-chloro-2,4-dinitrobenzene (CDNB) 1 mM. Changes in absorbance were recorded at 340 nm and enzymes activity was calculated as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6 × 10^3 M^-1 cm^-1.

**Glutathione reductase (GR) assay**

GR activity in tissue samples was analyzed as described elsewhere (Carlberg and Mannervik, 1975) with minor modifications. The reaction reagent 2 mL was made of of 1.65 mL phosphate buffer: (0.1 M; pH 7.6), 100 μL EDTA (0.5 mM), 50 μL oxidized glutathione (1 mM), 100 μL NADPH (0.1 mM) and 100 μL of homogenate. Activity of enzyme was monitored by recording the absorbance of the vanishing of NADPH at 340 nm at 25°C. Estimated of enzyme level was accomplished as nM NADPH oxidized/min/mg protein by employing molar extinction coefficient of 6.22 × 10^3 M/cm.

**Glutathione peroxidase (GPx) assay**

GPx activity was assessed as previously described (Jollow et al., 1974). Entire volume of 2 mL reaction solution comprised of 1 mM EDTA (100 μL), 0.1 M phosphate buffer (1.49 ml; pH 7.4), 1 mM sodium azide (100 μL), 1 IU/mLGR (50 μL), 1 mM reduced glutathione (GSH) (50 μL), 0.2 mM NADPH (100 μL), 0.25 mM H2O2 (10 μL) and tissue homogenate (100 μL). The loss of NADPH was recorded at 340 nm at room temperature. Enzyme level was estimated as nM NADPH oxidized/min/mg protein employing 6.22 × 10^3 M/cm molar extinction coefficient.

**Reduced glutathione (GSH) assay**

For this assays, GSH activity was checked as described by Jollow et al. (1974) using Ellman’s reagent (DTNB) as a substrate. The yellow color developed was read immediately at 412 nm and expressed as μmol GSH/g tissue.

**Assessment of oxidative stress markers**

**Estimation of Malondialdehyde (MDA)**

This assay was carried out following the previous protocol (Wills, 1966) with minor modifications. MDA is one of lipid peroxidation product that can be used as a marker of oxidative stress. The reaction mixture in a total volume of 1.0 mL contained 0.58 mL phosphate buffer (0.1 mol; pH 7.4), 0.2 mL homogenate sample, 0.2 mL ascorbic acid (100 mM), and 0.02 mL ferric chloride (100 mmol). The reaction mixture was incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by addition of 1.0 mL 10% trichloroacetic acid. Following addition of 1.0 mL 0.67% thiobarbituric acid, all tubes were placed in boiling water bath for 20 min and then shifted to ice bath before centrifuging at 2500×g for 10 min. The amount of thiobarbituric acid reactive substances (TBARS) formed in each of the samples was assessed by measuring optical density of the supernatant at 535 nm using a spectrophotometer against a reagent blank. The results were expressed as nmol TBARS/min/mg tissue at 37°C using a molar extinction coefficient of 1.56 × 105 M⁻¹ cm⁻¹.

**Statistical analysis**

The presented data in parametric tests are mean ± S.E. One-way analysis of variance (ANOVA) followed by Tukey’s test, when applicable. Kruskal-Wallis test followed by Dunn’s test was used to evaluate non-parametric results, which was expressed as median ± interquartile ranges. The Kolmogorov-Smirnov normality test was applied to verify the data normality. Moreover, power analysis was performed to determine all sample sizes. Differences were significant when p < 0.05, by using the GraphPad Prism version 8.00 for Windows (GraphPad Software, California, USA) program.

**RESULTS**

**Effect of STZ and A. ciliata extract on the locomotor activity of animals evaluated on open field test (OFT)**

Statistical analysis of the data obtained in the OFT revealed no difference between groups in the behavioral parameters recorded in this experiment (Figure 2). Our data show that the administration of treatments does not change the number of animal passages, indicating that it probably does...
Figure 2: Effect of the treatment with *A. ciliata* extract (50-150 mg/kg, p.o.), galantamine (3.0 mg/kg, i.p.), hexane fraction (100 mg/kg, p.o.) and spilanthol (10 mg/kg, p.o.) on animals with STZ-induced AD and submitted to Open Field Test (OFT). Values are expressed as means ± S.E.M. Statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc. Sham (fake-operated animals), which underwent the same procedures for i.c.v. STZ, but received artificial liquor.

not change the mobility of these animals in the memory tests.

**Effect of STZ and *A. ciliata* extract on discrimination index evaluated on novel object recognized (NOR)**

As can be seen by statistical analysis, the object discrimination index for animals in the sham group and animals treated with *A. ciliata* extract (50, 100 and 150 mg/kg), hexane fraction (100 mg/kg) spilanthol (10 mg/kg) and galantamine (3.0 mg/kg) was significantly higher than the discrimination index of objects in the vehicle-treated group (p < 0.05; p < 0.01; p < 0.01; p < 0.05 and p < 0.05 respectively) showed in Figure 3.

**Effect of STZ and *A. ciliata* extract on Inhibitory avoidance test (IAT)**

This assay investigated if mice developed accurate memory after they received footshock in inhibitory avoidance training trial. Therefore, mice were trained on the one-trial inhibitory avoidance task. Beneficial effects of treatments on cognitive deficits induced by STZ are best seen in panels B of Figure 4 which represented the inhibitory avoidance test sessions. Significantly, treatments with the extract 50 mg/kg (p < 0.01), 100 mg/kg (p < 0.01), 150 mg/kg (p < 0.01) and galantamine (p < 0.001) produced an increase in memory of the inhibitory avoidance when compared with the vehicle group. Thus, these findings indicate that treatment of mice with *A. ciliata* extract (groups 4 to 6) increased accurate memory like treatment with galantamine (group 3). Panel C demonstrate the positive effect of hexane fraction (p < 0.01) and spilanthol (p < 0.001). Also, demonstrated in this experiment that the pretreatment of the animals with capsazepine, an TRPV1 receptor antagonist blocked the nootropic effects of spilanthol when compared to the group that received the vehicle (Figure 4C) inferring that the spilanthol effects could be mediated by TRPV1 activating.
Figure 3: Effect of the treatment with *A. ciliata* extract (50-150 mg/kg, p.o.), galantamine (3.0 mg/kg, i.p.), hexane fraction (100 mg/kg, p.o.) and spilanthol (10 mg/kg, p.o.) on animals with STZ-induced AD and submitted to the novel object recognition test (NOR). The observation time ratio (recognition index) was determined for each mouse by dividing the time exploring the new object by the total exploration time. Values are expressed as means ± S.E.M. Statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc. * Denotes differences when compared to vehicle group (* p < 0.05 and ** p < 0.01). # Denotes difference compared to Sham group (fake-operated animals), which underwent the same procedures for i.c.v. STZ, but received artificial liquor (## p < 0.01).

**Effect of *A. ciliata* extract on brain oxidative status of mice**

The i.c.v. application of STZ significantly decreased the activity of antioxidant enzymes in the brain tissue of mice demonstrated by vehicle group compared to Sham group as seen in Figure 5 panel A for SOD (p < 0.01), panel B for CAT (p < 0.001), panel C for GR (p < 0.01), panel D for GST (p < 0.01), panel E for GPx (p < 0.001) and panel F for GSH (p < 0.01). On the other hand, in animals that received STZ and the treatment with ethanolic extract (150 mg/kg) significantly improved the activity the enzymes in brain tissue (p < 0.05; p < 0.001; p < 0.01; p < 0.001; p < 0.001 and p < 0.001 respectively). In addition, animals’ treatment with 150 mg/kg ethanolic extract was able to establish the activity of antioxidant enzymes to patterns like observed in Sham animals, group which did not receive treatment with STZ and did not have cognitive deficits. Results also demonstrate that STZ significantly increased oxidative stress markers, malondialdehyde (TBARS content; p < 0.001) and 150 mg/kg ethanolic extract was able to decrease these markers (p < 0.001) compared to vehicle group (Figure 6).

**DISCUSSION**

Since the introduction of galanthamine in Alzheimer's therapy, a phytoconstituent of *Galanthus nivalis*, natural resources, especially plants, have been investigated as
Figure 4: Effect of the treatment with *A. ciliata* extract (EEAC – 50, 100 and 150mg/kg, p.o.) and galantamine (3.0 mg/kg, p.o.) on animals with STZ-induced AD and submitted to inhibitory avoidance test (IAT). Plan A demonstrate the task of inhibitory avoidance and in plan B the results of the test session for better visualization. Plan C demonstrates the effect of administration of Capsazepine (40 mg/kg, i.p./TRPV1 receptor antagonist) 15 minutes before test. Data are expressed as medians interquartile ranges from (25%–75%). * Denotes differences in the test session compared to vehicle group (*p < 0.05; **p < 0.01 and *** p < 0.001). # Denotes differences compared to Sham group. ANOVA followed by the Kruskal-Wallis test between training and testing and the Dunnett’s test between groups. Sham (fake-operated animals), which underwent the same procedures for i.c.v. STZ, but received artificial liquor.
Figure 5: Effect of oral administration of A. ciliata extract (EEAC 150mg/kg, p.o.) on brain antioxidant status of animals with STZ-induced AD. The EEAC treatment significantly increased the activity of the enzymes SOD (A), CAT (B), GR (C), GST (D), GPX (E) and GSH (F). SHAM are the fake-operated animals, which underwent the same procedures for i.c.v. STZ, but received artificial liquor. Values are expressed as mean ± SEM (n=10). *Significant difference at p < 0.05 (*), p < 0.01 (**) and p < 0.001 (***) vs. control group respectively). # Denotes statistical differences in comparison with Sham animals (## p < 0.01 and ### p < 0.001). One way ANOVA followed by Tukey’s multiple comparison tests.

pharmacological targets for the treatment for this disease (Gregory et al., 2021). The pharmaceutical approaches developed to date do not alter disease progression. More than two hundred promising drug candidates have failed clinical trials in the past decade, suggesting that the disease and its causes may be highly complex. Medicinal plants and
herbal remedies are now gaining more interest as complementary and alternative interventions and are a valuable source for developing drug candidates for AD. Indeed, several scientific studies have described the use of various medicinal plants and their principal phytochemicals for the treatment of AD focusing not only on anticholinesterase activity, but also on other biochemical parameters involved in AD such as neuroinflammation, oxidative stress, and exacerbated activation of microglia, among others (Uddin et al., 2019; Stefanescu et al., 2020). The present work studied the protective effect of *A. ciliata* extract in a STZ (i.c.v.) induced model of dementia. AD is the most common type of dementia and represents one of the main health problems among other disorders of the CNS worldwide. Because it is a neurodegenerative pathological process in which the etiology is not fully established and the treatment is palliative, execution of adequate animal models is essential for understanding of its neurobiological bases and facilitates the approaches for the discovery of new therapeutic targets. The i.c.v. STZ model is an appropriate animal model used for study of sAD type dementia (Grieb, 2016; Jayant et al., 2016; Drummond and Wisniewski, 2017; Halawany et al., 2017; Ravelli et al., 2017; Berté et al. 2018).

Firstly, the model was developed for rats, but it was also standardized for mice (Ravelli et al., 2017). Glucose, its metabolites, and energy products metabolism (like ATP) are brain's main energy source and, therefore, normal glucose metabolism is fundamental for correct brain functions such as protein synthesis and cellular functions and molecular processes (Dienel, 2019). It has been widely reported in the literature that the i.c.v. STZ model of memory deficit shows an impaired glucose metabolism. In addition, various pathological aspects of AD like impaired brain glucose and energy metabolism are closely mimicked in animals after sub diabetogenic i.c.v. injection of STZ (Mayer et al., 1990; Lannert and Hoyer, 1998), which leads to progressive deficits in learning and memory in rats and mice (Grieb, 2016; Jayant et al. 2016; Drummond and Wisniewski, 2017; Halawany et al., 2017; Ravelli et al., 2017; Berté et al. 2018). It has also been reported in this model that the decrease in the levels of choline acetyltransferase in the hippocampus, leading to a decrease in acetylcholine (Costa et al., 2016), and that the septo-hippocampus system is also damaged which is drastic, since such brain structures are essential in the process of memory consolidation (Thomas, 2015; Pluta et al., 2021). In the present work, *A. ciliata* extract reversed deficit of memory and oxidative stress in STZ (i.c.v.) induced model of dementia. In this AD animal model, as expected, STZ caused a persistent memory deficit, as evidenced by the non-alteration in the behavior of animals in the inhibitory avoidance comparing the training and test sessions, as well as by the decrease in the index of recognition in the object recognition test, reproducing results obtained in several
works using this animal alzheimer model (Grieb, 2016; Jayant et al., 2016; Drummond and Wisniewski, 2017; Halawany et al., 2017; Ravelli et al., 2017; Berté et al., 2018; Denner et al., 2020). Curiously this memory deficit was reversed by treating the animals with different doses of the plant extract in study.

Oxidative stress might be the underlying cause related to the pathophysiology of AD or associated behavioral changes (Butterfield and Boyd-Kimball, 2018; Cassidy et al., 2020; Cazarin et al., 2021). In the present study, administration i.c.v. of STZ produced oxidative stress as evidenced by the significant increase in the level of TBARs and decrease in the GSH level, associated with decreased enzymes such as CAT, SOD, GST, GR and GPX. This increase in oxidative stress may be due to an increase in the level of glucose in the brain after STZ infusion (Butterfield and Halliwell, 2019). In a beautiful experiment, Pathan and colleagues demonstrated that brain slices from rats that received an STZ infusion showed reduced glucose consumption in the incubation medium compared to control rats with a hyperglycemic condition in the brain (Pathan et al., 2006). In addition, it has also been reported that oxidative stress, due to a hyperglycemic condition (Tailé et al., 2020) or direct effect of STZ, can cause endothelial dysfunction. So, in this model, impaired glucose metabolism and oxidative stress may be responsible for the endothelial dysfunction in animals’ brains. Impaired endothelial function is accompanied by decreased cerebral perfusion that has recently been associated with dementia (Wolters et al., 2017). Our results clearly demonstrated that treatment of animals with the extract of A. ciliata decreased oxidative stress and, consequently, the cognitive deficit caused by the i.c.v infusion of STZ. In our studies, both hexane fraction and isolated compound, the spilanthol were also administered to animals with STZ-induced sAD and evaluated in the inhibitory avoidance test. Our results showed that both treatments reduced animals’ cognitive deficits by improving the memory of inhibitory avoidance. As already reported, alkaloids represent a class of natural compounds that are highly active in the CNS as they produce antinoceptive (Rios et al., 2007; Gertsch et al., 2008; Déciga-Campos et al., 2010; Ong et al., 2011; Das et al., 2014), immunomodulatory (Gertsch, 2008), anticholinesterases and antioxidant effects (Tu et al., 2016). Previous studies suggest that these effects may be related to TRPV1 receptors modulation (Nomura et al., 2013; de la Rosa-Lugo et al., 2017). As previously reported TRPV1 has clinical relevance to neurodegenerative disorders as its activation can decrease neuroinflammation, diminish the production of cytokines (Jayant et al., 2016), cellular injury, and oxidative stress, prevents hyperphosphorylation of AD-associated tau protein (Xu et al., 2017), and participates in cognition, ischemic damage and neuroprotection (Gupta et al., 2014).

The relationship between alkaloids and TRPV1 receptors, among them and AD, made us think that the mechanism of action of the nootropic property found in the extract and hexane fraction of A. ciliata, as well as spilanthol could also be related to the activation of receptors TRPV1. So, we decided to check if the pretreatment of animals with the TRPV1 receptor antagonist, capsazepine, could reverse the effect of spilanthol in animals tested in the inhibitory avoidance. Our results showed that the nootropic effect of spilanthol may be related to the TRPV1 receptors since the beneficial effect of this alkaloid on the animals’ memory is not observed with the pretreatment of the animals with capsazepine. Our results corroborate other results in the literature (Jayant et al., 2016; Xu et al., 2017) and point out that positive pharmacological modulation of TRPV1 channels may be a potential research target for mitigating AD. Also, indirectly it can be verified that the reduction of oxidative stress in animals with STZ infusion and treated with the plant extract as observed in this study, can be related to the presence of the alkaloids present in the plant which would be modulating the TRPV1 receptors, however more experiments would be necessary to ascertain such hypothesis. In STZ induced memory deficit, there is a decreased activity of glycolytic enzymes resulting in a reduction in acetylcholine level (Sorial et al., 2017) which is intricately associated with cognition (Záborszky et al., 2018). Acetylcholine is degraded by AChE whose inhibition by AChE inhibitors is the most effective pharmacological approach for the symptomatic treatment of AD (Sorial et al., 2017; Záborszky et al., 2018; Weller and Hudson, 2018; Zhang et al., 2019). In the current study, the effect of treatments on AChE activity was not evaluated but we do not rule out the possibility that spilanthol, the compound we consider responsible for the plant’s nootropic effects, has AChE inhibiting activity since it is reported that alkaloids have such a property (Tu et al., 2016).

In AD the most important diagnostic symptom is the loss of memory and throughout the course of the disease, various types of memories are lost by the patient including declarative and procedural (Tu et al., 2016; Drummond and Wisniewski, 2017; Kozlov et al., 2017; Lane et al., 2018). As previously reported, STZ in addition to the biochemical changes produced in the brain of animals, induces important cognitive deficits such as the loss of different types of memory (Halawany et al., 2017; Ravelli et al., 2017; Berté et al., 2018). In the present study, to assess the effects of treatments on cognitive deficits induced by STZ, we used two memory tests that are well-known in the literature and widely used in the screening of substances with anti-AD potential (Crystal, 2016): the inhibitory avoidance test and the object recognition test. The object recognition task is a behavioral test used to access declarative memory in rodents, which is based on animal’s natural tendency to explore more the new object in detriment of the familiar, in a known context (Ennaceur, 2010; Myskiw et al., 2018; Reichelt et al., 2021). In inhibitory avoidance, the type of memory evaluated is emotional memory. In this test, the
animal learns to associate the context of the apparatus in which it finds itself, initially not aversive to receiving an electric shock in its paws (aversive) when it descends from a platform to explore the environment. Shocks occur in the training session. In the test session animals are replaced in the apparatus (on the platform) and the latency of descending the platform is timed. The difference between the latencies of descent between the training and test sessions are considered indices of memory (Izquierdo and Dias, 1983). Our results showed in both memory tests the deleterious effects of STZ as already reported in the literature (Lannert and Hoyer, 1998; Ennaceur, 2010; Pinton et al., 2010; Berté et al., 2018), as well as showing that treatment with the extract, the hexane fraction and the spilanthol which were obtained from the aerial parts of A. ciliata can significantly mitigate such effects, pointing out the pharmacological potential of the plant in AD.

CONCLUSION

In summary, A. ciliata demonstrated a promising therapeutic effect in the treatment of STZ-induced Alzheimer’s. The plant’s effect appears to be mediated by one of its phytochemicals, spilanthol. Our results together provide evidence that the therapeutic effect of the plant involves at least in part, inhibition of oxidative stress, which is involved in the pathogenesis of Alzheimer’s and activate TRPV1 type receptors.

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REFERENCES


On the participation of mTOR in recognition memory.


