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NEUROPROTECTIVE EFFECT OF EXTRACT OF *ANACARDIUM OCCIDENTALE* LINN ON A ROTENONE MODEL OF PARKINSON´S DISEASE

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ABSTRACT: In this study the neuroprotective activity extract hydroalcoholic of AO against behavioral and biochemical parameters induced by subcutaneous injection of rotenone was evaluated, important for the understanding of Parkinson´s disease, which is the most common neurodegenerative disorder affecting movement. Rotenone is a potent hydrophobic pesticide and a highly selective inhibitor of complex I in the electron transport chain. Anacardium occidentale (AO), popularly known as cashew, is a tropical tree native to north and northeast Brazil. Peduncles (pseudofruit) and nuts can be eaten raw or converted into various nutritional products (juice, tea, jam, and beverages). AO has been studied for its anti-inflammatory, antimicrobial, antitumor, molluscicides and antidiabetogenic activity. Behavior evaluations were performed using the models of open-field, rotarod, on horizontal bar and elevated T-maze. The in vivo antioxidant activity was evaluated in the substantia nigra (SN), cortex and striatal region by lipid peroxidation after behavioral tests. Systemic administration of rotenone produced hypolocomotion, muscle incoordination and memory deficit. AO administration (150 and 600 mg/kg, p.o.) improved rotenona induced dysfunctional behavior (locomotor, musculature coordination and memory retention). Biochemical analysis of the SN, cortex and striatum revealed that systemic rotenone administration significantly increased lipid peroxidation which was attenuated by daily treatment with AO. These results suggest that there are protective effects of AO on oxidative stress caused by rotenone mediated through its antioxidant activity.

INTRODUCTION: The increase in the elderly population has led to an increasing incidence of neurodegenerative diseases worldwide. Thus, interest in studies aimed at the prevention and cure of these diseases is very important.

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Natural constituents derived from plants are important to investigate, since studies have shown that these compounds exhibit a range of biological activities, with therapeutic potential, among them: antioxidant and/or anti-inflammatory.

The *Anacardium occidentale* (AO), popularly known as cashew, is a tree native to north and northeast of Brazil, well known for its trade in cashew nuts and fruit. The peduncle (pseudofruit) has high nutritional value, especially vitamins A, B, C and is rich in proteins, lipids, carbohydrates,

minerals, fiber, unsaturated fat and phenolic compounds [1]. The phenolic compounds present in the pulp of the cashew have antioxidant properties[2]. These properties are associated with the prevention of chronic diseases such as cancer, diabetes, cardiovascular and neurodegenerative diseases.

Neurodegenerative diseases such as Alzheimer's and Parkinson's (PD) are characterized by excessive neuronal atrophy and death in focal regions of the brain [3]. PD results from deficiency of the nigrostriatal dopaminergic system that is responsible for movement [4]. PD is defined by the presence of four cardinal signs: tremor when resting, rigidity, slowness of movement and shifting of balance. The main mechanisms proposed to explain the events culminating in neuronal death in PD are associated with mitochondrial changes, apoptosis and elevated levels of oxidative stress [5]. There is also a neuroinflammatory process with activation of glial cells and release of cytokines and nitric oxide [6]. Therefore, the process of degeneration is multifactorial. These events are a vicious cycle and could be initiated by any of them with neuronal death results [7].

Rotenone is a potent hydrophobic pesticide and exposure to rotenone mimics the clinical and pathological features of PD, such as neurochemical, behavioral and neuropathological changes, providing an animal model for PD [8]. It causes neurodegeneration via multiple mechanisms that include inhibition of complex I, induction of apoptosis, activation of microglia and oxidative stress [9]. It has been reported that the rotenone model is highly reproducible and may provide an excellent tool for testing new neuroprotective strategies [10].

The objective of the present work was to evaluate the possible neuroprotective actions of hydroalcoholic extract of *Anacardium occidentale* (AO) in oxidative stress induced by rotenone, as one model of experimental Parkinson´s disease in rats.

MATERIALS AND METHODS:

Animals

The study used adult male Wistar rats *(Rattus norvegicus* var. Albinus) weighing 250 to 300 g, from the Department of Physiology and Pharmacology, from the Federal University of Pernambuco (UFPE) and mice *(Mus musculus* var. Swiss) of both sexes, weighing between 25 and 30g, obtained from the Laboratory of Immunopathology Keizo Asami (LIKA), UFPE.

The animals were maintained under standard environmental conditions (12h light/dark cycle) and temperature (22±2°C) and fed with commercial feed (Labina®, Purina, Brazil) and water *ad libitum*. Experimental protocols were approved by the Ethics Committee on Animal Use at the Center for Biological Sciences UFPE, under license Nº. 23076.027073, according to the recommended standards by the Brazilian College for Animal Experimentation.

Sample and preparation hydroalcoholic extract

Samples of pseudofruit clone CCP-76 were from the Brazilian Organization for Agricultural Research (EMBRAPA) Brazil. These samples had been submitted to special treatment according to the protocol developed by EMBRAPA, described by Broinizi et al.[1]. Peduncle of the cashew samples was lyophilized and stored at -20ºC for further analysis. The hydroalcoholic extract was prepared according to the method described by Krygier and Solsulski[11] with some modifications.

The cashew peduncle was initially subjected to extraction with ethyl ether at a ratio of 1:5 (g sample: solvent mL) for one hour in a magnetic stirrer (Model RO15 power IKA® Staufen, Germany) until it reached room temperature, then the solution was vacuum filtered through a Buchner funnel. The resulting residue was extracted again with ethyl alcohol (90%) in the same proportion as before, following the same procedure, thus obtaining the hydroalcoholic extract. For administration to animals, hydroalcoholic rotaevapored (B525, Micronal) was extracted under vacuum at 30°C, in order to remove all the residual ethanol then resuspended in filtered water. All reagents and solvents used were of an analytical grade.

Experimental Groups

The animals were randomly divided into four experimental groups (n= 8). Rats from Group 1 received water and Tween 80 (2%, w/v) by oral

gavage (1 mL/kg) and one hour later a subcutaneous (s.c.) administration of the vehicle (sunflower oil 90%, w/v + DMSO 10%, w/v) for five days. Group 2 received water and Tween 80 (2%, w/v), the same as Group 1, and rotenone (3 mg/kg, s.c.) after one hour for five days. Groups 3-4 received the AO hydroalcoholic extract at 150 and 600 mg/kg respectively, and one hour later were given rotenone (3 mg/kg, s.c.) also over a period of five days. Twenty-four hours after the last day of treatment, the rats were evaluated with behavioral tests. After the behavioral tests, the animals were sacrificed for biochemical assays.

Assessment of acute toxicity of AO

In this test, male and female mice (n= 3/sex) were housed in cages and deprived of food overnight, with free access to water. A single dose of 5000 mg/kg body weight of AO was administered orally to the animals by gavage. In each case, the volume administered was 10 mL/kg. Following administration, the animals were carefully observed during the first 3 h, and thereafter, daily for 14 days, for any toxic signs and symptoms or death. Signs and symptoms of toxicity included: (1) autonomic effects, such as salivation, and piloerection; (2) central nervous system effects, such as tremors and convulsions; and (3) changes in activity levels, posture, strength, and strange behavior [12]. Body weight, food and water consumption were recorded daily.

Behavioral procedures

All animals were submitted to behavioral tests 24 hours after the processing.

Locomotors activity

To quantify the general activity, each rat was placed individually into the center of an open-field arena (a circular wooden box with a diameter of 100 cm and 40 cm high, with a floor divided into 19 regions). The frequency of locomotion (number of floor units entered by the animal with four paws); rearing frequency (number of times that the animal stood on its hind legs, with the trunk perpendicular to the floor of the arena); immobility time (time the animal stood without making any movement) and movement start (time to get out of the center of the open-field arena) were assessed for 5 min. The arena was cleaned with 5% alcohol, between the tests.

Rotarod activity

All animals were tested for motor skills and balance using a rotary axis according to Kumar and Kumar[13]. To perform this test, the animal was placed with all four paws on a bar with a diameter of 7.0 cm and set 25 cm above the floor, rotating at a speed of 25 rpm. Before division into experimental groups, the rats were trained in two sessions of 180 seconds each for acclimatization. The animals were placed on the rotating bar and the length of stay was recorded. A cut off time of 180 seconds was maintained throughout the experiment.

The elevated T-maze test for spatial memory

The test was performed according to the method by Kumar et al.[14]. The elevated T-maze consisted of one opposite open arms (50x10 cm) and two closed arms of the same dimensions, facing each other and a central area (10x10 cm) connecting the arms. The arms had walls of 40 cm. The rats were placed individually at the end of an open arm facing away from the central area.

The time taken by the animal to move from the open arm to either of the closed arms was registered 24 h after the last day of treatment and was called initial latency. Rats were allowed to explore the maze for 30 seconds after acquisition of the initial latency, and were then returned to their cages. The learning retention latency was evaluated again the next day.

The percentage of memory retention was calculated from the formula:

$$\frac{\text{Transfer of initial latency - Transfer latency repetition}}{\text{Transfer of initial latency}} \times 100$$

Catalepsy

The standard bar test was used to determine the intensity of catalepsy. The bar was made of wood and had a diameter of 1.2 cm and the height from the floor to the top of the bar was 9 cm. Both forelegs of a rat were gently placed on the horizontal bar and the animal was timed (in seconds, maximum 180 s) until one forepaw touched the floor. Each animal was used for the experiment only once.

Biochemical studies

Dissection and homogenization After behavioral assessments, the animals wereanesthetized and then

decapitated, the brains removed, placed on ice the striatum, cortex and substantia nigra dissected. These regions were weighed and homogenized in Potter Elvehjem type homogenizer with 1X phosphate buffered saline(10%, w/v), to which was added BHT (0.004%, w/v) to prevent autoxidation of the samples. The homogenate was centrifuged at 10000 g for 30 minutes at 4ºC and an aliquot of supernatant was separated for biochemical analyzes.

Measurement of lipid peroxidation

Quantitative measurement of lipid peroxidation in the dissected regions was assessed according to the method of Buege and Aust[15]. The amount of malonaldehyde (MDA) present in samples was quantified by the reaction with TBA. Aliquots of 500 uL of the supernatant were added to 1 mL TBA: TBA 0.38% (w/w), 250 mL of 1N chloridric acid (HCl), 15% TCA (w/w) and 20 mL of ethanolic BHT (2%, w/v).

The solution was heated at 100ºC for 15 minutes, followed by cooling in an ice bath. 1.5 mL of n-butanol was added, shaken and centrifuged to 3000 g. After centrifugation, the upper phase was collected and analyzed in a spectrophotometer (CARY 3E - UV - Visible Spectrophotometer Varian) at 532 nm. For calculations, we performed a standard curve with 1, 1, 3, 3-tetramethoxypropane. Results were expressed as nmol MDA/ mg protein.

Statistical Analysis

The results are expressed as mean ± standard error of the mean (SEM). The difference between groups was assessed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison/post-hoc test to determine the significance level. Statistical analysis was performed using Graph Pad Prism® 5.0. (GraphPad Software, Inc., La Jolla, CA 92037 USA).P-values less than 0.05 were considered statistically significant.

RESULTS:

Acute toxicity

During the 14-day experiment, no death, toxic signs and negative symptoms were observed in any mice after acute treatment by oral route with AO in a dose of 5000 mg/kg. No significant changes in food and water consumption or in the body weight were observed (data not shown). The LD_{50} could not be estimated, but it was possibly higher than 5000 mg/kg.

Effect of AO on locomotor activity

Administration of rotenone for 5 days significantly reduced the ambulatory activity and rearings and increased the duration of immobility and time of movement start of rats in the open field test. Treatment with extract of AO (150 and 600 mg/kg) for 5 days significantly improved locomotor activity and decreased the time of immobility and the movement start time in rats. **(Fig. 1)**

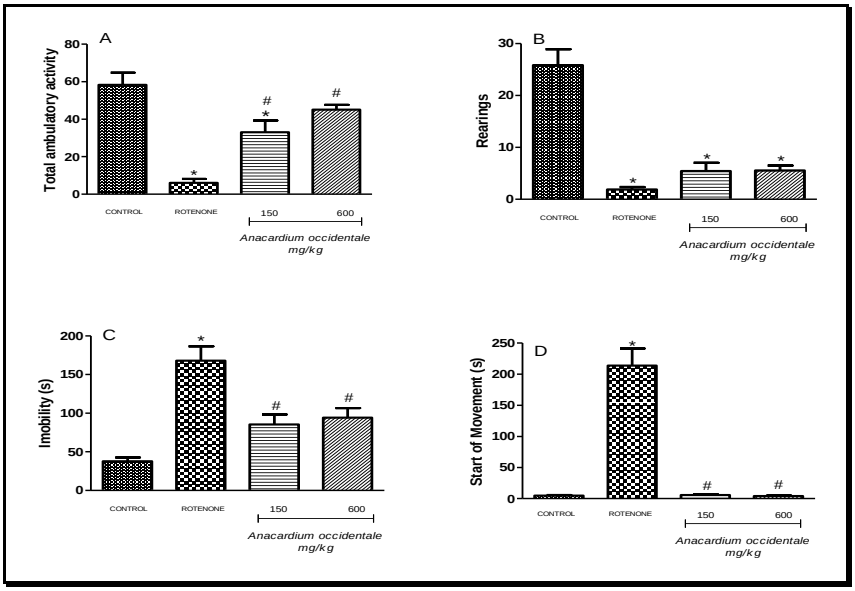

FIGURE 1: Effect of *Anacardium occidentale* (AO 150 and 600 mg/kg, p.o.) on locomotor activity in the open field test of rats. (A) Total ambulatory activity, (B) rearings, (C) duration of immobility and (D) start movement. Rotenone (3 mg/kg, s.c). Each bar represents the group mean ± SEM (n=8 per group). p <0.05 * Statistically significant compared to control # Statistically significant compared to rotenone (One-way ANOVA followed by Newman-Keuls test).

Effect of AO on rotarod test

Administration of rotenone caused a significant decrease in the time of permanency of the animals compared with control animals in the rotarod test. Daily treatment with extract of AO (600 mg/kg) for 5 days significantly increased the time of permanency compared with the group that received only rotenone **(Fig. 2)**.

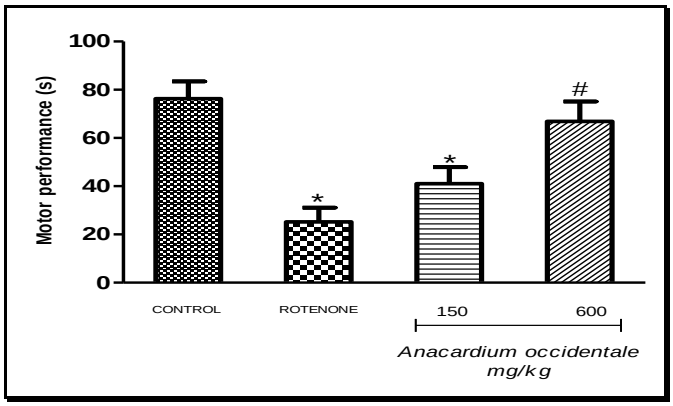

FIGURE 2: Effect of *Anacardium occidentale* (AO 150 and 600 mg/kg, p.o.) in the rotarod test in rats. Rotenone (3 mg/kg, s.c). Each bar represents the group mean ± SEM (n=8 per group). p <0.05 * Statistically significant compared to control # Statistically significant compared to rotenone (One-way ANOVA followed by Newman-Keuls test).

Effect of AO on memory performance in the elevated T-maze test

Treatment with rotenone caused marked memory loss as shown by the significant decrease in the percentage of memory retention compared with the control group in the elevated T-maze test. Daily treatment with extract of AO (150 and 600 mg/kg) 1 h before the administration of rotenone increased significantly the percentage of memory retention in rats, compared with the group that received only rotenone **(Fig. 3).**

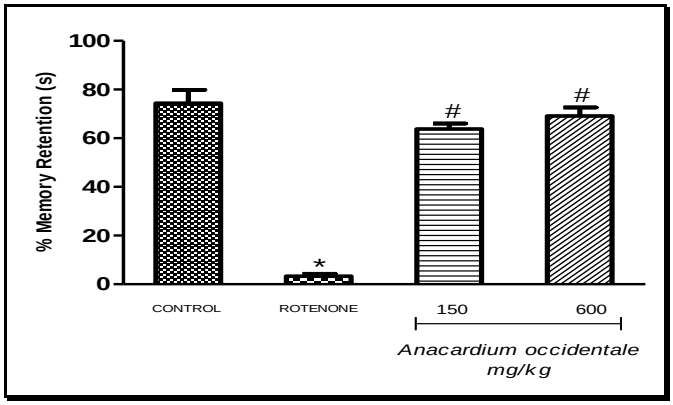

FIGURE 3: Effect of *Anacardium occidentale* (AO 150 and 600 mg/kg, p.o.) on memory performance in the elevated T-maze test in rats. Rotenone (3 mg/kg, s.c.). Each bar represents the group mean ± SEM(n=8 per group). p <0.05 * Statistically significant compared to control # Statistically significant compared to rotenone (One-way ANOVA followed by Newman-Keuls test).

Effect of AO on catalepsy

Treatment with rotenone caused a marked akinesia state as shown by the significant increase in motor deficiency compared with the control group. Daily treatment with the extract of AO (600 mg/kg) significantly improved the motor deficit in rats compared with the group that received only rotenone **(Fig. 4).**

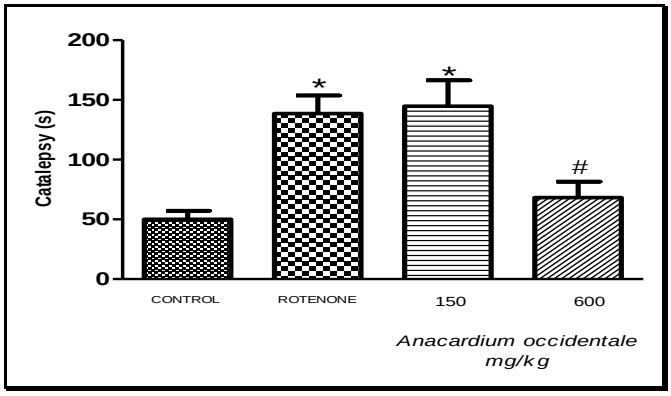

FIGURE 4: Effect of *Anacardium occidentale* (AO 150 and 600 mg/kg, p.o.) on akinesia test, in rats. rotenone (3 mg/kg, s.c). Each bar represents the group mean ± SEM (n=8 per group). p <0.05 * Statistically significant compared to control. # Statistically significant compared to rotenone (One-way ANOVA followed by Newman-Keuls test).

Effect of AO on brain lipid peroxidation

The administration of rotenone for 5 days induced oxidative stress as indicated by the significant increase of MDA concentration in the *substantia nigra*, cortex and striatum compared with the control group. Treatment with the extract of AO (600 mg/kg) 1 h before the administration of rotenone attenuated lipid peroxidation in the *substantia nigra*, cortex, and striatum, as shown by the decrease in MDA concentration **(Fig. 5)**.

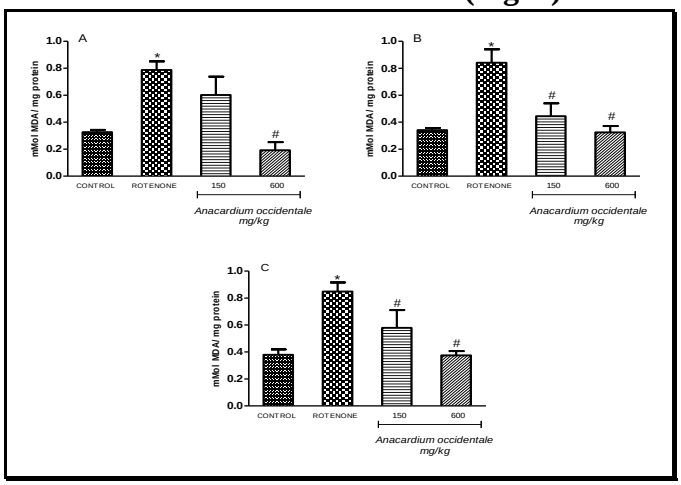

FIGURE 5: Lipid peroxidation levels (A) *substantia nigra* (B) cortex (C) striatum rats. Each bar represents the group mean ± SEM (n=8 per group). p <0.05. * Statistically significant compared to control # Statistically significant compared to rotenone (One-way ANOVA followed by Newman-Keuls test).

DISCUSSIONS: The current study investigated the hypothesis that hydroalcoholic extract of AO could have a potential antioxidant and neuroprotector action upon the rat in the central nervous system. Acute toxicity assessment in mice using a single AO dose of 5000 mg/kg shows for the first time that even in a high dose, this compound does not provoke toxic signs. According to Kennedy et al.[16], substances that present a lethal dose 50% (LD_{50}) higher than 5000 mg/kg by the oral route can be considered practically nontoxic.

Rotenone is a potent hydrophobic pesticide and highly selective inhibitor of complex I in the electron transport chain. This inhibition causes selective degeneration of nigrostriatal dopaminergic terminals[17, 18], reproducing many of the features of PD[8], such as reduced activity, flexed posture, rigidity and catalepsy correlated with the depletion of striatal dopamine and tyrosine hydroxylase[10,19] and neuroinflammation[20]. Sherer et al.[21] showed that the exposure to low doses of rotenone (2-3 mg/kg/day) caused highly selective nigroestriatal dopaminergic lesion.

There was no evidence of degeneration of other brain regions, such as the basal ganglia nuclei, such as the globus pallidus and subthalamic nucleus. Therefore, rotenone exposure to rodents is widely regarded as an experimental model for mimicking human PD.

In this study treatment with rotenone caused a marked akinesia state related to neural circuits associated with the basal ganglia function. Motor abnormalities, evidenced by decreased length of stay in the revolving bar rotarod, are mainly attributed to degeneration of striatal neurons, a region functionally connected by adhesions to the motor cortex[22]. Oral administration hydroalcoholic extract of AO was able to reduce motor abnormalities induced by rotenone and significantly promoted an improvement in the clinical state of the animals observed by their increased locomotor activity, percentage of memory retention and time of permanency on rotarod, suggesting a benefic systemic effect of this extract.

Sanders and Greenamyre[18] reported the important role of oxidative stress in the etiology of PD. Markers of oxidative stress are typically found in brain biopsies, peripheral cells, and biological fluids derived from patients with PD, indicating that indeed oxidative stress is a key factor in PD pathogenesis. Similarly, cumulative oxidative damage is the primary mechanism by which rotenone exposure leads to degeneration of dopaminergic neurons[23].

Brain cells are particularly susceptible to oxidative damage due to reduced activity of antioxidant enzymes and the large amount of polyunsaturated fatty acids, which may affect the ability of neurons to respond to stress[24]. In postmortem brains from PD victims markers of lipid peroxidation are found[6]. Malodyaldhyde (MDA) is a specific marker of lipid peroxidation, which is largely a result of the peroxidation of polyunsaturated fatty acids with more than two double bonds[18]. In our study, administration of rotenone promoted increased levels of MDA in substantia nigra, striatum and cortex, suggesting an increased lipid peroxidation and, thus, oxidative stress. The treatment with AO decreased the levels of MDA and this effect was probably due to the antioxidant capacity of this extract [1, 2].

CONCLUSIONS: Taken together, our data suggested neuroprotective effects of AO, probably mediated through its antioxidant activity.Thus the present work brought out new insights for the treatment of neurodegenerative diseasesand highlighted the pharmacological properties of AO, suggesting its effective use in clinical conditions in human. However, the clinical relevance of AO for the treatment of PD warrants further studies.

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