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Chronic administration of D-galactose induces memory loss, cholinergic system impairment, and oxidative damage in mice: Protective effects of *Piliostigma reticulatum* leaves extract

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ABSTRACT

Previous study showed that D-galactose (D-gal) increased production of Reactive Oxygen Species and resulted in impairment of memory and cholinergic system. *Piliostigma reticulatum* has been reported to have many benefits and medicinal properties. In this study, we evaluated the protective effect the aqueous extract of *Piliostigma reticulatum*against D-galactose-induced memory loss, impairment of cholinergic system and oxidative damage. Our results displayed that *Piliostigma reticulatum* administration significantly improved behavioral performance of D-gal-treated mice in morris water maze task and open field test. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione content (GSH) in D-galactose-treated mice were enhanced, while the content of the lipid peroxidation product malondialdehyde (MDA) was decreased by the plant administration. Furthermore, our results also showed that *Piliostigma reticulatum* significantly inhibited cholinesterase (AchE) activity, increased the level of acetylcholine in the brain of D-gal-treated mice, which could help restore impairment of brain function.

INTRODUCTION

World population is aging due to longer life expectancy and lower fertility rates [1]. This aging is accompanied by an increase of the prevalence of chronic diseases related to age such as dementia [2]. Africa today faces an unprecedented demographic aging and one of the greatest socio-health and economic risks of the 21st century [3]. In less than 40 years, the proportion of people aged 60 years and over is expected to quadruple, from 56 million in 2010 to 215 million in 2050 [4].

Dementia is defined by Hill et al., [5] as "a clinical syndrome characterized by a progressive impairment of cognitive and emotional abilities enough severe to disrupt daily functioning and diminish quality of life." It is estimated that 46.8 million people worldwide have dementia in 2015, with just over half (58%) living in low- and middle-income countries. Each year, there are approximately 9.9 million new cases. This issue would continue to increase because, according to statistics, 74.7 million people would have dementia in 2030 and this number would increase to 131.5 million in 2050 [6]. This increase is mainly due to an increase in the incidence of dementia in low- and middle-income countries; particularly those in sub-Saharan Africa where the number of demented males could increase by 215% between 2015 and 2050. It is therefore a real public health problem, it is important and long-lasting, and it is important to be aware of the challenges that this evolution implies all the more that the African populations are aging in a difficult socio-economic context. There are several pharmaceutical and non-pharmaceutical treatments for dementia; however, until now, no basic curative therapy has been established [7] and [8].

Piliostigma reticulatum, medicinal plant commonly used in the Benoué basin (North, Cameroon), is known to have various health benefits. The antioxidative and anti-inflammatory effects of this plant have been reported [9], and such effects may contribute to neuroprotection.

The objective of this work is to demonstrate the promising effects of aqueous extract of *P. reticulatum* leaves and its properties on memory loss, oxidative stress and alteration of the

cholinergic system induced by D -galactose.

MATERIALS AND METHOD

Plant material

Leaves of *P. reticulatum* used were collected in Cameroon in the immediate vicinity of Ngaoundéré, during the dry season. A voucher specimen of the plant (4498/Geerling) was authenticated by the Botanist *Mapongmetsem and deposited at the National Herbarium of Cameroon in Yaoundé. Dried leaves of P. reticulatum were* ground. 100 g of this powder were boiled in 500 ml of distilled water for 20 min. After cooling, the supernatant was collected, filtered with a Watman N°1 filter paper and was evaporated to dryness using a Rota vapor at a temperature of 60°C.

Animals and drugs administration

30 adult mice (*Mus musculus* Swiss; 28 ± 4g) were used for this study. The animals were housed in standard cages, at 25°C, on a 12/12 h light-dark cycle. They were supplied with food and water *ad libitum*. Mice were randomly divided into 6 groups of 5 animals each: one neutral control group received distilled water as vehicle, one negative control group received D-galactose and four test groups received different doses of aqueous extract,excepted the neutral control group, mice were injected subcutaneously with D-galactose (SigmaAldrich, MO, USA) at the dose of 500 mg/kg body wt. /day for 42 days. From the 21st day, mice for the test groups received, respectively, plant extract at 35, 87.5, 175 and 350 mg/kg body wt./day by oral gavage for three weeks after injection of D-galactose. Animals were sacrificed after the behavioral test for biochemical assay. Treatments were administered orally in a volume of 10 ml/kg of mice body weight. The study was conducted in accordance with the nationally (N°.FWA-IRB00001954) and internationally accepted principles for laboratory animal use and care as found in the US guidelines (NIH Publication No. 8023, revised 1996).

Behavioral testing

The Morris water maze

The Morris water maze (MWM) test was as described [10]. The experimental apparatus consisted of a circular water tank (150 cm in diameter, 35 cm in height), containing water at 23-25 °C, which was rendered opaque by adding powder milk, to a depth of 15 cm. A platform (13 cm in diameter, 20.5 cm in height) was submerged 1 cm below the water surface and placed at the midpoint of one quadrant (the target quadrant). The pool was located in a test room that contained various prominent visual cues. Each mouse received two training periods per day for 4 consecutive days. For each trial, a mouse was placed in the water facing the wall at one of four starting positions and released. The time required for the mouse to find the hidden platform was recorded. The probe test was done on day 5; the platform was removed and the time that a mouse spent swimming in the target quadrant and the number of times, the mouse crossed over the platform site was recorded.

Open field test

Locomotors activity was quantified in an open field, a black tank 70x70 cm with its floor divided into 49 squares. Each mouse was gently placed in the very center of the tank and activity was scored as a line crossing when a mouse removed all four paws from one square and entered another. Rears were scored when a mouse raised both front paws from the floor. The animals were left to explore it freely for 5 min, during that time the number of line crossings, grooming and rears were counted.

Preparation of brain tissue and determination of biochemical parameters

After behavioral testing, all mice were deeply anesthetized and sacrificed by decapitation. Brains were promptly dissected and perfused with 50 mM (pH 7.4) ice-cold phosphate buffer saline solution (PBS), then homogenized in 1/10 (w/v) PBS containing a protease inhibitor cocktail (Sigma-Aldrich). The homogenates were divided into two portions and one part was centrifuged immediately at 8000 g for 10 min to obtain the supernatant for assaying brain catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) activities, MDA level and protein content. The second part of the homogenates was sonicated four times for 30 s with 20 s intervals using a VWR Bronson Scientific sonicator, centrifuged at 5000 g for 10 min at 4°C, and the supernatant collected for determination of superoxide dismutases (SOD), acetylcholinesterase (AchE) enzyme activities and level of acetylcholine (Ach).

Briefly, the activity of SOD was measured according to Misra and Fridovich [11]. The GPx activity assay was based on the method of Mizuno and Ohta [12]. The procedure to estimate the GSH level followed to the method as described by Ellman [13]. The MDA level in brain was determined according to the method described by Wilbur and al, [14]. The Ach level and AchE activity were determined according to Hestrin method [15] and the method of Ellman and collaborators [16] respectively. The CAT activity was determined according to the method of Beer and Sizer [17].

Statistical analysis

All data are expressed as mean ± SD. Data were statistically analyzed using one or two-way analysis of variance (ANOVA) using the Xlstat software package. The Turkey's t-test was applied for the detection of significance between different groups with respect to control. $P<0.001$, $P<0.01$, $P<0.05$ are considered as extremely significant, significant and moderate significant from the control. $P>0.05$ is considered as non-significant with respect to control.

RESULTS

Effects of *P. reticulatum* on the behavior of D-gal-treated mice

Morris water maze

Morris water maze test was used to assess the spatial learning and memory ability of animals. Two-way ANOVA showed that the escape latencies were significantly higher in D-gal-treated mice than in control mice [$(F (27, 112) = 15; P < 0,001)$]. This result indicated that the spatial learning and memory ability in D-gal-treated mice was impaired. The Fig.1A shown that, the latency to find the platform declined progressively during the training days in all groups. However, the D-gal-treated group mice had longer latency to find the platform throughout the training period than did control mice, showing poorer learning performance due to chronic administration of D-gal. Plant treatment significantly shortened the latency as compared with the D-gal treatment mice. Statistical analysis of these data revealed that both training days and treatments had significant effects on long-term spatial memory [$(F (6, 3) = 14; P <0.001)$; $R2 = 78.57\%$] and [$(F (6.3) = 100; P <0.001)$; $R2 = 78.52\%$], respectively.

In the probe trial, D-gal-treated mice failed to remember the precise location of the platform, the numbers of target crossing was significantly reduced in the D-gal-treated mice indicating a spatial navigation deficit, *P. reticulatum* treatment significantly [(F (6, 28) = 7 ; P < 0,001)] reversed these spatial navigation deficits as seen in Fig. 1B. Furthermore, the time and the percentage of time spent in the target quadrant were increased by the administration of *P. reticulatum* as compared with the D-gal-treated mice (Fig. 1C & D) [(F (6, 28) = 6; P < 0,001)]. All these results revealed that both *P. reticulatum* could improve spatial learning and memory in D-gal-treated mice.

Open-field behaviors

Based on spontaneous exploration of a novel environment, the open field is one of the most widely used behavioral tests expressed by rearing, grooming, and line crossing. The data showed that the spontaneous behavior including rearing, grooming, and line crossing decreased in mice treated D-galactose. *P. reticulatum*, significantly [(F (5, 24) = 16; P < 0,001)]; [(F (5, 24) = 8; P < 0,001)] and [(F (5, 24) = 7; P < 0,001)] respectively, inhibited the decrease of rearing, grooming, and line crossings in these mice (Fig. 2AC). The results demonstrate that *P. reticulatum* improved the changes of spontaneous behavior related with aging in our model of mice given D-galactose.

Effects of *P. reticulatum* on SOD, CAT, GPx, and AchE activities in the brain of the D-galactose-treated mice

In comparison with neutral control group mice, the presented data (Fig. 3A) showed a significant decrease in SOD activity in the brain of the D-gal-treated mice [(F (5, 24) = 6; P < 0,001)]. *P. reticulatum* treatment resulted in a remarkable elevation of enzyme activity in D-gal-treated mice (p<0, 01).

The responses of CAT activity to D-gal, *P. reticulatum* treatment was similar to SOD activity (Fig. 3B). The CAT activity in the brain of D-gal-treated mice declined as compared with the neutral control group mice, a dose of *P. reticulatum* (350 mg/kg body wt.) significantly the activities of CAT [(F (5, 24) = 5 ; P < 0,01)].

The activity of GPx in the brains of D-gal-treated mice was significantly lower as compared with the neutral control group (Figs. 3C). *P. reticulatum* treatment resulted in a significant elevation in the activity of this enzyme [(F (5, 24) = 6 ; P < 0,001)].

Compared with the neutral control, D-gal-treated mice displayed a remarkable increase in AchE activity (P<0.001). However, this increase in Ache activity was restored in the brain of the plant-treated mice. *P. reticulatum* shown significant effect [(F (5, 24) = 9; P <0.001)]. As compare to D-gal-treated mice.

Effects of *P. reticulatum* on MDA, GSH, Ach and Total protein contents in the brain of D-gal-treated mice.

D-gal-treated mice showed a significant increase in MDA level as compared with the neutral control group (Table. 1). This increase in MDA was attenuated in the brains of plant-treated mice. MDA-reducing effect of *P. reticulatum* was significantly different as compared to D-gal-treated mice [(F, 5, 24) = 12, P <0.001].

It is apparent from theTable. 1, that D-gal significantly decrease GSH content. A comparison between the D-gal-treated group and the *P.reticulatum*/D-gal group showed that *P. reticulatum* could reversed GSH content of D-gal-treated mice [(F (5, 24) = 12; P < 0,001)].

Compared with the neutral control, D-gal treatment caused a significant decrease in Ach level in the brain (P<0.001) (Table 1). The D-gal induced decline in the Ach level was significantly attenuated by the presence of *P. reticulatum* [(F (5, 24) = 12; P < 0,001)].

Table 1 also described the content of protein in the brain of all groups of mice. Compared with the neutral control, D-gal model mice displayed a remarkable decrease in protein content (P<0.001). However, this decrease was restored in the brain of plant-treated mice. *P. reticulatum* significantly increase the protein content in brain of D-gal-treated mice [(F (5, 24) = 11211; P < 0,001)].

The correlation between the biochemical parameters and cognitive Parameters in mouse brain

In order to examine the relationship between latency on the fourth day of the water maze test and the activities of SOD, CAT, GPx , AchE and the MDA, protein, Ach and GSH levels in mouse brain, the data obtained from different treatments were analyzed statistically as follows: Y = A + B x X.

In which Y indicates latency to find the platform, and X indicates the activities of SOD, CAT, GPx, Ache and the level of MDA, protein, Ach and GSH levels, R- regression coefficient and p Probability.

As shown in Table 2, latency to reach the platform on the fourth day was negatively correlated with the activities of CAT (R = -0.540). Such negative correlation between latency and GPx and SOD activities and protein, Ach and GSH levels also existed in the mouse brain. But the level of MDA and AchE activity were positively correlated with latency on the fourth day (R= 0.689 and 0.686) respectively.

DISCUSSION

D-galactose (D-gal) is a reducing sugar that can be metabolized. However, at high thresholds, it readily reacts with amino-free amines in proteins and peptides in vivo to form advanced glycation end products (AGEs) [18]. Research shows that EFAs induce the production of Oxygen Reactive Species and increase carbonyl protein levels, this aggravates and accelerates the aging process and triggers the early phases of age-related diseases such as diabetes, arteriosclerosis, nephropathy, Alzheimer's disease [19], [20] and [21]. Recent work has also shown that continuous subcutaneous injection of D-gal induces the production of free radicals and the decrease of antioxidant enzymes in rodents, leading to decreased expression of proteins related to memory and deterioration of learning and the memory function that could be associated with alteration of the basal cholinergic nuclei of the pro-encephalon [22], [23], [24] and [25].

In the present study, D-gal-induced memory loss test shows a small variation in the latency of the platform in D-gal-treated mice compared to the animals in the neutral control group. On the other hand, the mice treated with the aqueous extract of *P. reticulatum* show a gradual decrease and following the days of training and the group of animals of the latency to find platform. This decrease in exhaust latency marks an improvement in memory [26] and an antagonism of the aqueous extract on the effects of D-gal [27] and [26].

D-gal significantly decreased the time and percentage of time spent in the target quadrant compared to mice treated with distilled water. The aqueous extract of *P. reticulatum* to significantly and dose-dependent increased the time spent in the

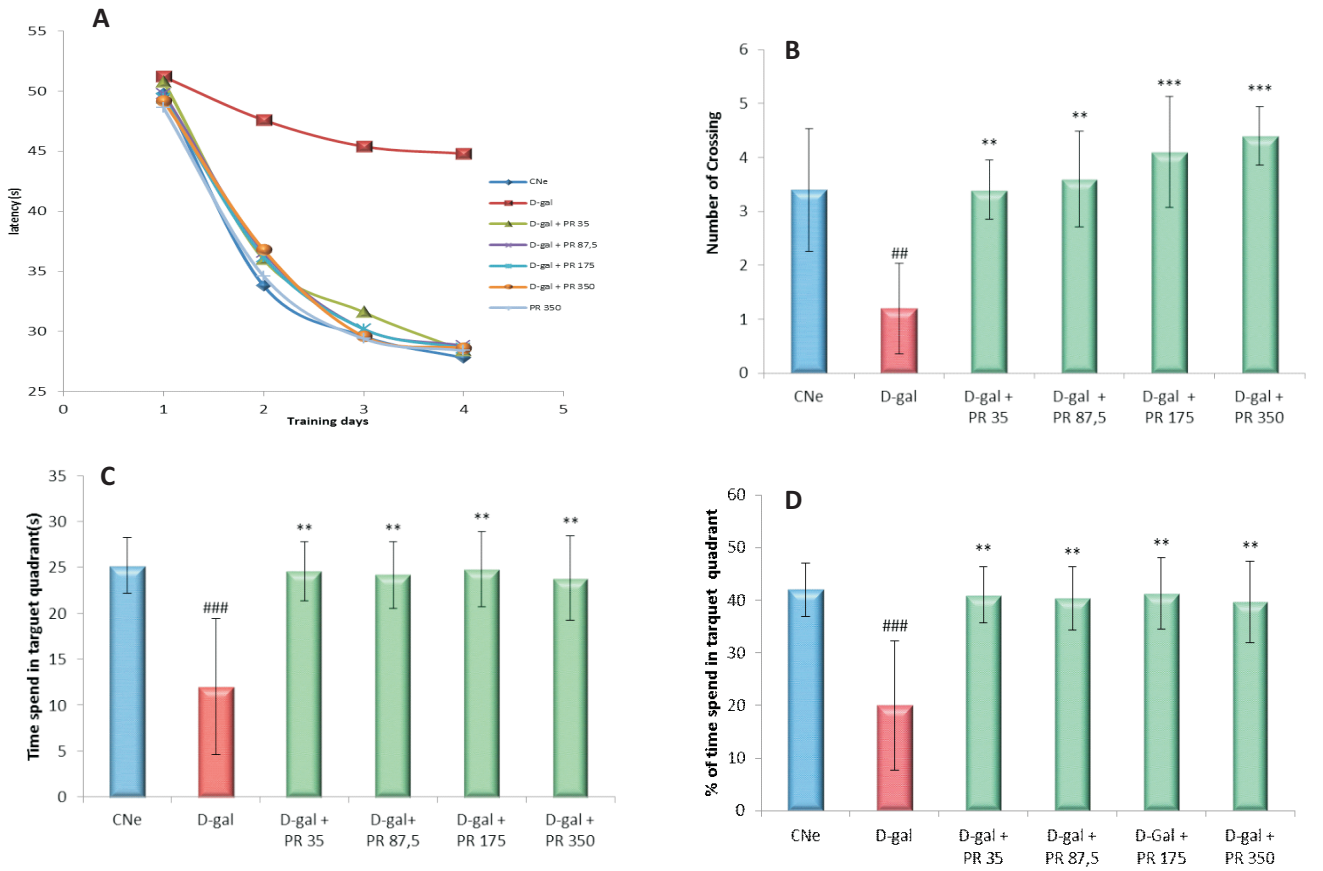

Fig. 1. : Effects of D-gal and *P. reticulatum* on the spatial learning and memory of mice in the Morris water maze. (A) Latency to find the hidden platform along 4 consecutive days. (B) Number of crossing. (C) Time spends in the target quadrant. (D) Percentage of time spent in the target quadrant in which the platform had previously been located during acquisition. Each value represents the mean ± ESM, n = 5, ** p <0.01, *** p <0.001, significant difference from the negative control. ## p <0.01, ### p <0.001significant difference from the neutral control. ANOVA followed by the Turkey test. CNe: Neutral control (distilled water). D-gal: Negative control (D-galactose (500 mg / kg)). PR: *Piliostigma reticulatum*

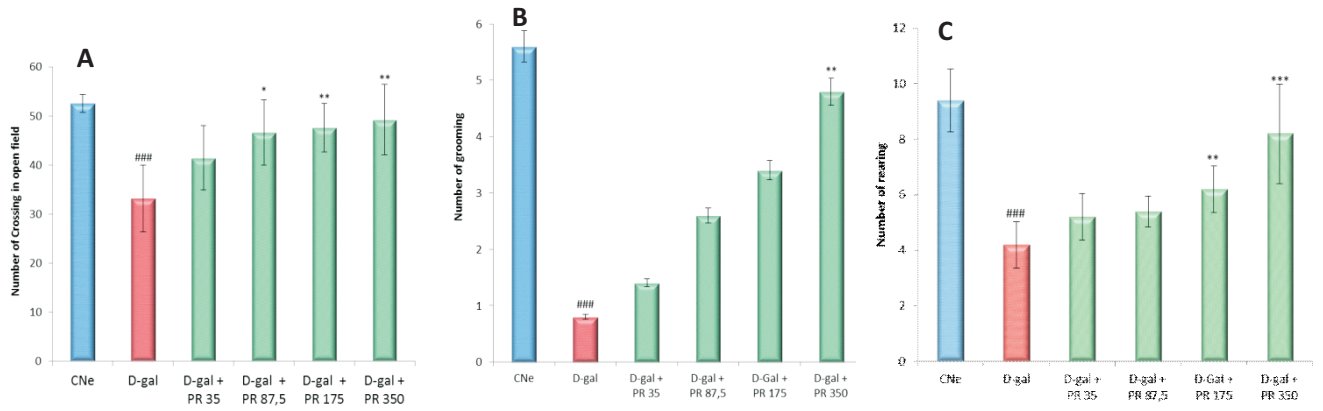

Fig. 2. : Effects of D-gal and *P. reticulatum* on the spontaneous exploration of mice in the open field. (A) Number of crossing. (B) Number of grooming. (C) Number of rearing. Each value represents the mean ± ESM, n = 5, * p <0.05, ** p <0.01, *** p <0.001, significant difference from the negative control. ### p <0.001, significant difference from the neutral control. ANOVA followed by the Turkey test. CNe: Neutral control (distilled water). D-gal: Negative control (D-galactose (500 mg / kg)). PR: *Piliostigma reticulatum*

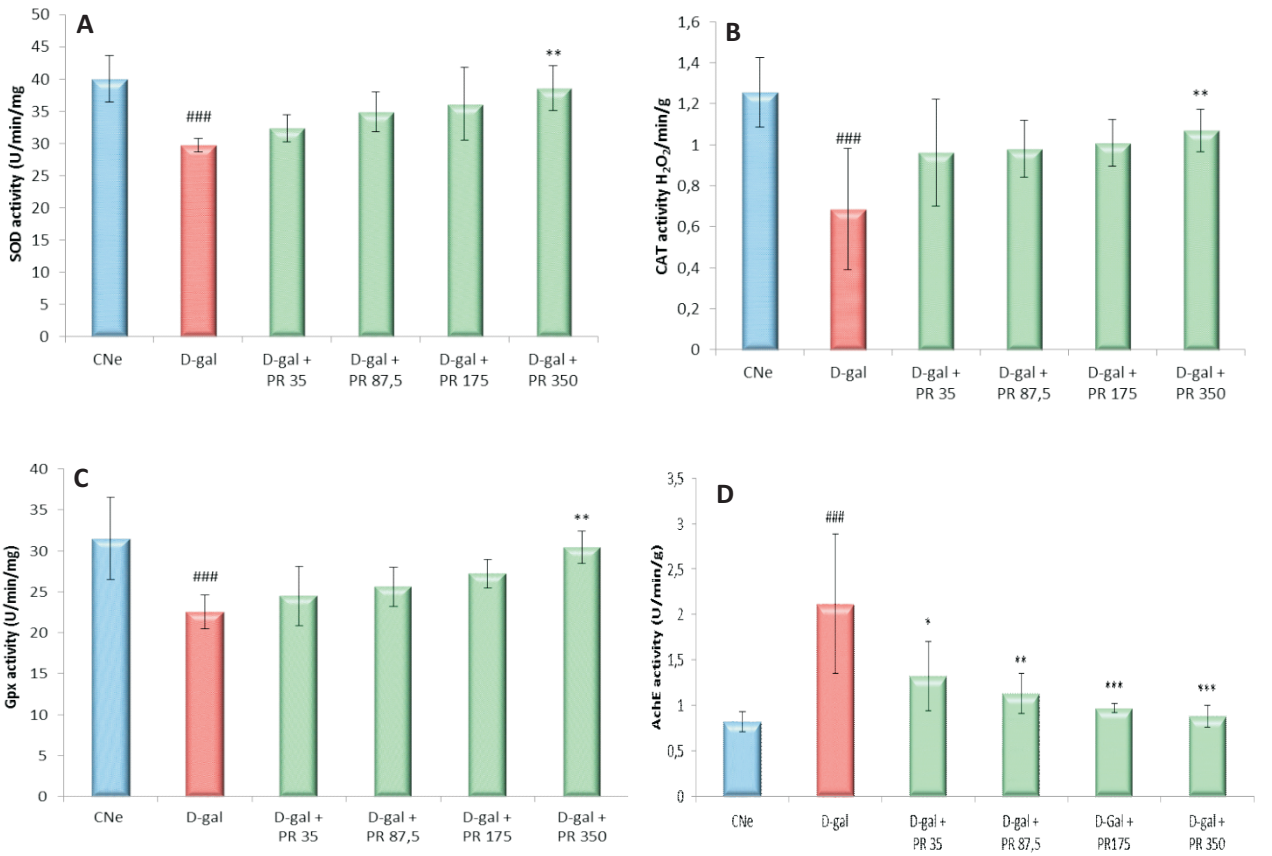

Fig. 3. : Effects of *P. reticulatum* on SOD (A), CAT (B), GPx (C) , and AchE (D) activities in the brain of the D-galactose-treated mice. Each value represents the mean ± ESM, n = 5, * p <0.05, ** p <0.01,*** p <0.001, significant difference from the negative control. ### p <0.001, significant difference from the neutral control. ANOVA followed by the Turkey test. CNe: Neutral control (distilled water). D-gal: Negative control (D-galactose (500 mg / kg)). PR: *Piliostigma reticulatum*

Table 1: Effects of *P. reticulatum* on MDA, GSH, Ach and Total protein contents in the brain of D-gal-treated mice.

			Doses de *P. reticulatum* mg/kg			
	CNe	**D- galactose (500 mg/kg)**	**D-gal + PR 35**	**D-gal + PR 87,5**	**D-gal + PR 175**	**D-gal + PR 350**
MDA (µmol/g)	13,18 ± 1,93	19,46 ± 1,57 ###	16,57 ± 1,11	14,23 ± 2,18***	13,73 ± 1,34***	13,27 ± 1,40***
GSH (µmol/g)	38,41 ± 4,43	20,96 ± 1,19###	30,27 ± 2,58*	31,85 ± 2,39**	34,13 ± 3,49***	36,44 ± 6,90***
Ach (µg/mg)	6,06± 0,97	3,40± 0,70###	4,83± 0,72*	5,23± 0,65**	5,54± 0,44***	6,05± 0,07***
Protéines totales mg/ml	10,44 ± 0,05	2,88 ± 0,05###	5,64 ± 0,04***	8,44 ± 0,04***	9,20 ± 0,06***	9,34 ± 0,10***

Each value represents the mean ± ESM, n = 5, * p <0.05, ** p <0.01, *** p <0.001, significant difference from the negative control. ### p <0.001, significant difference to the neutral control. ANOVA followed by the Turkey test.: CNe: Neutral control (distilled water). Negative control (D-galactose (500 mg / kg)). PR: *Piliostigma reticulatum*.

Table 2: The correlation between the biochemical parameters and cognitive Parameters in mouse brain

Relations coefficient	Regression equation	Regression	Probability
The latency and the MDA content	Y= 5,99 + 1,67X	R= 0.689	P<0.001
The latency and the CAT activity	Y= 45,63 - 14,55X	R= -0.540	P<0.01
The latency and the GPx activity	Y= 49,04 - 0,66X	R= -0.423	P<0.01
The latency and the SOD activity	Y= 58,50 - 0,77X	R= -0.554	P<0.01
The latency and the GSH content	Y= 46,15 - 1,96X	R= -0.778	P<0.001
The latency and the Ach content	Y= 7,537 - 0,0925X	R= -0.525	P<0.01
The latency and the AchE activity	Y= 21,29 + 8,18X	R= 0.686	P<0.001
The latency and the protein content	Y= 54,74 - 0,74X	R= -0.749	P<0.001

target quadrant. The removal of the platform generally induces a swimming tendency preferentially in the zone where the latter was. The control groups and those receiving the D-gal antagonist substance spend more time in the target quadrant whereas animals treated with D-gal in the different quadrants [26].

Similar results were also obtained on the number of crossing of the target quadrant. Indeed, D-gal significantly decreases the number of crossing of the target quadrant compared to the neutral control. The aqueous extract of the plant significantly and in a dose-dependent manner increased the number of crossing of the target quadrant. The increase in the number of crossing of the target quadrant by mice treated with aqueous extract compared with those treated with D-gal explains the neuroprotective effect of the plant [26].

In the same experiment, the aqueous extract of *P. reticulatum* inhibited the activity of AchE and increased the level of Ach. While D-gal significantly increased the activity of AchE and decreased the level of Ach. The cholinergic system is one of great importance in the processes of learning and memory [28] and [26]. Research has shown that learning and memory deficits in several neurodegenerative disorders are related to degradation of the cholinergic system [28] and [26]. Previous studies have shown that the administration of D-gal induces a significant increase in the activity of AchE in the brain of animals which leads to a dysfunction of the cholinergic system [29]. Our data confirmed these results. Also, administration of the aqueous extractof *P. reticulatum* restored AchE activity and concentration of Ach.

Our work revealed that D-gal induces a decrease in the level of proteins and GSH and the activity of Gpx, CAT and SOD on the one hand and the increase of MDA on the other hand. Previous work has shown that continuous subcutaneous injection of D-gal induces the production of free radicals and the decrease of antioxidant enzymes in rodents, leading to decreased protein expression [22], [30], [25], [24] and [31]. The aqueous extract of *P. reticulatum*, on the other hand, caused the increase in the level of proteins and GSH and the activity of Gpx, CAT and SOD and a decrease of the rate of MDA. Brain aging is a risk factor for neurodegenerative diseases such as Alzheimer's disease. Damage caused by oxidative stress plays an important role in cerebral aging [30]. These changes are similar, at least in part, to the characteristics of the normal aging process. Therefore, pharmacological substances that antagonize the effects of D-gal contribute to decreasing the rate of aging of the brain [22], [32] and [23]. Numerous studies have shown the role of oxidative stress in the pathogenesis of neurodegenerative diseases [32]. Indeed, oxidative stress causes damage to several molecules such as DNA, proteins and lipids of cell membranes [33], the accumulation of such damaged cellular macromolecules leads to malfunctions [34]. The brain, with high oxygen demand, a high level of unsaturated lipids, and relatively deficient in the antioxidant defense mechanism, is the organ most susceptible to oxidative damage. Similarly, peroxidation of lipids in the brain tissue contributes to learning and memory deficits [35]. Thus, antioxidant therapy has a great importance for the management of neurodegenerative diseases [30].

Based on the spontaneous exploration of a new environment, the open field test is the most used behavioral test to observe motor behavior in laboratory animals. We observed a significant decrease in the number of crossing, rearing and grooming in D-gal treated mice. Treatment at different doses of *P. reticulatum* led to an increase in the number of crossing, rearing and grooming. These results suggest that the injection of D-gal causes motor abnormalities and altered behavior of exploration induced by novelty. The data also indicated that administration of the aqueous extract of *P. reticulatum* is capable of reversing the alteration of D-gal-induced behavior. Similar observations were reported by Zhang et al. [36], exploration being part of learning [36], we can suggest that the aqueous extract of *P. reticulatum* improves memory.

CONCLUSION

In conclusion, the present study indicated that administration of D-gal caused memory loss, cholinergic system impairment and a decrease in antioxidant enzyme activities and an increase in the MDA level in mice. *P. reticulatum* significantly reversed the cognitive loss, cholinergic system impairments and improved the activities of antioxidant enzymes in the mouse brain. These properties could explain the use of this plant in traditional medicine in Africa. Therefore *P. reticulatum* may have potential as an antiaging therapy or in the treatment of neurodegenerative

diseases.

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