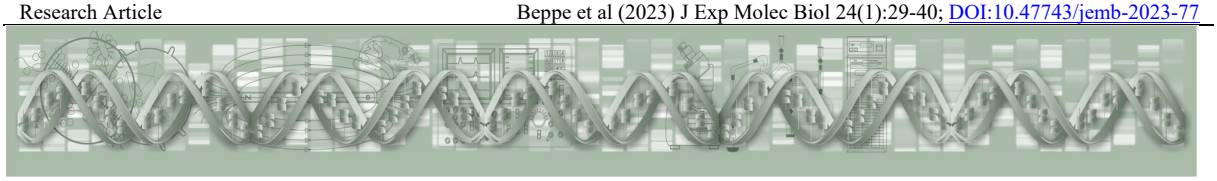

EVALUATION OF THE NEUROPROTECTIVE EFFECTS OF HYDROETHANOLIC EXTRACT OF *DIOSPYROS MESPILLIFORMIS* TRUNK BARK (EBENACEA) ON DIAZEPAM-INDUCED AMNESIA IN MICE

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Abstract

Diospyros mespilliformis (DM) is a plant of the Ebonaceae family that is commonly used in traditional medicine to treat epilepsy and schizophrenia. Radial arm mazes (RAM), Y mazes, and the novel object recognition test (NOR) were used to assess working and reference memory in mice. After the different tests, the hippocampus of the animals was separated for the determination of biochemical activities of markers such as acetylcholinesterase (AChE), malondialdehyde (MDA), and superoxide dismutase (SOD). The plant extract produced a significant rise in the percentage of alternation of mice in the Y-maze test, the time to observe the new object at the dose of 50 mg/kg ($p<0.01$) and the discrimination index ($p<0.001$) in all animals treated with the extract compared to the negative control. The result of the RAM test showed a significant decrease ($p<0.05$) in the number of errors on the reference memory in animals treated with the extract at 100 mg/kg. The extract significantly decreased AChE activity, and MDA concentration, and significantly increased SOD activity compared to the negative control group. These effects of the hydroethanolic extract on working and reference memories of DM extract would be related to the phenolic compounds quantified in the plant extract.

Keywords: *Diospyros mespilliformis*, amnesia, Diazepam, working memory, reference memory

Introduction

Amnesia is the pathological inability to learn new information or remember previously acquired information (Esther 2008). Amnesia is part of dementia, an estimated 46.8 million people worldwide suffered from dementia (Prince et al. 2013), among which 4 million were in Africa (ADI 2015). Many forms of brain damage can harm memory: physical aggression affecting the brain, diseases (brain tumors and infection), smoking (Ho et al. 2012), and medications such as benzodiazepines (Brault 2014). Diazepam's binding to GABA-ergic receptors results in chloride ion entry into the target cell and its hyperpolarization, which decreases postsynaptic excitability; thus, they have an inhibitory effect on signal transmission (Trincavelli et al. 2012). Benzodiazepines thus induce anterograde amnesia (Esther 2008). Diazepam induces alterations in brain structures with abnormal phosphorylation of the Tau protein which leads to the release of several free radicals (Ennaceur et al. 2009). However, the drugs used in the treatment of amnesia have several adverse effects and are unable to restore damaged structures because they cannot induce neurogenesis. Thus, research needs to promote the use of medicinal plants which

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can be an alternative solution. Therefore, plants are an interesting source of new compounds in the search for bioactive molecules (Mohammedi 2013). This is the case of plants such as *Daniella oliveri* (Beppe et al. 2020), *Vigna subterranea* (Ngatanko et al. 2020), and *Ziziphus mucronata* (Foyet et al. 2019) whose therapeutic effects have been proven on neurological pathologies. *D. mespilliformis* belongs to the Ebenacea family and is used in traditional medicine for the treatment of epilepsy (Ali et al. 2021) and schizophrenia (Arbonnier 2008). The general objective of the present study was to evaluate the effects of hydroethanolic extract of *D. mespilliformis* trunk stem bark on diazepam-induced amnesia in mice.

Materials and Methods

Chemical substances: Diazepam, piracetam, and ketamine were obtained from Alfa-sigma (France); piracetam was dissolved in distilled water.

Plant material and extraction protocol

The bark of the trunk of *D. mespilliformis* was collected in Maroua, then authenticated at the School of Fauna of Garoua by comparison with a sample found there on the number HEFG/01404. Four thousand five hundred grams (4500 g) of fresh plant material was shade-dried for 16 days and then ground to powder. Then 500 g of the powder was macerated in 5 L of an ethanol/water mixture (80/20 v/v) for 72 h. The filtrate was concentrated with a rotary evaporator at 50°C and then oven-dried at 50°C. Forty-two grams (42 g) of crude extract was obtained after drying which lead to an extraction yield of 8.4%.

Animal matériel

Forty-two male mice between 9 and 10 weeks of age weighing between 25 and 30 g were used. These animals were purchased from the National Veterinary Laboratory (LANAVET) in Garoua.

Quantitative phytochemical analysis of the hydroethanolic extract of the trunk bark of *Dispyros mespilliformis*

Determination of total phenols: The determination of total polyphenols was performed according to the method of Folin-Ciocalteu (Mahmoudi et al. 2013).

Determination of flavonoids: Flavonoid was evaluated using the method of (Mimica-Duckic 1999).

Determination of tannins: The method by Bainbridge et al. (1996) was used to perform Tannins concentration

Determination of saponins: Saponins were performed using the method by Mohammedi (2013).

Animal material and experimental protocol

Forty-two mice (42 mice) were randomized into 6 groups of 7 animals each. They were treated for 14 days with the different solutions used in the experiment. However, diazepam. The animals were treated as follows: a normal control group received distilled water (DW; 10 mL/kg, p.o), a negative control group received distilled water (10 mL/kg, p.o) and diazepam (3 mg/kg, i.p) (DW + DZP), a positive control group received diazepam (3 mg/kg) and piracetam (200 mg/kg, p. o) (DZP + PIR), three test groups received diazepam (3 mg/kg) and subsequently, *D. mespilliformis* extract at different doses (50, 100 and 150 mg/kg; p.o) (DZP + 50 mg/kg, DZP + 100 mg/kg and DZP + 150 mg/kg). After all tests, the animals were sacrificed, and the hippocampi were removed for the determination of some biochemical parameters and histological studies.

Behavioral tests

Y-maze test: The Y-maze is used to assess the working memory of animals (Wolf et al. 2016). The alternation percentage is defined by the following relationship:

[(total number of alternations) / (total number of entries-2)] x 100.

Novel object recognition test: The novel object recognition test is a widely used test to access episodic-type memory (Sandrine 2018).

Radial arm maze test: The radial arm maze is an 8-armed device numbered 1 to 8 (48 cm x 12 cm) with a circular center of 32 cm, it is used to test working memory and spatial reference. At the end of four of the eight arms is a food reinforcer placed at least 50 cm from the center and out of sight of the animal (Hritcu and Nabeshima 2009).

Biochemical analysis of oxidative stress parameters

Hippocampal sampling: After behavioral testing, animals were anesthetized with ketamine (50 mg/ml) and diazepam (10 mg/2 ml). The brains were completely isolated and only the hippocampi were used for the preparation of the homogenate in triphosphate buffer (0.2 M; pH 7.4) kept cool at 4 °C.

Measurement of acetylcholine esterase (AChE) activity: The measurement of AChE activity was based on the Ellman method by spectrophotometry (Ellman 2007).

Determination of some parameters of oxidative stress

Determination of Malondialdehyde (MDA): The determination of the amount of MDA in the hippocampus was performed following the technique described by Wilbur et al. (1959).

Superoxide Dismutase (SOD) assay: The SOD assay was performed according to the principle described by Mishra 1972.

Realization of histological sections: Twenty-four hours (24 h) after behavioral experiments, mice were euthanized by decapitation under ketamine and diazepam anesthesia (10 mg/kg and 50 mg/kg B.W. i.p., respectively); hippocampi were harvested and fixed in 10% formalin for histopathological evaluation. Portions of the hippocampus were dehydrated, immersed in kerosene, deparaffinized, rehydrated, and stained with hematoxylin and eosin. The sectioned parts of the brain were then filmed, and the images were captured using a digital camera connected to an optical microscope (Scientifico, Haryana, India).

Statistical analysis

The results obtained were expressed as mean ± MSE. Data were analyzed by one-factor ANOVA (Y-maze) and two-factor ANOVA (NOR and RAM) followed by Dunnett's and Bonferroni's post-tests, respectively. All analyses were performed using Graph Pad Prism version 8.0.1 for Windows. Results were considered significant for $p<0.05$.

Results and discussion

Quantitative phytochemical test of the hydroethanolic extract of *Diospyros mespilliformis* trunk bark

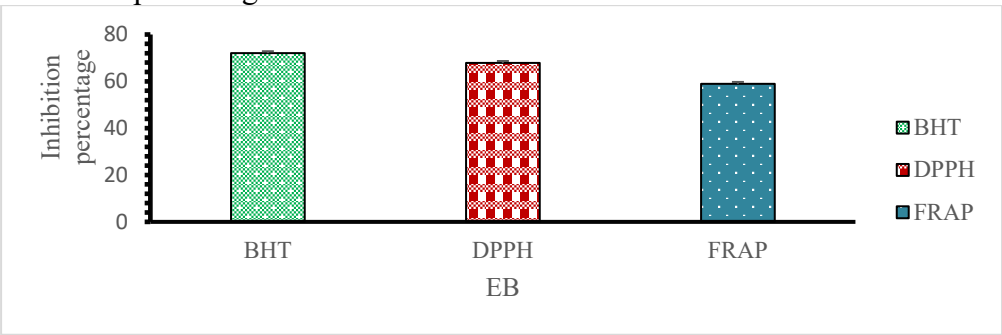
The phytochemical screening performed on the hydroethanolic extract of *D. mespilliformis* trunk bark showed that flavonoids are the most abundant polyphenols with an amount of 55.119 equivalent (eq) of quercetin/100 g of dry extract followed by saponins with an amount of 38.803 equivalent (eq) of galactose/100 g of dry extract (Table 1). Tannins were the least abundant polyphenols in the extract with an amount of 5.239 equivalent (eq) of catechin/100 g dry extract.

Table 1. Results of quantitative phytochemical screening of selected secondary metabolites of the hydroethanolic extract of *Diospyros mespilliformis* trunk bark.

Secondary metabolite class	Secondary metabolite concentration
Total polyphenols	73.146 mg Eq gallic acid/100 g ES
Flavonoids	55,119 mg Eq quercetin/100 g ES
Tannins	5,239 mg Eq catechin/100 g ES
Saponins	38,803 Eq galactose/100 g ES

Eq = equivalence, ES = dry extract

In vitro antioxidant activity: The antioxidant potential of the hydroethanolic extract of *Diospyros mespilliformis* trunk bark was measured using the DPPH and FRAP tests (Fig. 1). The results of these two tests revealed that the extract has a DPPH inhibition percentage of 67.87% and FRAP of 58.94% in comparison to the positive control (BHT) which has an inhibition percentage of 72.05%.



BHT: positive; DPPH: inhibition of DPPH extract; FRAP: inhibition of FRAP extract; Each histogram represents the mean ± MSE.

Effect of hydroethanol extract of *Diospyros mespilliformis* trunk bark on short-term memory

Figure 2 shows the percentage of alternation of animals. Animals treated only with diazepam showed a significant decrease ($p < 0.001$) in the percentage of alternations (56.5 ± 2.02%) compared to animals in the normal control group (90.0 ± 0.49%). The hydroethanolic extract of *D. mespilliformis* bark significantly ($p < 0.001$) increased the percentage of alternation at all doses (92.70 ± 1.86 %, 84.7 ± 2.91%, and 74.00 ± 1.04 % at 50, 100, and 150 mg/kg, respectively) compared to the negative control group. The reference substance piracetam significantly ($p<0.01$) increased the percentage of alternation of the positive control (68.9±0.84%) compared to the negative control.

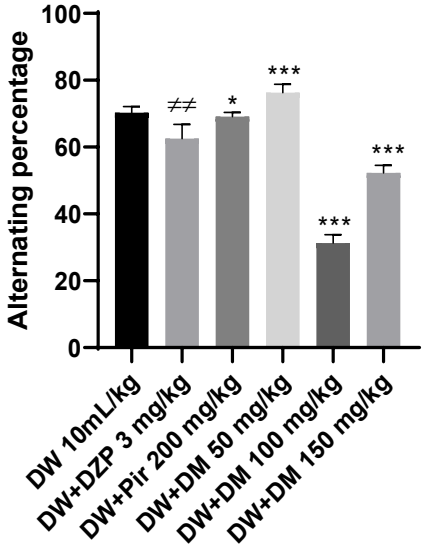


Figure 2. Effect of hydro-ethanol extract of *Diospyros mespilliformis* barks on the percentage of alternation of the Y-Maze test after 14 days of treatments. Each histogram represents the mean ± MSE, DZP = diazepam for negative control; DW= distilled water; Pir = piracetam for positive control; DM= the hydro-ethanolic extract of *Diospyros mespilliformis*; $^{###}P < 0.001$ significant difference of the negative compared to the normal control; $^{***}P < 0.001$, $^{**}P < 0.01$ significant difference compared to the negative control

Effect of hydroethanolic extract of *D. mespilliformis* trunk bark on exploration time and discrimination index in the novel object recognition test

The results in Figure 3 below show the exploration time (Figure 3A) and discrimination index (Figure 3B) of animals. Animals in the negative control group showed a significant decrease in novel object exploration time ($p<0.001$) and discrimination index ($p<0.01$) compared to animals in the normal control group. On the other hand, the hydroethanolic extract of *D. mespilliformis* induced in the animals a significant increase ($p<0.01$) in the exploration time of the novel object at the dose of 50 mg/kg as well as a significant increase ($p<0.001$) in the discrimination index at all doses tested, in comparison with the negative control. Piracetam induced a significant increase ($p<0.001$) in the time to explore the novel object and in the discrimination index of the animals compared to the negative control.

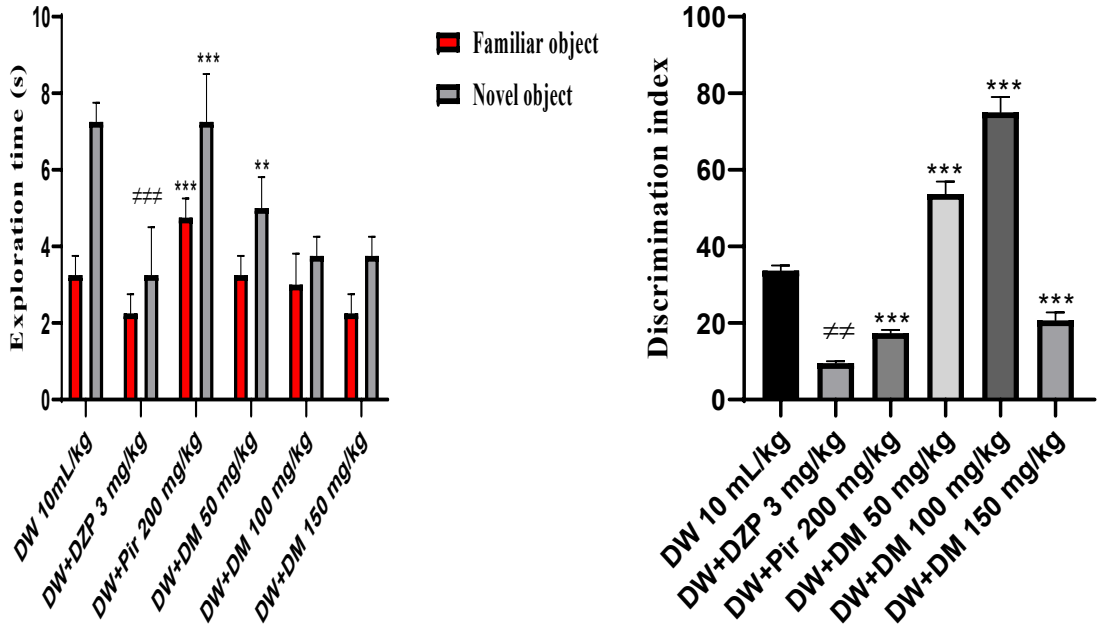


Figure 3. Effect of hydroethanol extract of *Diospyros mespilliformis* trunk bark on scanning time (A) and discrimination index (B). Each histogram represents the mean ± MSE. (n=5), DZP = diazepam; DW= distilled water; Pir = piracetam positive control; DM= the hydroethanolic extract of *Diospyros mespilliformis*; $^{\#\#}P < 0.01$ significant difference of the negative control compared to the normal control; $^{***}P < 0.001$ a significant difference of the test batches compared to the negative control

Effect of the hydroethanol extract of *Diospyros mespilliformis* barks on baseline memory in the 8-arm maze test

Figure 4 below shows the effect of the extract on the baseline memory. These results show that diazepam induced a significant increase ($p<0.05$ on days 5 and 6) and ($p<0.001$ on days 7) in the number of errors in reference memory of animals in the negative control group compared to animals in the normal control group. The hydroethanolic extract of *D. mespilliformis* barks at the dose of 50 mg/kg significantly ($p<0.05$) reversed the number of errors (4.00, 2.75, 2.00, and 1.25) on the reference memory of the animals in comparison to the animals of the negative control group (5.75, 5.75, 5.75, and 5.00) at days 4; 5; 6 and 7 respectively. Similarly, the 100 mg/kg dose extract significantly reversed the number of errors (2.25 and 2.50) in baseline memory on days 6 and 7. Piracetam, the reference product, induced a significant decrease ($p<0.05$) in the number of errors (2.00) in the reference memory of animals in the positive control group compared to the negative control on day 7.

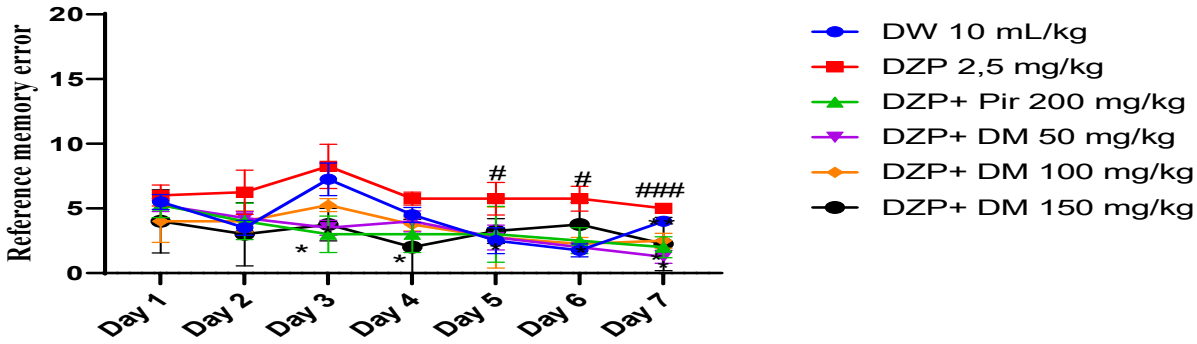


Figure 4. Effect of hydroethanol extract of *Diospyros mespilliformis* trunk bark on baseline memory. Each point represents the mean ± MSE. DZP = diazepam negative control; DW= distilled water normal control; Pir = piracetam positive control; DM= the hydroethanolic extract of *Diospyros mespilliformis*; $^{###}P < 0.001$ significant difference of the negative control compared to the normal control; $^{*}P < 0.05$ significant difference of the test and positive batches compared to the negative control

Effect of hydroethanolic extract of *Diospyros mespilliformis* bark on acetylcholine esterase activity

Figure 5 represents the activity of AchE in the hippocampus of mice. It appears that AchE activity was significantly ($p<0.001$ and $p<0.05$) decreased in animals treated with the hydroethanolic extract of *D. mespilliformis* bark at doses of 50 mg/kg and 100 mg/kg, respectively, compared to the negative control. The reference substance piracetam significantly ($p<0.001$) decreased the percentage of alternation in the positive control compared to the negative control.

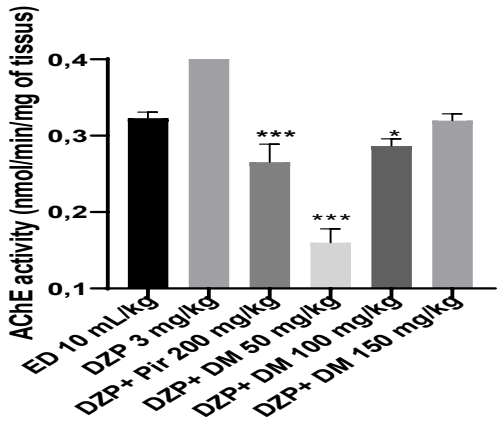


Figure 5. Effect of hydroethanol extract of *Diospyros mespilliformis* bark on acetylcholine esterase activity. Each bar represents the mean ± MSE. *P < 0.05 a significant difference from the negative control. AchE= acetylcholine esterase; DZP = diazepam for negative control; DW= distilled water; Pir = piracetam for positive control; DM= the hydro ethanolic extract of *Diospyros mespilliformis*.

Effect of hydroethanol extract of *Diospyros mespilliformis* barks on the concentration of Malondialdehyde (MDA), superoxide dismutase (SOD) activity

Animals in the negative control group showed a significant increase ($p<0.05$) in MDA concentration compared to animals in the normal control group after 14 days of treatment (Figure 6A). The hydroethanolic extract of *D. mespilliformis* bark at the dose of 50 mg/kg, 100

mg/kg, and 150 mg/kg significantly ($p<0.001$) decreased the concentration of MDA and significantly ($p<0.001$) increased the SOD activity of the animals compared to the animals in the negative control group (Fig. 6B). The reference substance piracetam significantly ($p<0.05$) decreased the MDA concentration and significantly ($p<0.001$) increased the SOD activity of the animals compared to the negative control.

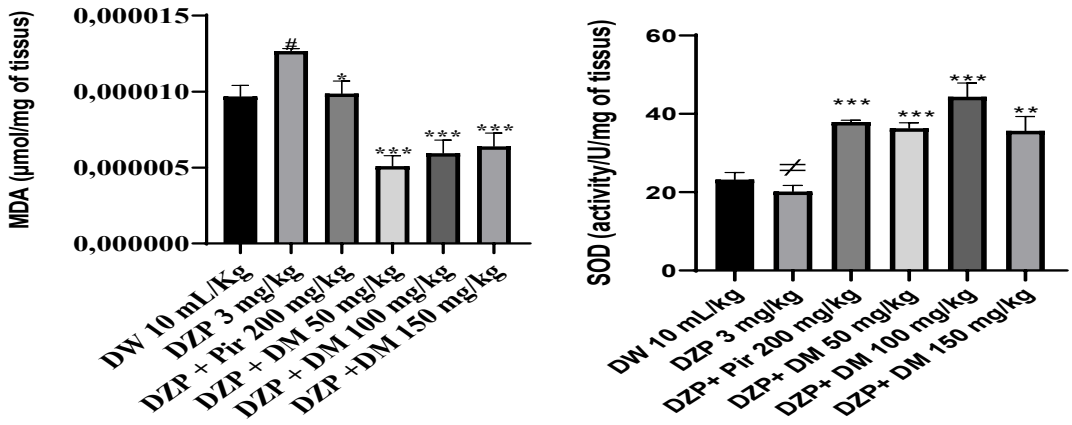


Figure 6. Effect of hydroethanolic extract of *Diospyros mespilliformis* trunk barks on MDA concentration (A) and SOD activity (B) in mouse hippocampus. Each histogram represents the mean ± MSE. DZP = diazepam for negative control; DW= distilled water for normal; Pir = piracetam for positive control; DM= the hydro ethanolic extract of *Diospyros mespilliformis;* $^{\#}P < 0.05$ significant difference of the negative compared to the normal control; $^{***}P < 0.001$ and $^{*}P < 0.05$ a significant difference of the test and positive batches respectively compared to the negative control

Effect of hydroethanolic extract of *Diospyros mespilliformis* trunk bark on hippocampal sections

Histological analysis (Figure 7) shows in the normal control a normal architecture of the hippocampus, with neurons of intact appearance in the different layers (GD, CA1, CA2, and CA3). In the negative control animals that received diazepam, a pathological modification (neuronal vacuolization) was observed in the different hippocampal layers. The animal groups that received the plant extract (at different concentrations) and piracetam showed a microstructure of the hippocampus close to that of the normal control.

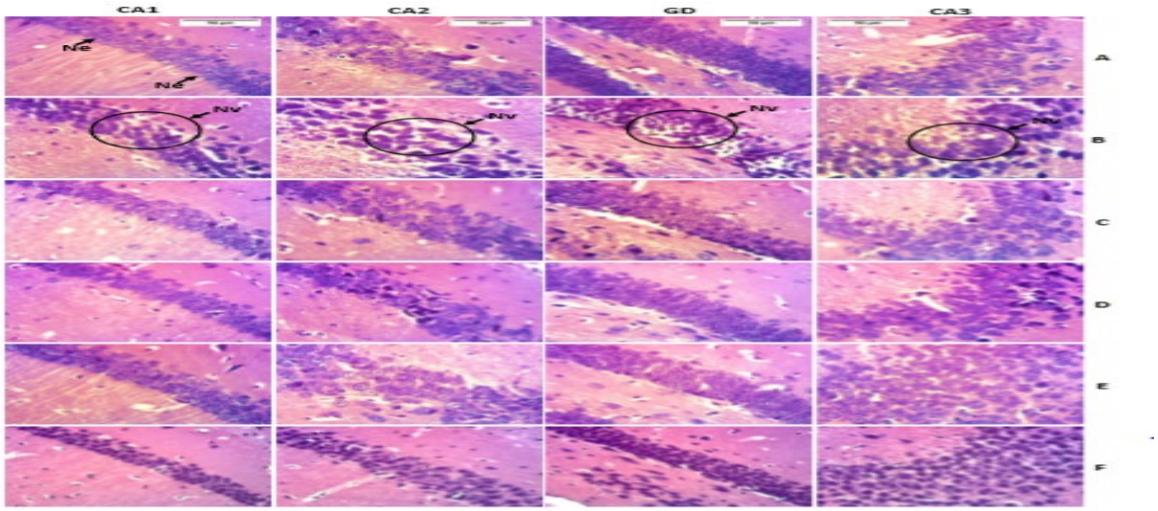


Figure 7. Microphotographs of the dentate gyrus (x250) and Ammon horns 1, 2, and 3 (X250) of the hippocampus; Hematoxylin-eosin staining. CA1, 2, 3 = Ammon corn 1, 2 and 3; GD = Dentate gyrus; Ne = Neuron; Vn = Vacuolated neuron; A = Normal control; B = Negative control; C = Positive control; D, E, F = Test batches receiving the extract at doses of 50, 100 and 150 mg/kg respectively

The aqueous extract of the plant was used to assess the cognitive aspect of memory through behavioral tests such as the Y-maze, radial arm maze, and novel object recognition test. Subsequently, biochemical analyses were performed to verify the observations of the behavioral studies. The Y-maze is used to measure the impact of a substance on short-term memory (Tolman 1924). An increase in the percentage of alternations reflects an improvement in short-term memory (Krishna et al. 2016). Treatment with the plant extract at all doses resulted in a significant increase in this percentage, compared to the negative control. Diazepam reduced the memory capacity of the negative control animals by binding to GABA-ergic type A receptors, resulting in a massive entry of chlorine ions into the target cell, leading to hyperpolarization and a decrease in neuronal excitability (Trincavelli et al. 2012). Further tests such as the arm maze test are needed to confirm these observed effects. This test measures the learning abilities in the environment where the animal is placed, as well as its short and long-term memory abilities (Beppe et al. 2014). The number of working memory errors in this test did not show any significance between the groups of animals. This could be because these animals were able to retain residual memory. Cohen and Squire (1980) showed that in the case of severe memory loss, short-term memory is preserved. Pretreatment with DM hydroethanolic extract significantly reduced the number of errors in baseline memory in mice. Beppe et al (2014) showed that the decrease in the number of errors on reference memory could be related to memory improvement in parkinsonian rats. The novel object recognition test is regularly used to demonstrate episodic-type memory (Sandrine 2018). Diazepam-treated animals (3 mg/kg) showed a significant decrease in novel object viewing time and discrimination index. Diazepam could have caused alterations in brain structures leading to memory impairment in this negative control group, as reported by Ennaceur et al (2009). Indeed, novelty recognition is based on the natural preference for novelty displayed by rodents to assess cognitive alterations in animal models of neurodegenerative disorders (Ennaceur et al. 2009). Treatment of animals with DM extract at all doses showed an increase in the discrimination index compared to the negative control. Acetylcholine is one of the most important neuromodulators in the cerebral cortex (Jaffard 1994). It is importantly and involved in the neural circuits of working memory, attention, episodic memory and spatial memory (Brault 2014). This

neuromodulator is degraded at the synaptic cleft by AChE to choline and acetate (Sanson 2009). Benzodiazepines such as diazepam primarily stimulate GABA receptors, and thus hyperpolarise and reduce serotonin and acetylcholine levels in the brain. A significant decrease in AChE enzyme activity was noted in animals treated with DM extract at 50 mg/kg and 100 mg/kg. The decrease in acetylcholine esterase activity could improve the cholinergic transmission process and therefore also improve learning and memory (Kouémou et al. 2017, Kim et al. 2018). Stress implies in the process of brain damage, as it causes direct denaturations of the blood-brain barrier (BBB) junction proteins (Elizabeth 2010). Diazepam induces alterations in brain structures with abnormal phosphorylation of the Tau protein resulting in the release of several free radicals (Ennaceur et al. 2009). MDA is derived from lipid peroxidation which leads to an increase in the permeability of the blood-brain barrier in vitro (Mertsch et al. 2001) and thus, hypermethylation of the promoter region of the adhesion protein E-cadherin, resulting in its loss of expression, as well as an alteration of the BBB function (Lim et al. 2008). Concerning protein damage, it has been suggested that reactive species can modulate calcium channel function and thus mediate dysfunction via Ca^{2+} cytotoxicity (Brown and Davis 2002). Animals treated with DM hydroethanolic extract at all doses showed a significant reduction in MDA concentration compared to the negative control. Antioxidants are substances capable of neutralizing or reducing free radical damage in the body while allowing the maintenance of non-cytotoxic concentrations of ROS (reactive oxygen species) at the cellular level (Mohammedi, 2013). The flavonoids and tannins in this DM extract could have acted to decrease the concentration of MDA. The extract might possess the ability to inhibit the transformation of hydrogen peroxide to hydroxyl radical by reducing ferric iron (Fe^{3+}), which is essential for the formation of hydroxyl radical to ferrous iron (Fe^{2+}) in vitro. This reducing power could be due to the presence in the extract, of phenolic compounds including flavonoids and hydrolyzable tannins (Alioune et al. 2015). The presence of the hydroxyl group on the chemical structure of flavonoids and tannins allows them to trap radical species by giving up an electron (Boubekri 2014). Once their electrons are surrendered, they become radicals but remain stable due to their aromatic system which allows them to capture other radical species (Dai and Mumper 2010). All the above statements taken together further confirm the antioxidant potential of the plant extract evaluated in this study. Mohammedi (2013) showed that flavonoids are antioxidants capable of scavenging free radicals and that tannins contain significant antioxidant properties that act as scavengers and proton donors against lipid free radicals produced during lipid peroxidation; yet MDA is a product of lipid peroxidation. SOD is the first line of antioxidant defense (Sfar et al. 2013). The assay from homogenates of animals treated with the extract at all doses showed a significant increase in SOD activity compared to the negative control.

In this work, DZP caused alterations in different structures of the hippocampus. Pretreatment with *D. mespilliformis* hydroethanolic extract protected the hippocampus from the neurotoxic effects of DZP. Flavonoids protect vulnerable neurons, improving existing neuronal functions and stimulating neuronal regeneration (Vauzour et al. 2010).

Conclusions

This work was undertaken to evaluate the impact of the hydroethanolic extract of *Diospyros mespilliformis* bark on diazepam-induced amnesia in mice. The hydroethanolic extract of DM trunk bark improved the memory of amnesic mice by decreasing MDA levels, increasing SOD activity and decreasing acetylcholine esterase levels in the hippocampus of animals. The antioxidant properties of this extract are thought to be due to the secondary metabolites it contains, which mainly protect the hippocampus against the neurotoxic effects of diazepam.

Abbreviatons: CA: Cornu Ammonis; DZP : diazepam; *D. mespilliformis*: *Diospyros mespilliformis*; BW: Body weight; YM: Y-maze; AChE: Acetylcholine esterase; MDA: Malondialdehyde; SOD: Superoxide dismutase.

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