Neuroprotective, behavioral and biochemical effects of aqueous extract of *Hibiscus cannabinus* L. on aluminum chloride induced Alzheimer's disease in male Wistar rats

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**ABSTRACT**

Alzheimer's disease (AD) is the most common form of dementia affecting the cognitive functions of the aged populations. The present study was conducted to assess the anti-amnesic effects of the aqueous extract of *Hibiscus cannabinus* seeds on aluminum chloride (AlCl₃) induced cognitive impairment in male Wistar rats. The aqueous extract (125, 250 and 500 mg/kg) was chronically administrated during 28 consecutive days in separate groups of AlCl₃ treated rats. The T-maze, radial-arm maze and the novel object recognition task were used for the exploration of cognitive functions. The hippocampus was isolated for biochemical assay of Malondialdehyde (MDA) and reduced glutathione (GSH) levels, catalase (CAT) and acetylcholinesterase activities (AChE). Whole brains, liver, kidneys and testes were isolated for histological studies. The aqueous extract of *H. cannabinus* seeds significantly reversed amnesia symptoms induced by chronic AlCl₃ administration in rats by decreasing the time spent in the discriminated arm, the reference memory errors and the working memory errors in the radial-arm maze. The exploratory time in the novel object recognition task was also significantly (p<0.05) ameliorated. Moreover, the plant extract significantly increases the CAT (p<0.05) and GSH but significantly reduced MDA (p<0.05) and acetylcholinesterase (p<0.01) levels in hippocampi homogenates. A reorganization of the pyramidal density of the hippocampus, a regression of vascular congestion in the liver and a disappearance of distal and proximal tubule clarification in kidneys has been observed. The amelioration of all these parameters confers potential anti-amnesic and antioxidant properties to the aqueous seeds extract of *H. cannabinus*.

**Key words:** *Hibiscus cannabinus*, Aluminum, memory, acetylcholinesterase, hippocampus.

**ABBREVIATIONS**

AD; Alzheimer's disease, AlCl₃; Aluminum chloride, MDA; Malondialdehyde, GSH; Reduced glutathione, CAT; Catalase, AChE; Acetylcholinesterase activities, Aβ; Amyloid-β, HFGN; Herbarium of the National School of Fauna, DI; Discrimination index, HCl; Chloridric acid, ANOVA; Analysis of Variance, SEM; Standard error of the mean, ZS; Zinc sulphate, HC; *Hibiscus cannabinus*, CA; Amon Horn, GD; Dentate Gyrus, AI; Aluminum.

**INTRODUCTION**

Alzheimer's disease (AD) is a neurodegenerative disease associated with aging. AD is characterized by the deterioration of memory, learning and other cognitive functions due to the progressive loss of neural function (Kim and Choi, 2015; Konar et al, 2019). Although the etiology of AD remains elusive, multiple factors such as low levels of acetylcholine (ACh), oxidative stress, accumulation of misfolded amyloid-β (Aβ) and neurofibrillary tangles in brain tissues and hyperphosphorylation of tau protein in neurons play definitive roles in the pathophysiology of AD.
(Novak et al., 2018). AD manifests anatomically with neuronal and synaptic loss, particularly in the hippocampus, frontal cortex, temporal lobe, parietal lobe and cingulate gyrus (Scott et al., 2015; Berté et al., 2018). AD is estimated at 70% of dementia and is the fourth leading cause of death in the elderly, after heart disease, cancer and stroke. In sub-Saharan Africa, AD is the most common type of dementia with prevalence up to 21.60% (Olaniyi and Mbuyi, 2014). Metal dyshomeostasis has been implicated in many neurodegenerative disorders. Metals play a major catalytic role in the production of free radicals and much attention was given to the role of many metals including iron, mercury, copper and aluminum in AD. Although AD causes are not fully known, Aluminum has been suggested as a causal factor (Manap et al., 2019). Aluminum is used in manufacturing some cooking utensils and food preservatives, in water and naturally in dietary products.

It is also used in pharmacological drugs as anti-acid against gastric ulcers and as an antiperspirant (Abdulmalek et al., 2015). Aluminum does not have a biological role in the body. However, it is a well-established neurotoxin and it is suspected to be linked with various neurodegenerative diseases including Parkinson’s dementia, amyotrophic lateral sclerosis and Alzheimer's disease (Masahiro et al., 2011). Chronic aluminum intoxication reproduces neuropathological marks of AD which to date remains without cure. Existing drugs for Alzheimer’s disease such as memantine, donepezil, rivastigmine and galantamine offer only symptomatic relief by providing temporary palliative therapy. Besides, these medications are endowed with side effects such as fatigue, anxiety, confusion, sleep disturbances, vomiting, nausea and drug resistance after prolonged treatment (Ba et al., 2019). Medicinal plants have historically proven their value as a source of molecules with therapeutic potential and still represent an important pool for the identification of novel drug leads (Atanasov et al., 2015). Bilgic et al. (2018) reported that polyphenols and fatty acids are extensively shown to possess promising antioxidative actions in Alzheimer’s diseases. Species of the genus Hibiscus such as Hibiscus sabdariffa and Hibiscus sinensis have improved memory disorder in rats and mice respectively due to the presence of flavonoids and saponins which acts as antioxidant (Nade et al., 2010; Bayani et al., 2018).

*Hibiscus cannabinus* (H. cannabinus), a member of the Malvaceae family, has been used in traditional medicine for the treatment of various inflammatory diseases (Chaudhari et al., 2015; Ayadi et al., 2016). The *H. cannabinus* seed contains a variety of different compounds such as phenolic compounds, flavonoids, essential oils, mono and polyunsaturated fatty acids (Kai et al., 2015; Jaihyun et al., 2017). Moreover, in a preliminary study carried out within our laboratory (unpublished results), the aqueous extract of *H. cannabinus* seeds completely reversed behavioral disorders and some organ dysfunctions induced by chronic exposure to lead acetate in rodents. This extract could therefore have beneficial effects on certain neurological pathologies induced by heavy metals. The present study was thus to investigate the neuroprotective effects of the aqueous extract of *H. cannabinus* seeds on aluminum chloride induced Alzheimer’s disease in male Wistar rats. Further we evaluated the potential of this extract to prevent toxic effects of the aluminum on some important organs of the body.

**Materials and Methods**

**Plant material and preparation of the extract**

*H. cannabinus* seed was bought in a local market of Maroua (Far-North Region, Cameroon) in January 2017. A voucher specimen was identified by Mr. Emmanuel VOUNSERBO and deposited at the Herbarium of the National School of Fauna (no. HEGF 5338), Garoua, Cameroon. After washing and grinding, 150 g of the obtained powder was macerated in 600 mL of distilled water for 12 h at room temperature. The combined solutions were filtered with Whatman paper No. 4 and evaporated using an oven (50°C, during 24 h) to yield 6.3 g of aqueous crude extract.

**Phytochemical analysis**

**Total Phenolic content**

The Folin-Ciocalteu assay as described by Makni et al. (2018) was used for the determination of total phenolics present in the *H. cannabinus* seeds extract. Briefly, 10 µL of 3 appropriately diluted extracts or standard gallic acid solutions was mixed with 20 µL of a Folin-Ciocalteu reagent solution in a 96-well plate and mixed gently. After five min, 30 µL of freshly prepared 20% sodium carbonate was added followed by 158 µL of distilled water. The reaction mixture was kept in dark for 2 h and the absorbance of the blue coloration formed was measured using the spectrophotometer at 765 nm against the blank solution prepared by the same procedure described above except the extract solution was substituted by 10 µL of ethanol. The total phenolic content was expressed as mg gallic acid equivalent (mg GAE/g).

**Determination of total Flavonoid content**

Total flavonoids in the extracts were determined using the method described previously by Makni et al. (2018). A 30 µL aliquot of appropriately diluted sample solution was mixed with 180 µL of distilled water in a well plate, and subsequently 10 µL of a 5% aqueous NaNO₂ solution was added. After 6 min, 20 µL of a 10% of aluminum chloride
solution was added and allowed to stand for 6 min; then 60 l 
of 4% NaOH solution was added to the mixture and stood 
for another 15 min. Absorbance of the mixture was
determined at 510 nm versus a prepared water blank. Total 
flavonoids were calculated with respect to quercetin
standard compound (12.5, 25, 50, 75, and 100 µg/mL). All
values were expressed as milligrams of quercetin
equivalents per 1 g sample (mg QEEq/g sample).

**Determination of Flavonol content**

The flavonol content was measured using the method of
Makni et al. (2018). The rutin calibration curve was
prepared in a well of plate by mixing 40 µL of various
concentrations of ethanolic solutions of rutin with 40 µL
(20 mg/mL) aluminum trichloride and 120 µL (50 mg/mL)
sodium acetate. The absorbance at 440 nm was read after
2.5 h. The same procedure was used for 40 µL of plant
extract instead of rutin solution. All determinations were
carried out in triplicate. The flavonol content was
calculated using a standard curve obtained from various
concentrations of rutin (0-50 µg/mL). All values were
expressed as milligrams of rutin equivalents per 1 g sample
(mg REEq/g sample).

**Determination of condensed Tannin content**

The condensed Tannin content in the extracts and its
fractions were determined using the modified vanillin
assay. 10 µL of appropriately diluted sample solution was
mixed with 120 µL of 4% vanillin solution (in methanol) in
a well plate, and then 60 µL of concentrate HCl was added
and mixed. After 15 min, the absorbance of the mixture was
determined at 500 nm against a blank solution, which was
prepared by the same procedure described above except
the extract solution was substituted by 10 µL of water.
Different concentrations of catechin ranging from 25 to 300
g/ml were used as standard compound for the
quantification of total condensed tannins. All values were
expressed as milligrams of catechin equivalents per 1 g sample
(mg CEeq/g) (Makni et al., 2018).

**Experimental animals**

Male Wistar albino rats (n = 36), weighing (206 g) at the
beginning of the experiment, were obtained from the
animal breeding facility of the laboratory of the University
of Ngaoundéré, Cameroon. The animals were housed in
polyacrylic cages (6 animals/cage) and maintained in a
temperature and light-controlled room (25 ± 2°C, natural
cycle of light and dark succession). The animals were
acclimated to laboratory condition for 14 days before the
start of experiment. Prior to treatment, the animals were
fasted for 12 h. However, all animals were allowed to drink
water ad libitum. Rats were treated following the guidelines
of the Cameroonian bioethics committee (Reg N° FWA-
IRB00001954) and in accordance with NIH-Care and Use of
Laboratory Animals manual (8th Edition). The present
study was approved by the Ethic Committee of the Faculty
of Science of the University of Maroua (Ref. N°14/0261/
Uma/D/FS/VD-RC), Cameroon. Efforts were also made to
minimize animal suffering and to reduce the number of
animals used in the experiment. Each animal was tested in
only one behavioral test. The experiments were performed
in the morning (8-12 h).

**Drug Administration**

The rats were divided into six groups (six animals per
group): (1) control group received distilled water
treatment (10 mL/kg); (2) aluminum chloride (AlCl₃, 17
mg/kg) - alone - treated rats, as negative control; (3)
aluminum chloride - treated rats + zinc sulphate [ZS (5
mg/kg, p.o.)], as positive control; (4) aluminum chloride -
treated rats + the aqueous extract of H. cannabinus
treatment [HC (125; 250; 500 mg/kg, p.o.)]. H. cannabinus
dissolved in distilled water and administered daily by
oral gavage (p.o.) for twenty-eight consecutive days (1 h
prior to experimental manipulations). The different doses
of H. cannabinus (125; 250 and 500 mg/kg) used in this
experiment were selected from the previous study
conducted by (Nyang et al., 2016). Aluminum chloride (17
mg/kg) was dissolved in distilled water and was
administered once a day by oral gavage (p.o.), 30 min prior
to administration of the aqueous extract of H. cannabinus
seeds (Fatemeh et al., 2013). Zinc sulphate (5 mg/kg, p.o.)
dissolved in distilled water and was administered for
twenty-eight consecutive days (Kockaya et al., 2012). This
treatment schedule is seen in Table 1.

**Behavioral tests**

**Radial arm-maze task**

The radial arm-maze used in the present study consists of
eight arms, numbered from 1 to 8 (48 - 912 cm), placed
radially around a central platform (32 cm in diameter). The
apparatus was placed 50 cm above the ground. At the end of
each arm, there was a food cup (3.5 cm in diameter)
where the bait is to be deposited. The observer is placed in
corner so as not to distract the animal and no objects
should be moved during the experiment for orientation
purposes (Foyet et al., 2016). Before the performance of the
maze task, the animals were kept on restricted diet and
body weight was maintained at 85% of their free-feeding
weight over a week period, with water being available ad
libitum. In this test, the animals were accustomed to
the environment, placed on the central platform and allowed to
explore the maze for 5 min. The food was initially available throughout the maze, but was progressively restricted to the food cup. The animals were trained for 7 days to run to the end of the arms and consume the bait. Animals were trained for maze task performance by conducting daily training trial for 5 min.

Criterion performance was defined as consumption of all four baits or until 5 min had elapsed. The completely trained animals were chosen for the study. Briefly, each animal was placed individually in the center of the maze and subjected to working and reference memory tasks, in which same four arms (1, 3, 5 and 7) were baited for each daily training trial. The other four arms (2, 4, 6 and 8) were never baited. An arm entry was considered when all four limbs of the rat were within an arm. A reference memory error was recorded if the rat visited an unbaited arm. A working memory error was recorded if the rat returned to a corridor that it had already visited during the current test. Reference memory is regarded as a long-term memory for information that remains constant over repeated trials (memory for the positions of baited arms), whereas working memory is considered a short-term memory in which the information to be remembered changes in every trial (memory for the positions of arms that had already been visited in each trial). At the end of each trial the maze was cleaned with 70% ethanol to avoid residual odors.

**T-maze**

The T-maze test is a widely used for assessing cognitive abilities in rodents. It is a behavioral approach to study some aspects of cognition as alternation behavior, place recognition, object recognition, spatial discrimination, working memory and reference memory (Deacon and Rawlins, 2006). It consists of a departure compartment and two arrival corridors. Opaque guillotine doors fixed at the exit of the starting compartment as well as at the entrance of each of the arrival corridors make it possible to control access to the different areas of the labyrinth. At the end of each of the arrival compartments is a feed trough that can contain a food booster. The rats were placed one after the other in the starting arm of the T-Maze. After each passage, the device was cleaned with ethanol at 70° to remove as much as possible, the odorous traces left by the previous rat. This operation took place during three days following three phases: habituation, acquisition and retention. In the habituation phase, 1 h after the treatment, the rats become familiar with the T-maze for a period of 5 min. Food was placed in each of the inlet arms to promote exploration. Each rat was placed in the departure arm. After 15 s, all guillotine doors were opened. The animal could then choose one of the corridors of the arrival arms of the device indicating its preference. The arrival arm chosen the first time was called the preferred arm (right arm) and the arm not chosen was called the discriminated arm (left arm).

After the 5 min of observation, the rat was returned to its cage and the experimental device was cleaned with ethanol at 70° before the next rat was introduced. The following parameters were noted: the latency time for selecting the preferred arm, the time spent in the discriminated arm, and the number of times the rat returned to the starting arm. The acquisition phase began 24 h after the habituation session and 1 h after treatment. The corridor of the arm discriminated by the animal was closed then a booster (food) was placed in the arrival corridor of the arm preferred by the animal. The rat was placed in the departure compartment and allowed to move to the open lane (preferred lane during habituation). This exercise was done in 5 min for each animal. The device was cleaned with ethanol at 70° before the next rat was introduced. The following parameters were noted: the latency time to enter the preferred arm, the time spent in the preferred arm, the number of times back in the starting arm. The retention phase began 24 h after the acquisition session and 1 h after treatment. Each animal was placed in the device for 5 min and all incoming arms were opened. Reinforcements were placed in both corridors. The following parameters were noted during the 5 min observation time for each rat: the latency time to select the discriminated arm and the time spent in the discriminated arm.

**Novel object recognition test**

The principle of this test is based on the natural exploratory behavior of rodents. Animals naturally preferentially explore an unknown object (Ennaceur and Delacour, 1988).

### Table 1: Treatment schedule.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
<th>Doses (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal control treated with distilled water</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>Negative control treated with aluminum chloride</td>
<td>17 mg/kg, p.o</td>
</tr>
<tr>
<td>III</td>
<td>Aluminum chloride + zinc sulphate</td>
<td>17 mg/kg + 5mg/kg, p.o</td>
</tr>
<tr>
<td>IV</td>
<td>Aluminum chloride + H. cannabinus</td>
<td>17 mg/kg + 125 mg/kg, p.o</td>
</tr>
<tr>
<td>V</td>
<td>Aluminum chloride + H. cannabinus</td>
<td>17 mg/kg + 250 mg/kg, p.o</td>
</tr>
<tr>
<td>VI</td>
<td>Aluminum chloride + H. cannabinus</td>
<td>17 mg/kg + 500 mg/kg, p.o</td>
</tr>
</tbody>
</table>

*H. cannabinus: Hibiscus cannabinus, p.o: per os.*
The object recognition test was conducted over 3 days: the first day was the habituation phase (for 5 min), the next day was the training phase (for 5 min) and the third day was the test phase (for 5 min). During the habituation phase, the rat was placed in the arena without any object and explored the arena. Then, during the training phase, the rat was subjected to a first test (T1), during which two identical objects were placed in the arena in opposite directions. The rat was placed in the arena and was left there until it explored the two identical objects. Twenty-four (24) h after the first test (T1), the second test was carried out (T2) during the test phase. In this second test, a new object (N) replaced one of the objects presented in the first test, and the rat was exposed this time to two different objects: the familiar (F) and the new (N). The animal is considered to be exploring when it points its snout towards the object at least 2 cm away from it. After each passage, the device was cleaned with ethanol at 70° to eliminate as much as possible the odorous traces left by the previous rat. The distinction between the familiar object (F) and the new object (N) at T2 was measured by comparing the time spent exploring the familiar object (F) with the time spent exploring the new object (N). The discrimination index (DI), which represents the difference of the exploration time over the total time spent exploring the two objects at (T2), was determined according to the formula:

$$DI = \frac{N - F}{N + F}$$

Estimation of biochemical parameters brain tissue preparation

The rats were decapitated on day 28 after the last behavioral testing. The skull was cut open and the brain was exposed from its dorsal side. The whole brain was immediately removed and a 10% (w/v) homogenate of brain samples (0.03 M sodium phosphate buffer, pH 7.4) was prepared. The homogenate was centrifuged (15 min at 3000 rpm) and the supernatant was used for assays of CAT activities, total GSH content and MDA level. In addition, the level of AChE was estimated.

Estimation of antioxidant enzymes

Estimation of reduced glutathion

The reduced glutathion was measured following the procedure previously described by Ellman (1959). Briefly, the homogenate was added with equal volume of trichloroacetic acid (TBA, 0.67 %) containing 1 mM EDTA to precipitate the proteins. The mixture was kept for 5 min prior to centrifugation. The supernatant (200 μL) was then transferred to a new set of test tubes and the added 3 ml of the Ellman’s reagent (5, 5′-dithio bis-2-nitrobenzoic acid) (0.1 mM) was prepared in 0.3 M phosphate buffer with 1% of sodium citrate solution. Then all the test tubes rose to the volume of 2 mL. After the completion of the total reaction, the solutions were measured at 412 nm against blank. Absorbance values were compared with a standard curve generated from standard curve from known GSH.

Determination of CAT

Catalase (CAT) activity was assayed following the method of Sinha et al. (1977). The reaction mixture consisted of 750 μL phosphate buffer (0.1 M, pH 7.5), 50 μL supernatant. Reaction was started by adding 50μL H₂O₂ 0.16 M, incubated at 37°C for 1 min and reaction was stopped by the addition of 2000 μL of dichromate acetic acid reagent. The tubes were immediately incubated in a boiling water bath for 10 min and then cooled in tap water. Optical densities were read at 570 nm using a spectrophotometer. The catalase level in the samples was obtained from a calibration curve previously established. Catalase activity was expressed as μ moles of H₂O₂ consumed/min/g of tissue.

Determination of Malondialdehyde

MDA, which is a measure of lipid peroxidation, was spectrophotometrically measured using the thiobarbituric acid assay. 500 μL of supernatant added and briefly mixed with 250 ml of 1% trichloroacetic acid in 50 mM HCl and 500 μL of 0.67% thiobarbituric acid. Samples were maintained at 90°C for 10 min. Furthermore, samples were centrifuged at 3000 rpm for 10 min and supernatants were read at 530 nm. The results were expressed as nmol/g protein.

Estimation of acetylcholinesterase (AChE)

Acetylcholinesterase activity was estimated by using an artificial substrate, acetylthiocholine (ATC). In the medium, thiocholine released due to the cleavage of ATC by AChE is allowed to react with the -SH reagent 5,5′-dithiobis -2 nitrobenzoic acid (DTNB), which is reduced to a yellow colored anion called thionitrobenzoic acid, measurable at the wave length 412 nm (Ellman et al., 1961). Concentration of thionitrobenzoic acid was spectrophotometrically detected and taken as a direct estimate of the AChE activity.

Histopathological studies

On day 28, whole brains, livers, kidneys and testes were collected, fixed in 10% formalin for a week. The histomorphology was assessed from 5 μm sections of
Table 2: Concentrations of the secondary metabolites of *H. cannabinus* seeds extract.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Concentrations</th>
<th>Units</th>
</tr>
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<tbody>
<tr>
<td>Total phenolic</td>
<td>510.07± 9.85</td>
<td>mg Eq Gallic Acid/g dry weight</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>259.32 ± 41.29</td>
<td>mg Eq Quercetin/g dry weight</td>
</tr>
<tr>
<td>Favonols</td>
<td>130.38 ± 3.46</td>
<td>mg Eq Rutin/g dry weight</td>
</tr>
<tr>
<td>Cendensed tannins</td>
<td>64.83±5.62</td>
<td>mg Eq Catechin/g dry weight</td>
</tr>
</tbody>
</table>

Every value represents the concentration ± ESM of the different metabolites: mg: milligrams, eq: equivalent.

paraffin-embedded tissues following hematoxylin-eosin staining.

**STATISTICAL ANALYSIS**

All the results were expressed as mean ± SEM. Data for the T- maze were analyzed by analysis of variance (ANOVA) followed by Tukey's post hoc test in GraphPad Instat package version 8.00 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. Two-way repeated measures (mixed model) ANOVA followed by Bonferroni post tests were used to compare the two objects in the object recognition task and the radial-arm parameters in GraphPad Prism (version 8.00). Data for the biochemical tests were analyzed using two-way analysis of variance (ANOVA) followed by post hoc multiple comparisons using Bonferroni tests.  *p* < 0.05 was considered significant.

**RESULTS AND DISCUSSION**

**Results**

**Phytochemical screening**

The qualitative phytochemical screening of the aqueous extract of *H. cannabinus* seeds revealed the presence of phenols, flavonoids, saponins, tannins, triterpenes and cardiac glycosides. The concentrations of the principal secondary metabolites are mentioned in Table 2.

**Effects of *Hibiscus cannabinus* on working memory in the radial maze**

During all the experiment period in the radial arm - maze test, the number of working memory errors was significantly high (*p* < 0.05) in the aluminum chloride-treated rats compared to the distilled water-treated rats (Figure 1A). Chronic administration of *H. cannabinus* seeds extract decreased the number of working memory errors in rats, especially at the doses of 250 and 500 mg/kg on day 4 of treatment, but was maintained until the day 7 of the experiment (*p* < 0.05). The radial arm - maze test (Figure 1B) revealed a high number of reference memory errors in animals of all groups at the beginning of the experiment (Day 1). The number of errors drops from the second day, but for those exposed to aluminum chloride it remains significantly high from day 3 until the end of the experiment (*p* < 0.05, days 3 to 6; *p* < 0.001, day 7) while compared to normal control group. Administration of the aqueous extract of *H. cannabinus* seeds at the dose of 250 mg/kg significantly reduced reference memory errors on day 2 (*p* < 0.01), day 4 (*p* < 0.01) and day 5 (*p* < 0.05). The dose of 500 mg/kg significantly reduced the number of errors on day 2 (*p* < 0.05) and day 5 (*p* < 0.01).

**Effects of *Hibiscus cannabinus* on memory in the T-maze test**

Figure 2A shows the effects of *H. cannabinus* on long-term memory in the T- maze. This figure showed a significant reduction (*p*<0.001) of the time taken to choose the discriminated arm in rats exposed to aluminum chloride as compared to the distilled water treated group. This time increased significantly following the administration of zinc sulphate (*p*<0.001) as well as after 28 days treatment with the aqueous extract of *H. cannabinus* seeds, especially at a dose of 500 mg/kg (*p*<0.05). Besides this, animals treated with aluminum chloride spent more time in the discriminated arm than those receiving distilled water (Figure 2B). This time was reduced after administration of the aqueous extract of *H. cannabinus* seeds, but the significant reduction was noticed only at the dose of 500 mg/kg.

**Effects of the extract on memory in the novel object recognition**

All rats treated with the aqueous extract of *H. cannabinus* seeds and zinc sulphate, as well as the control animals took more time to explore the new object on day 28. However, the time taken by the animals subjected to aluminum chloride administration solely was significantly lesser than that of normal animals, indicating the impairment of the
memory by this heavy metal. *H. cannabinus* (250 mg/kg) significantly (p<0.05) ameliorated the exploratory time of the rats in this task as compared to aluminum chloride group (Figure 3A). Although the exploratory time increased at the dose of 125 and 500 mg/kg, these increments were not significant as compared to aluminum chloride group. Regarding the discrimination index data, ANOVA revealed overall differences between the groups. The results show that the aluminum chloride significantly (p<0.01) decreased the value of the discrimination index as compared to the distilled water treated group (Figure 3B). Nevertheless, the administration of the aqueous extract of *H. cannabinus* seeds, especially the dose of 250 mg/kg, significantly increase (p<0.05) the value of the
discrimination index with respect to the group of rats treated with aluminum chloride.

**Effect of the aqueous extract of *Hibiscus cannabinus* seeds on brain lipid peroxidation and antioxidant enzymes**

**CAT activity in the brain**

As shown in Figure 4A, aluminum chloride administration to the rats brought about a significant reduction (p < 0.001) in CAT level in the rat's hippocampus as compared to rats treated with distilled water. The administration of zinc sulphate to the aluminum chloride exposed rats just like the plant extract (500 mg/kg) resulted in a significant (p < 0.05) increase in CAT activity when compared with the group of rats exposed to aluminum chloride solely.

**GSH activity in the brain**

The effect of aqueous extract of *H. cannabinus* on glutathione content in the brain is summarized in Figure 4B. The GSH level of hippocampus homogenate in aluminum chloride group was found to be significantly lower (p < 0.01) than the GSH level in normal control group. At the end of the treatment period with *H. cannabinus* (125, 250 and 500 mg/kg), GSH level was found to be increased but not significant. Zinc sulphate restored the glutathione level to normal despite aluminum chloride exposure.

**MDA level in the brain**

As shown in Figure 5A, the administration of aluminum chloride solely significantly increased (p < 0.05) the MDA level. However, this trend was significantly reversed after 28 days of treatment of the AlCl₃ exposed rats with the aqueous extract of seeds of *H. cannabinus* at the doses of 250 and 500 mg/kg (p < 0.05). The standard drug zinc sulphate also significantly (p < 0.01) reduced the MDA expression in the brain tissue.

**Effect of aqueous extracts of *Hibiscus cannabinus* on brain AChE activities**

Aluminum induced memory impairment by decreasing cholinergic function, as measured by acetylcholinesterase (AChE) activities, the key enzymes involved in the degradation of acetylcholine. Aluminum chloride treatment significantly increased AChE activity (Figure 5) in hippocampus as compared to control rats. However, aqueous extracts of *H. cannabinus* seeds (250 and 500 mg/kg) co-treatment significantly attenuated the AChE hyperactivity in this region of brain as compared to control rats (p < 0.05; p < 0.01 respectively). It was observed that zinc sulphate was the most potent treatment in the reduction of AChE activity.

**Histopathological study**

Effects of the aqueous extract of *H. cannabinus* seeds on
Figure 3: Effect of *H. cannabinus* on memory (A) and discrimination index (B) in the object recognition test. Data was analyzed by one-way ANOVA followed by Tukey post-hoc test. Each column represents mean ± S.E.M. of 6 animals. **p < 0.01; ***p < 0.001, when compared to normal animals, δp < 0.05 when compared to Aluminum chloride treated group, AlCl₃: Aluminum chloride; ZS: Zinc sulphate; HC 125, 250, 500 : *Hibiscus cannabinus* (125, 250, 500 mg/kg).

铝氯化物诱导的海马组织结构紊乱。海马的结构异常（红色框），细胞密度几乎相同（C）和不同剂量的*H. cannabinus*种子（D, 125 mg/kg), (E, 250 mg/kg), (F, 500 mg/kg)。
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**Figure 4:** Effect of the aqueous extract of *H. cannabinus* administration on CAT (A) and GSH (B) level of the brain tissue. Data was analyzed by one-way ANOVA followed by Tukey post-hoc test. Each column represents mean± S.E.M. of 6 animals. **p < 0.01; ***p < 0.001 when compared to normal animals, δp < 0.05 when compared to Aluminum chloride treated group, AlCl₃ : Aluminum chloride; ZS : Zinc sulphate; HC 125, 250, 500 : *Hibiscus cannabinus* (125, 250, 500 mg/kg).

**Figure 5:** Effect of the aqueous extract of *H. cannabinus* administration on MDA (A) an AChE (B) level of the brain tissue. Data was analyzed by one-way ANOVA followed by Tukey post-hoc test. Each column represents mean± S.E.M. of 6 animals. *p < 0.05; **p < 0.01 when compared to normal animals, δp < 0.05; δδp < 0.01; δδδp < 0.01 when compared to Aluminum chloride treated group, AlCl₃: Aluminum chloride; ZS: Zinc sulphate; HC 125, 250, 500: *Hibiscus cannabinus* (125, 250, 500 mg/kg).

500mg/kg). However, a distortion of the pyramidal cell layer of the CA1 zone in an animal exposed to aluminum chloride (Figure 6B) but treated with zinc sulphate (red arrow) was noted (Figure 6C). In all groups, it was observed that the dentate gyrus cells (Figure 6D, 6E, 6F) appear to be intact (black arrow).

**Effects of *Hibiscus cannabinus* seeds aqueous extract on the structural architecture of liver, kidneys and testes**

The architecture of the livers (Figure 7), was normal (A) in the group of rats treated with distilled water. Following treatment with aluminum chloride, there was vascular...
congestion in the liver (green arrow). Nevertheless, the vascular congestion regressed following the treatment with the aqueous extract of *H. cannabinus* seeds particularly with the dose 250 mg/kg (7E) as well as with zinc sulphate (7C). Kidneys (Figure 8A) and testes (Figure 9A) showed normal architecture in the group of rats treated with distilled water. In the aluminum chloride treated group, there was clarification of the distal and proximal tubules.
Figure 7: Liver tissue of rats (H&E, X 40) showing (A) control group with normal histological, (B) AlCl₃ treated group with histopathological changes in the hepatocytes and vascular congestion, (C) Zinc sulphate treated group without deviation from normal histological structure, (D-E-F) Sections in liver of a rat treated with H. cannabinus extract (125, 250 and 500 mg/kg respectively). 1: central vein; 2: hepatocytes; 3: biliary duct; 4: hepatic artery; sinusoids spaces (blue arrow).

(yellow arrows) with light expand. At the base of the seminiferous tube membrane (9B), cellular disorganization (blue arrow) was observed. Spermatozoa of aluminum chloride-treated rats were smaller in number than those of rats treated with distilled water. Following the treatment with the aqueous extract of H. cannabinus seeds, clarification disappeared and a reorganization of the base of the seminiferous tube membrane was observed.
DISCUSSION

The current study investigated the effect of the aqueous extract of *H. cannabinus* seeds (125, 250 and 500 mg/kg, 28 consecutive treatment days) in the aluminum rat model for Alzheimer’s disease using the radial-arm maze, T-maze and novel object recognition task for assessing memory function. A previous results obtained in our laboratory clearly indicated that the aqueous extract of this plant could effectively prevent some neurological disorders including...
memory impairment induced by a heavy metal such as lead (unpublished results). The animal model employed in this current study is also through a heavy metal, known to mimic AD more closely than the scopolamine mouse model. The model of aluminum chloride was based on the fact that aluminum (Al) has the potential to be neurotoxic in humans.

Figure 9: Histology of testes X40 - Hematoxylin & Eosin. (A) Section in testis of a control rat showing normal seminiferous tubules. S, sperms; IT, interstitial tissue. (B) Section in testis of a rat treated with Aluminum chloride showing an increase of the lumen of seminiferous tubules without sperm cells; total alteration of testicular morphology (Al) and degenerated interstitial tissue (De). (C) Sections in testis of a rat treated with Zinc sulphate showing normal seminiferous tubules but with some alterations. (D-E-F) Sections in testis of a rat treated with *Hibiscus cannabinus* extract (125, 250 and 500 mg/kg respectively) showing increase of spermatogenic cells and lumen of seminiferous tubules with sperm stream (S).
and animals (Fatma et al., 2015). Aluminum is a ubiquitous metal that has been implicated in the etiology of neurodegenerative disorders and cognitive dysfunction, which exacerbates brain oxidative damage, causes neuronal inflammation and induces impairment in working memory, visuoception, attention and semantic memory. Aluminum exposure is known to be neurotoxic and can induce cognitive deficiency and dementia (Lin et al., 2015).

Radial - arm maze test is purely a hippocampal-dependent test to measure the hippocampal-dependent spatial working memory, but also long term memory in rodent (Al-Amin et al., 2016). In the maze, the target was not visible with the direct eyesight and the animals had to depend on the hippocampal encoding and retrieval of spatial memory. In the present study, animals exposed to aluminum chloride made more mistakes in finding the target, visiting the non-baited arms and returning regularly to the arms already visited. This result suggests that aluminum treatment causes impaired spatial working memory formation as well as reference memory formation. Conversely, rats exposed to AlCl₃ but regularly treated with the aqueous extract of *H. cannabinus* seeds at the doses of 250 and 500 mg/kg showed a significant reduction in the number of working memory errors and reference memory errors as compared to animals exposed to AlCl₃ solely. This suggests an inhibition of the neurotoxic action of AlCl₃ on the learning and memorisation process. T-maze and novel object recognition test have been utilized for the assessment of learning and memory in rats (Prabhu et al., 2014). In the recognition test, normal rats spend more time exploring a novel object than a familiar object (Romberg et al., 2012; Scott et al., 2013). Aluminum chloride exposure was responsible for a marked decline in cognitive performance of rats in the T-maze test, with a significant reduction (p < 0.001) of the time taken to choose the discriminated arm associated with an increase in the time spent in that arm.

Moreover, the aluminum-treated rats have increased the time taken to explore the familiar object as compared to the novel object in the NOR test. Indeed, high levels of aluminum in the brain can impair long period potentiation, which is thought to be the major physiological basis of learning and memory (Alawdi et al., 2016). Hippocampal fast learning relies on the mechanisms of synaptic long-term potentiation (LTP) (Nehls, 2016). The effect of aluminum chloride on memory is attributed to its ability to interfere with downstream effector molecules, such as GMP, involved in long-term potentiation (Lakshmi et al., 2015; Auti and Kulkarni, 2019). This disruption could thus explain the memory impairment and neurobehavioral deficits observed. In this work, the treatment of rats with *H. cannabinus* seeds increases the latency time to choose the discriminated arm and decreases the time spent in the discriminated arm. The increase in time spent to choose the discriminated arm is an indicator of improvement learning in rodents. The administration of aqueous extract of *H. cannabinus* in the aluminum chloride-treated rats resulted in a significant increase of the discrimination index along with a significant increase of the exploration time of the novel object. These results corroborate those obtained above in the radial maze task and suggest that the aqueous extract of *H. cannabinus* seeds possesses memory enhancing activities. This could be due to the presence of certain molecules of the aqueous extract likely to protect against the action of aluminum chloride. Taken together, behavioral test results suggest that the aqueous extract of *H. cannabinus* seeds would possess protective properties against aluminum chloride induced neuronal toxicity.

In this animal model, administration of AlCl₃ significantly increased the AChE activity. Studies have shown that aluminum chloride-mediated acetylcholinesterase hyperactivity, affects cholinergic neurotransmission and leads to learning disability and memory loss in experimental rats (Khalifa et al., 2019). Aluminum can also induce AChE activity by affecting its peripheral sites and changing its secondary structure (Prema et al., 2016). AChE is an enzyme which, by degrading ACh, directly impacts cholinergic transmission and is thus involved in the pathophysiology of AD (Hu et al., 2019). The increasing of the AChE level indicates a gradually decrease of cognitive function (Chunyue et al., 2019). The decrease of AChE concentration prevents the loss of ACh thereby increasing the cholinergic transmission and therefore used for diminishing the symptoms of AD patients (Fu et al., 2020). The aqueous extract of *H. cannabinus* seeds significantly decreased the concentration of AChE and especially at doses 250 and 500 mg/kg. In the radial maze paradigm, the extract significantly enhanced long-term memory by reducing the number working and reference memory errors. This hypothesis was further confirmed in the T-maze and novel object recognition test. Moreover, as oxidative damage is mediated by free radicals, it was necessary to investigate the status of endogenous antioxidant enzymes. In fact, endogenous antioxidant enzymes are the first line of defence against free radical damage under oxidative stress conditions and the lipid peroxidation which is one of the main manifestations of oxidative damage.

In the present study, the administration of AlCl₃ to the animals also showed a significant increase in the MDA and significantly decreases GSH (p<0.01) and CAT (p<0.001) activity. The elevation of lipid peroxidation in brain of Aluminum chloride treated rats was thus evidenced by increased production of MDA. It has been reported that AlCl₃ restrains the NADH activity and results in the increase of reactive oxygen species level, which reduces the antioxidant defense mechanism. Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) are produced by normal metabolism and are involved in various physiological and pathological conditions. When there is an imbalance between the antioxidants and oxidants, the free radicals accumulate leading to vigorous damage to
macromolecules such as nucleic acids, proteins and lipids (Rather et al., 2019). Moreover, AlCl3 can also potentiate the activity of ferrous (Fe2+) and ferric (Fe3+) ions to cause oxidative damage, leading to neurodegeneration (Nemat et al., 2013). Zinc sulphate treated rats showed a significant increase in reduced GSH and CAT content compared to aluminum chloride-treated rats, suggesting antioxidant effects. In addition, administration of zinc sulphate in aluminum chloride-treated rats resulted in a significant (p < 0.01) decrease in MAD relative to aluminum chloride treated rats, indicating its role in the decrease in lipid peroxidation and its protective role in oxidative damage. The treatment of rats with *H. cannabius* seeds significantly decreased (p < 0.05) the MDA concentration and significantly elevated (p < 0.05) the CAT and GSH activity in the hippocampus, especially at the dose of 500 mg/kg. MDA is a naturally occurring endogenous product of lipid peroxidation and prostaglandin biosynthesis and its value in an organ is a measure for the ability of this organ to handle the oxidative stress it is exposed to.

Catalase is the enzymatic antioxidant, while GSH is the most abundant intracellular non-enzymatic antioxidant which is capable of removing ROS in the brain. The results obtained in the present study suggest that aqueous extract of *H. cannabius* seeds might reduce lipid peroxidation and elevate the antioxidant enzyme status in the hippocampus of rats exposed to aluminum chloride. Since aluminum accumulates in all regions of the brain with the maximum being in hippocampus, one of the most vulnerable regions and key site of learning and memory, histological analysis was done (Fatma et al., 2015). In the present study, histopathological analysis revealed a decrease in the population of pyramidal cells and cellular disorganization at the CA1 and CA4 region of the hippocampus of the rats exposed to AlCl3 solely while the cell density was almost intact in zinc sulphate-treated animals and in those treated with the aqueous extract of *H. cannabius* seeds (250 and 500 mg/kg). Neurons in the CA1 and CA3 areas are critically important in establishing the correct route during the learning period while CA1 neurons and dentate gyrus network are active in the acquisition of spatiotemporal information and memory (Nehls, 2016). It is known that aluminum chloride is responsible for neuronal death in the hippocampus (Alawdi et al., 2016). Furthermore, aluminum salts have also been reported to cause cell depletion in the hippocampus and degeneration of cholinergic terminals in the cortical areas (Azza et al., 2016). It was observed that sections of rat brains treated with AlCl3 (17 mg/kg) for 4 weeks revealed a marked shrinkage in size of pyramidal cells with pyramidal cells loss of in CA1 and CA3 regions of the hippocampus caused by a chronic inflammation and oxidative damage (Fatma et al., 2015).

In the other organs (liver, kidneys and testes), the treatment with the aqueous extract of *H. cannabius* seeds maintained the integrity of the hepatic, renal and testicular parenchyma. These results are in perfect agreement with the behavioral and biochemical data which showed the protective effect of this plant. The beneficial effects of the aqueous extract of *H. cannabius* seeds in this study may be due to its antioxidant activity. The quantities of phenolic compounds obtained in *H. cannabius* seeds are greater than those of other plants used in the same region for various therapies like *Tetrapleura tetraptera* (273.48 ± 1.82 mg QE/g DE for Polyphenols) (Saague et al., 2019). Recent study by Jaihyunk et al. (2017) identified 5 major compounds in the aqueous extract of *H. cannabius* seeds: p-hydroxybenzoic acid, vanillin, p-coumaric acid, syringic acid and gallic acid. These compounds have all the anti-inflammatory and antioxidant properties. The neuroprotective effects of *H. cannabius* could therefore be explained by the presence of these phenolic compounds.

**CONCLUSIONS**

In conclusion, our results demonstrated that aqueous extract of *H. cannabius* seeds suppresses the AlCl3 induced learning and memory impairments, oxidative stress, AChE hyperactivity and other toxic effects. These effects may be due to the synergistic action of its phenolic components. However, supplementary pharmacological and phytochemical investigations will be necessary to clarify the molecular mechanisms of neuroprotection.

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**REFERENCES**


