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INTERFERENCE OF BRAIN OXIDATIVE STRESS MARKERS PROFILE AND AMNESTIC TENDENCY IN WISTAR RATS TREATED WITH DOSES OF CANNABIS SATIVA LEAVES

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ABSTRACT
The uncontrolled repeated use of Cannabis sativa remains a challenge for the potential medical usefulness of the plant. Cannabis being a psychoactive substance with different physiological properties, the onset and extent of its effects are often a factor of the mode of consumption. This present study is aimed at investigating the effects of daily oral ingestion of C. sativa on spatial memory and its changes in oxidative stress marker activities in wistar rats. Twenty-five albino wistar rats were acclimated to laboratory condition for fourteen days, following which they were separated into 5 groups of 5 animals each. Group I were used as control receiving only distilled water orally, Group II-IV were administered with 0.1ml, 0.2ml and 0.3ml cannabis via oral route respectively for 21days, while group V animals were administered with epinephrine. Cognitive activities were assessed using Passive Avoidance test, Barnes maze test and Navigation maze test. The brain oxidative stress markers that were used to determine stress activities include Superoxide dismutase, catalase, reduced glutathione and malondialdehyde. Results obtained were analyzed and some activities were statistically significant in comparison to the control group. Cannabis significantly ($p< 0.05$) decreased the activity of Superoxide dismutase, reduced glutathione and malondialdehyde and an increase in catalase activity when compared to the control. From the results obtained, it can be concluded that the animals that were administered with cannabis displayed significantly reduced anxiety, increased stress and impairment of memory compared to the control. Observations from the result shows that cannabis modulated appreciable oxidative stress tendencies due to increase in reactive oxygen species production characterized of stress.

KEWYWORDS: Cannabis sativa, memory, superoxide dismutase, catalase, glutathione, malondialdehyde.

INTRODUCTION
According to Maurga (2004)[1] some plants have been noted to have medicinal value and has been used worldwide since ancient times in folkloric medicine for treatment of various human ailments and is still prevalent in developing countries till date. This use of medicinal plants in the treatment of diseases and dysfunctions which goes back to several millennia, has contributed immensely to the development of pharmaceuticals since about 25% of modern drugs are derived from plants.[2] As stated by the World Health Organisation (WHO), about 80% of the world populations (over 4 billion) today depend on plant-based medicine for their health care needs.[3] as several plants contain active principles that have been proven to be beneficial through extensive laboratory tests and repeated clinical trials.[4] Hence, they have been extensively used in production of synthetic drugs as about 25% of active compounds in synthetic drugs currently prescribed were first identified in plant sources. Some plants have also been said to have some neuroactive effects on human. One of the most widely known plant with a long history of use both as a medicinal agent and intoxicant is the *Cannabis sativa* L.[5] It is also known as marijuana in America and hashish in the Middle East.[6] It is an annual herbaceous flowering plant indigenous to eastern Asia but now of cosmopolitan distribution due to widespread cultivation.[5] It has been cultivated throughout recorded history, used as a source of industrial fiber, seed oil, food, recreation, religious and spiritual moods and medicine.[7][8][9][10] However, the species *C. sativa* is generally consumed for its psychotropic effects and is

seen as the most widely used illicit medicinal plant with an estimated number of 119–224 million users worldwide.[11][12] It is majorly abused by adolescence and young adults all over the globe [5] in their various preparations such as pot, cannabis, grass, weed, hemp and joint.[13]

Though Cannabis has several effects on multiple organ systems, its effect is mostly exerted on the CNS as a psychoactive agent due to presence of its main psychoactive ingredient, Tetrahydrocannabinol (THC), which is responsible for most, if not all, of the effects associated with the use of cannabis.[14] [15] The brain is one of the most metabolically active tissues and is particularly sensitive to toxicity especially those associated with neurotoxic effects of drugs of abuse which is commonly associated with oxidative stress,[16,17] mitochondrial dysfunction, apoptosis and inhibition of neurogenesis, in addition to other mechanisms.[18] Oxidative stress from oxidative metabolism cause disruptions in normal mechanisms of cellular signaling[19], base damage, strand breaks in DNA[20] as well as linked to certain cardiovascular diseases, chronic fatigue syndrome and hyperoxia[21], and suspected to be important in neuro-degenerative diseases such as Lou Gehrig's disease, Parkinson's disease, Alzheimer's disease, Huntington's disease, depression, and multiple sclerosis.[22] Hence, as a critical phase for cerebral development, exposure to addictive substances during the adolescence phase of life leads to various alterations in brain functions that can be translated into functional consequences throughout life.[23] In recent years, there has been noticeable increase in cannabis and its products consumption among teenagers and young adults.[24]

Though cannabis is broadly supposed to be a safe recreational drug, its use is increasing among adolescents, which upon repeated exposure may develop some unfavourable effects on the brain's functional connectivity, intelligence, and cognitive performance.[25,26,27] Hence, the need to investigate the effect of cannabis in inducing oxidative stress and amnestic tendency. The regular use of cannabis is of great concern since it is associated with an increased possibility of deleterious consequences.[28] This is coming at the back of several evidences that shows that exposure to *cannabis* can lead to health challenges such as; motor skills impairment.[29][26]

MATERIALS AND METHODS

Animal

A total of twenty-five male wistar albino rats weighing 130-150g were obtained from animal house of the University of Port Harcourt. The rats were kept in clean disinfected wooden cages with saw dust as beddings in the animal house, with 12hours light/dark cycle and 50-60% humidity at a temperature of about 30^{o}C and were allowed to acclimatize to the new environment for two weeks, with free access to clean water and animal feed. The rats were weighed using an analytical weighing balance at commencement of the experiment.

Cannabis Sativa Leave

Dried leaves of 500g of cannabis sativa were blended into fine powdered form. The powdered form was soaked in 2 litres of distilled water and was evaporated to yield extract of total weight of 30g, which was stored at room temperature until ready for use.

Experimental Design

A total of twenty five albino wistar rats were weighed and divided into five groups of five animals in each group.

Groups	Number of Animals	Treatment
Group I (control)	5	Feed + water ad libitum
Group II	5	Feed + water ad libitum + 100g rat cannabis
Group III	5	Feed + water ad libitum + 300g rat cannabis
Group IV	5	Feed + water ad libitum + 500g rat cannabis
Group V	5	Drug Epinephrine- treated (0.3ml/100g)

After 7 days of treatment with different doses of cannabis sativa in the test groups, neuro-cognitive behavioural test commenced beginning with passive avoidance test, then to Barnes maze and finally navigational task in no particular order every experimental day. At the end of three weeks, all the animals were sacrificed by cervical dislocation, and the brain were rapidly dissected and then prepared for biochemical assay for the oxidative stress markers (SOD, MDA, GSH and CAT).

Blood sample preparation

After the animals were sacrificed and blood collected from the randomly selected test animals, the blood samples were centrifuged for 10 minutes at 4^{o}c.

Brain Oxidative Stress Marker Determination

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) are a group of metalloenzymes that are found in all living things. SODs are the front line of defence against reactive oxygen species mediated injury (Kangralkar, 2010). Superoxide dismutase activity was determined according to the method of McCord and Fridovich (1969). Briefly, 0.01 ml of the brain homogenate was mixed with 0.2 ml of 0.1 M EDTA containing 0.0015 % NaCN, 0.1 ml of 1.5 mM NBT and phosphate buffer with pH 7.8 to a total volume of 2.6 ml. On adding 0.05 ml of riboflavin, the absorbance of the solution was measured against distilled water at 560 nm. All the tubes were illuminated uniformly for 15 minutes and absorbance of the blue

color formed was measured again. Percent of inhibition was calculated after comparing absorbance of sample to the absorbance of control (the tube containing no enzyme activity). The volume of the sample required to scavenge 50 % of the generated superoxide anion was considered as 1 unit of enzyme activity and expressed in U/L protein.

Malondialdehyde (MDA)
The level of lipid peroxidation was measured as malondialdehyde (MDA) according to the method of Ohkawa et al. (1979). Lipid peroxidation: The level of lipid peroxidation in the tissue was measured as malondialdehyde (MDA) according to the method of Ohkawa et al. (1979). Absorbance of the clear supernatant was measured at 532 nm against butanol: pyridine mixture. The MDA level was calculated and is expressed in µmol/L.

Catalase (CAT)
CAT is a common and very important antioxidant enzyme which catalyses hydrogen peroxide to water and oxygen (Ossowski 1993). Catalase breaks down two hydrogen peroxide molecules into one molecule of oxygen and two molecules of water in a two-step reaction (Deisseroth 1970). Catalase activity was evaluated according to sadauskiene et al (2018). The obtained result was expressed in U/ml.

Glutathione peroxidase (GPx)
Glutathione peroxidase activity was determined according to the method of Hafeman et al. (1974). The absorbance of the yellow colored complex was measured at 412 nm after incubation for 10 minutes at 37 o C against distilled water.

Cognitive Tests
Passive avoidance test
The passive avoidance test is a fear elevated test used to access memory and learning in rats. In this test, subjects learn to avoid an environment in which an aversive stimulus was previously delivered. The passive avoidance task is useful for evaluating the effect of novel chemical entities on learning and memory as well as studying the mechanisms involved in cognition.

Principle of Operation
The passive avoidance box is a trough shaped alloy divided into a light and a dark compartment. The white compartment is free of aversive stimulus and a dark compartment is equipped with shocks. Basically, passive avoidance principle involves timing of transition i.e time that the animal take to move from the white compartment to the dark compartment.

Procedures
- The animals were placed in the light compartment and allowed to roam. Here, a flash of light occurs which makes the rats uncomfortable and causes them to move into the dark compartment.
- When the animal steps into the dark compartment, a mild foot shock which last for about 1 to 2 seconds is given forcing the animal to exit the dark compartment.
- The animal is monitored and the transition time it took the animal to re-enter the dark compartment was recorded for a maximum of 300 seconds.
- The procedure was repeated for all animals for three consecutive weeks with three trials on each day.
- At the end of the 5 minutes, the animal was returned to its home cage and the box was wiped down using cotton wool dipped in 70 %ethanol, and left to dry before another animal was introduced into the box.

Barnes maze test
The Barnes maze is a behavioural test that was originally developed to study spatial learning and memory in rats (Barnes, 1979). It is a hippocampal-dependent task where animals learn the relationship between distal cues (place learning) in the surrounding environment and a fixed escape location (Williams, 2003).

Principles of operation
The typical Barnes maze setup consists of an elevated circular platform with evenly-spaced holes around the perimeter. An escape tunnel is mounted underneath one hole while the remaining holes are left empty. Rats find bright light and open spaces aversive and would therefore want to escape to somewhere dark. The escape tunnel is maintained at a fixed location for the duration of training, which involves multiple daily trials spread over several days. The time it takes to escape into the dark hole is then recorded.

Procedures
- The maze was set up by attaching the escape tunnel to the platform. The maze should be kept away from any noise distraction.
- The maze was cleaned with 70% ethanol before the start of the test to remove any smell or dirt.
- Rats were placed at the centre of the elevated circular platform.
- The circular surface was then spin clockwise with minimal force.
- When the circular surface stopped rotating, the time at which it took the rat to escape from the open space to the dark tunnel was recorded for a maximum of 300 seconds.
- The procedure was repeated for all animals for three consecutive weeks with three trials on each day.
- At the end of the 5 minutes, the animal was returned to its home cage and the maze was wiped down using cotton wool dipped in 70 %ethanol, and left to dry before another animal was introduced on the maze.

Navigation maze test
The navigation maze test is used to examine spatial learning and memory. It is used in assessment of

exploration, path planning and navigation which depends on learning and memory capacities to form cognitive maps. It is used to test the effects of lesion to the brain in areas concerned with memory.

Principles of operation

The apparatus has two doors, an entrance and an exit door. It is made of fine wood and glass. The objective was to test whether the animals could return to a home site using the sense of direction.

Procedures

- The animals were placed at the entrance door of the navigational maze box and the stop watch was started.
- The animals were allowed to find their way to the other door.
- The time the animal to find its way back was recorded for a maximum of 300 seconds.
- The procedure was repeated for all animals for three consecutive weeks with three trials on each day.
- At the end of the 5 minutes, the animal was returned to its home cage and the maze was wiped down using cotton wool dipped in 70 % ethanol, and left to dry before another animal was introduced into the box.

Statistical Analysis

The quantitative data were presented in charts while qualitative data were represented in tables. Data obtained for the different sets of tests were analysed using Analysis of Variance (ANOVA) and Hoc test, $P<0.05$ was considered to be statistically significant. The analysis was performed using statistical package for Social Sciences (SPSS).

RESULTS

Table 2: Pattern of response of oxidative markers after 21 day-treatment with cannabis sativa.

Groups	Malondialdeyde (mmol/ml ± sem)	Catalase (U/ml ± sem)	Superoxide dismutase (U/ml ± sem)	Glutathione reductase (U/ml ± sem)
Control group	9.67 ± 0.26	105.08 ± 3.18	96.33 ± 0.82	4.97 ± 0.57
100mg/kg cannabis	5.77 ± 0.78	108.23 ± 8.97	93.33 ± 2.04	4.01 ± 0.34
300mg/kg cannabis	6.49 ± 0.60*	111.29 ± 1.36	92.67 ± 2.45	4.83 ± 0.062
500mg/kg cannabis	8.60 ± 0.26	128.24 ± 0.91*	93.99 ± 0.41	4.62 ± 0.26
Epinephrine group	9.35 ± 0.21m,	125.23 ± 1.65*	86.67 ± 4.08*	4.49 ± 0.16

Values are presented in mean ± sem. N=5. * means values are statistically significant when compared to the control values.

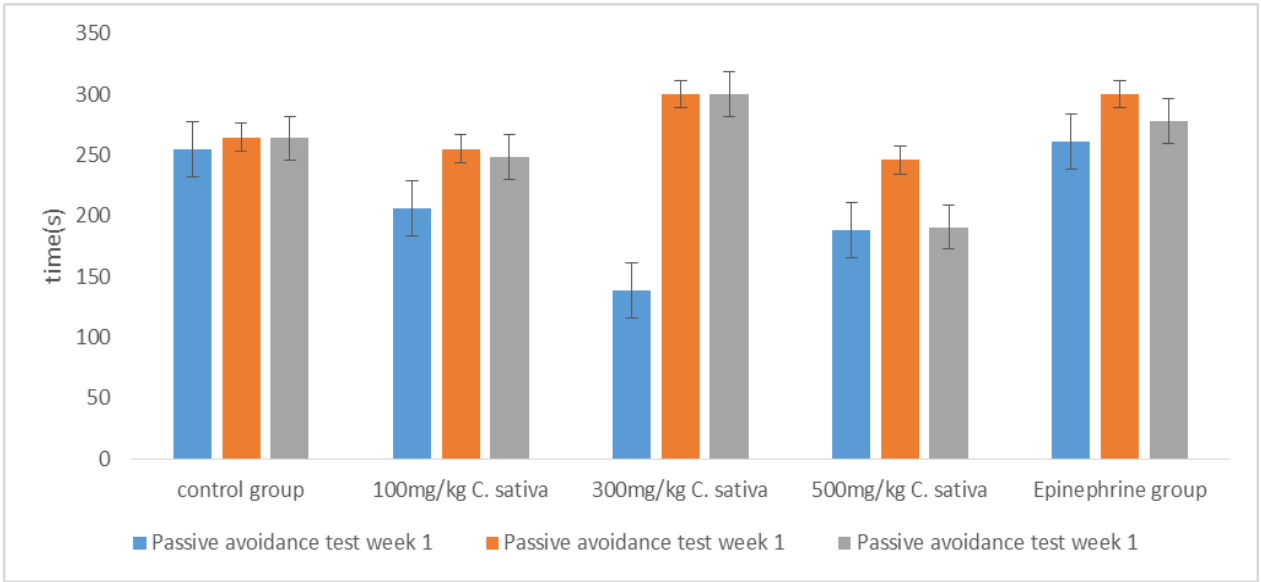

Figure 1: Response pattern in amnestic tendency in the test and control groups in week 1.

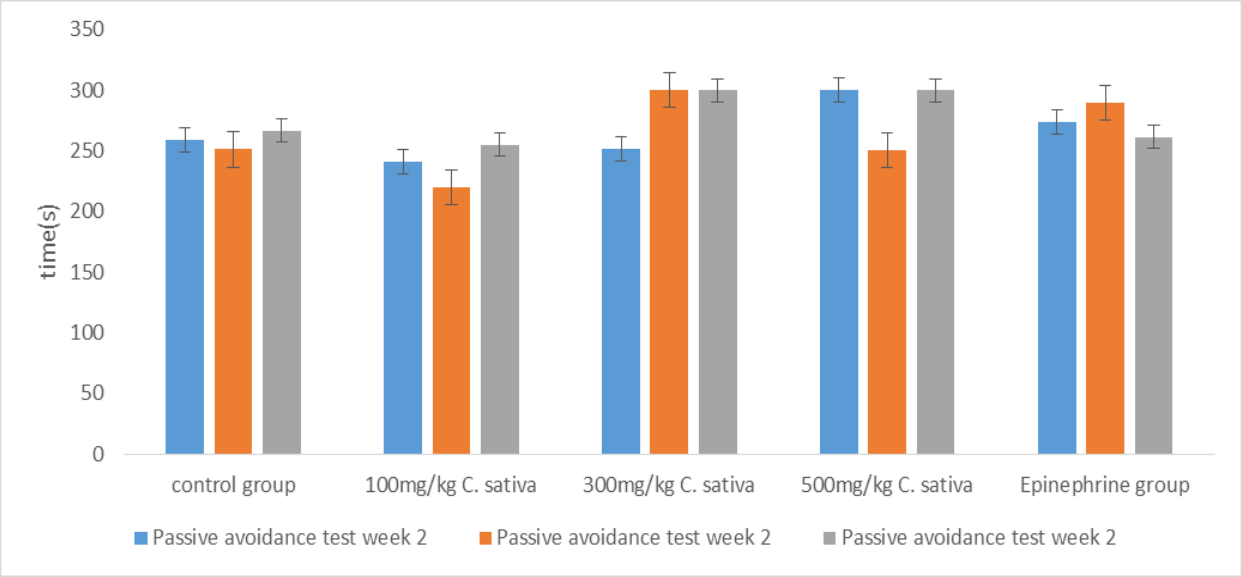

Figure 2: Response pattern in amnestic tendency in the test and control groups in week 2.

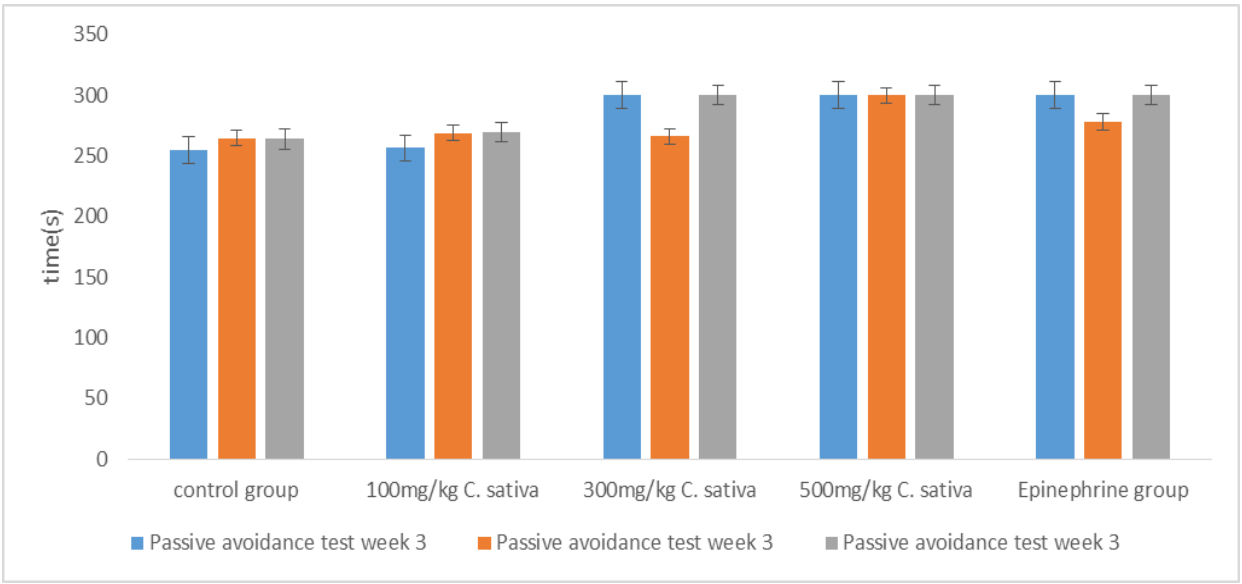

Figure 3: Response pattern in amnestic tendency in the test and control groups in week 3.

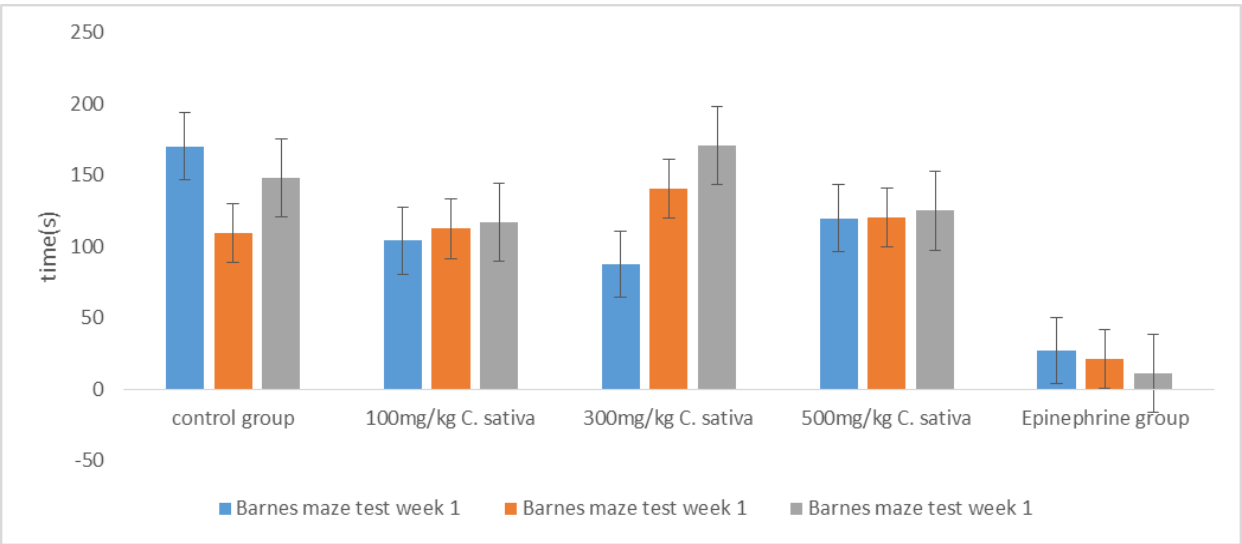

Figure 4: Pattern of response in spatial memory assessment in the test and control groups in week 1.

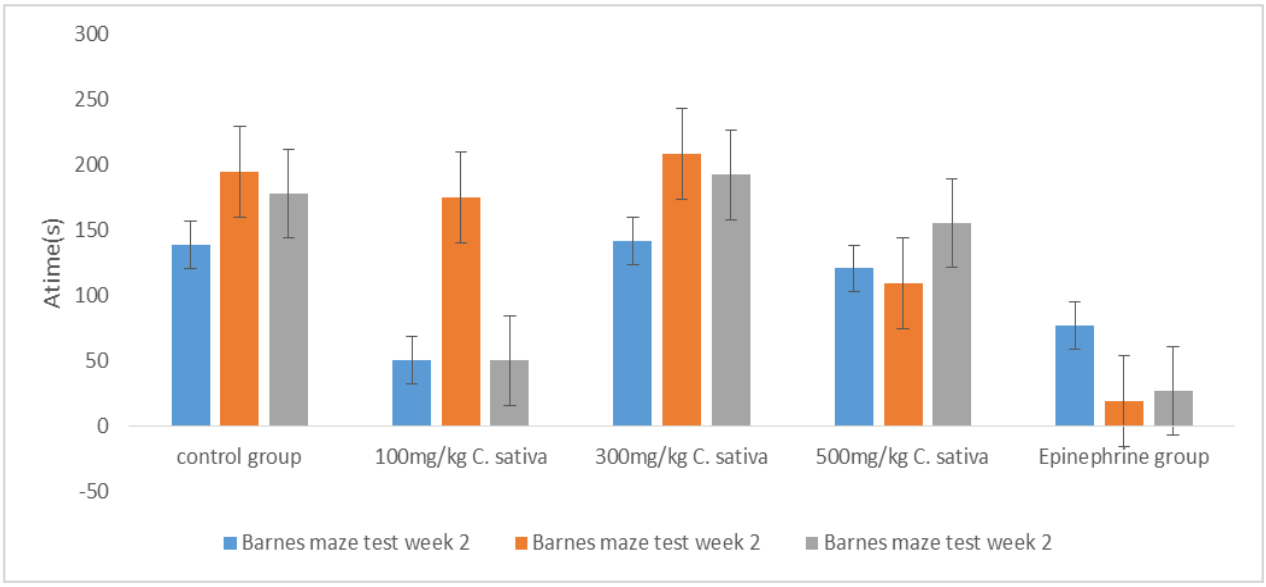

Figure 5: Pattern of response in spatial memory assessment in the test and control groups in week 2.

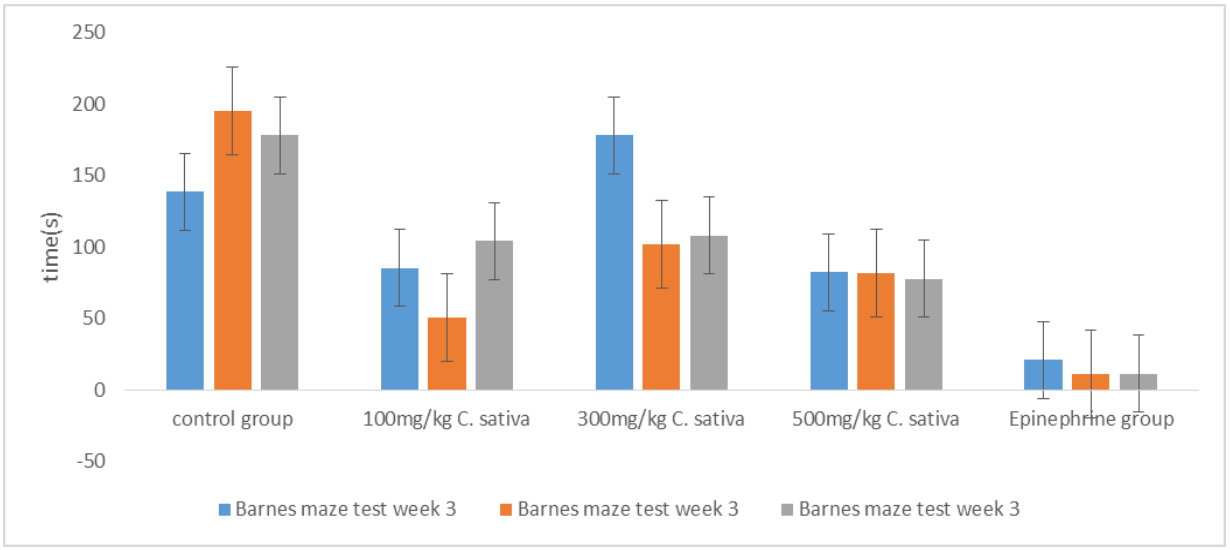

Figure 5: Pattern of response in spatial memory assessment in the test and control groups in week 3

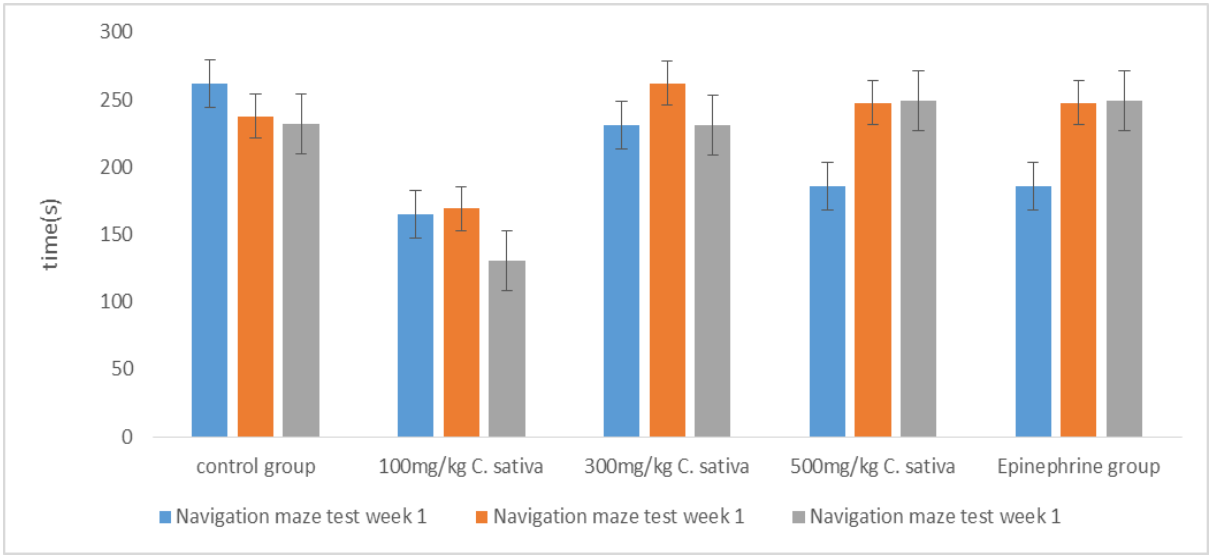

Figure 7: Pattern of adaptive locomotion assessment in the test and control groups in week 1.

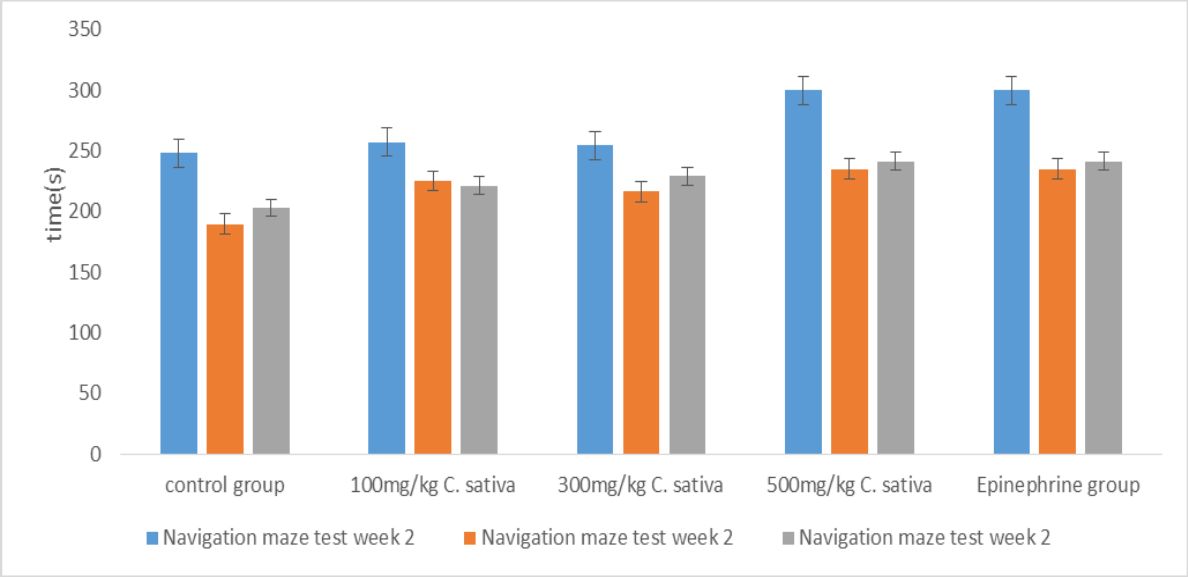

Figure 8: Pattern of adaptive locomotion assessment in the test and control groups in week 2

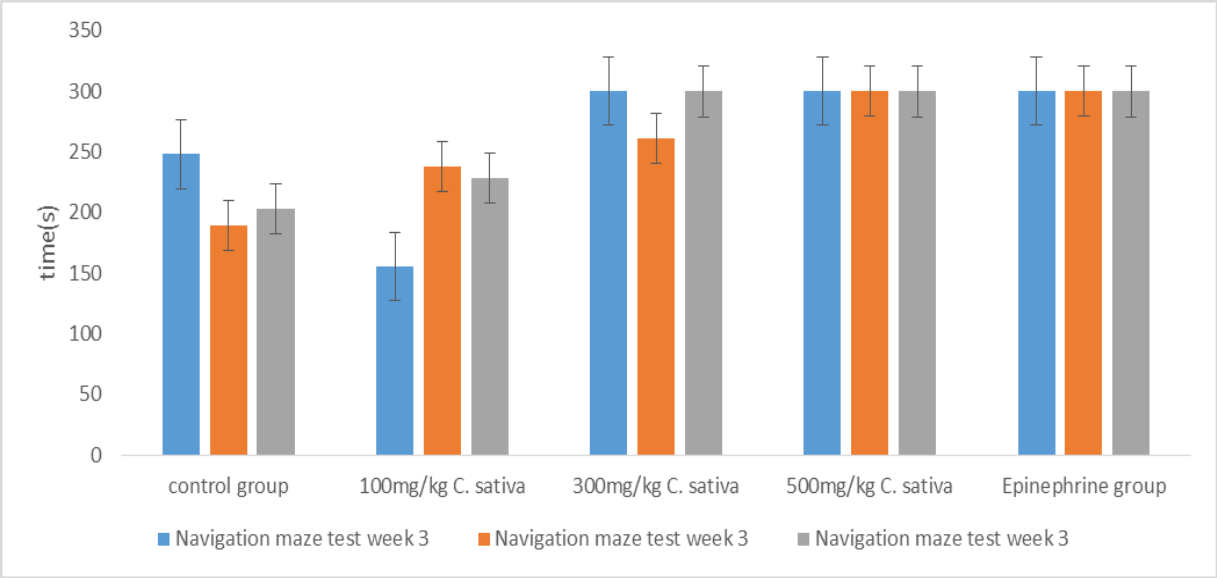

Figure 9: Pattern of adaptive locomotion assessment in the test and control groups in week 3

DISCUSSION

C. sativa is a known psychoactive compound used to improve a wide variety of health conditions [30]. Cannabis consumption has a variety of health effects, some are beneficial while other effects are not. The present study was designed to investigate brain oxidative stress markers and Memory in albino wistar rats administered with cannabis. The procedure was done using a series of test which are Superoxide dismutase, catalase, malondialdehyde and reduced glutathione for brain oxidative stress markers and Navigational maze test, passive avoidance test, Barnes maze test for memory activities. Increased pacing and exploratory activities in passive avoidance test, Barnes maze and navigation maze test suggested intact motor system and low anxiety The locomotor function and aggressive behaviour of the test animals were moderate relative to the controls in this study. The malondialdehyde levels in blood (plasma) from test animals that were fed with various doses cannabis was significantly reduced compared to the control group. However, for the epinephrine group there was no significant difference in MDA level when compared to the control.

The results from table 2 showed a decrease in SOD activity from the test animals that received cannabis when compared to the control. The epinephrine group revealed a much significant decrease in SOD levels when compared to the mean. The levels of superoxide dismutase in animals that consumed Cannabis were significantly lower relative to control mice. The significant loss of superoxide dismutase activity is a manifestation of oxidative damage of the brain.[31] Likewise, there was a dose-dependent decline in the levels of malondialdehyde in the cannabis diet groups, another indication of the oxidative damage potential of

Cannabis consumed orally in this study. Interestingly, the changes recorded in the different antioxidant biomarkers assayed in brain tissues of the study animals did not alter to any noticeable extent the behaviour of the mice.

The result obtained on the activity of catalase showed significant increase in CAT activity in all test groups and epinephrine when compared to the control group. The increase in catalase activity in plasma shows that cannabis sativa is capable of regulating oxidative stress and this is in accordance with[32] who observed in other studies the ability of hemp seed to increase CAT levels in plasma. The results obtained revealed that GSH level was lower in the plasma of test animals that were exposed to 0.2ml, 0.3ml and epinephrine when compared to the control group. 0.1ml group experienced much lower decrease in GSH level in comparison to the control. This may be possible by the initiation of repair to traumatically injured brain tissues as previously demonstrated in transgenic mice.[33]

The navigation maze test is used to examine spatial learning and memory. It is used in assessment of exploration, path planning and navigation which depends on learning and memory capacities to form cognitive maps. It is used to test the effects of lesion to the brain in areas concerned with memory is also capable of accessing damages to cortical regions of the brain. From the current study the navigational test involving three trials for the total period of three weeks.

For week one, results obtained from figure 6-9, there is a significant difference in test animals treated with 100mg/kg cannabis in comparison with the control group. It took the animals exposed to dose shorter time to navigate its way from the entrance to the exit door therefore showing heightened memory. However, there is no significant difference in test animals treated with higher doses and epinephrine when compared to different shock levels and control. The time taken to perform the navigational is a clear reflection of how alert the animal in challenging situations.

For week two from figure 8, there was no significant difference in 100 and 300mg/kg lcannabis treated animals when compared to control group. But in the group treated with 500mg/kg cannabis, there was a slight increase in time spent when compared to the control group. Similar pattern of response was recorded in week 3.

The passive avoidance test is used to teach subjects to avoid an environment in which an aversive stimulus was previously delivered. This also serve as a test for amnestic tendency in the rats.

For week one, results obtained (figure 1) there was a significant increase in time spent post aversive experience and latency in step through test animals treated with 300mg/kg cannabis and epinephrine at trial 2 in comparison with the control group. However, there is no significant difference in test animals treated with 100mg/kg when compared to control. For week two from table 4.2 (figure 4.2.2); there is a slight increase in time spent in 0.2ml and 0.3ml cannabis treated animals when compared to control group. Higher doses of cannabis led to preponderance and degree of awareness with no obvious sign of amnesia in week three (figures 2 & 3). This result suggested significant ($p< 0.05$) increase in spatial learning and memory at week 3 compared to week 1 and 2.

The Barnes maze is a behavioural test that was developed to study spatial learning and memory in rats (Barnes, 1979). It is a hippocampal-dependent task where animals learn the relationship between distal cues (place learning) in the surrounding environment and a fixed escape location (Williams, 2003). Different trails of this test allow to measure spatial learning, response preservation and memory.

Week 1 results obtained (figure 4) showed that there was a lower time to locate the escape box for all cannabis treated rats compared to the control group. The epinephrine group showed significantly ($p<0.05$) lower time to locate the escape box compared to the control. This significantly lower time to locate the escape box in the cannabis treated rats compared to the control rats indicates increased spatial learning and memory. Heavy marijuana use has been associated altered spatial learning and memory.[32]

Results obtained from week two (figure 5) showed a significant decrease in exploration to discovery time was noticed in trial 1 and 2 of 100mg/kg cannabis treated group when compared to control. Epinephrine group showed statistically significantly lower time to locate the escape box.

In week three (figure 6), pattern of activity was both time and dose-dependent as it was observed that 500mg/kg cannabis group had the least time to locate the escape box in comparison to control group.

CONCLUSION

Administration of whole Cannabis plants may not adversely influence or significantly modify neurobehavioural patterns with regards to pronounced amnesia and cognito/motor actions in rats. A trade-off between the generation of oxidative radicals and oxidative defence mechanisms in the brain may have been elicited by different constituents of Cannabis. There were no correlations between the mild changes in behavioural patterns and oxidative stress differentials in rats that consumed Cannabis within study period.

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