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Effects of Oral Ingestion of Flunitrazepam and Alcohol Mixture on the Cerebellum of the Adult Wistar Rat

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Abstract
Flunitrazepam is a central nervous system depressant in a class of drugs called benzodiazepines. Alcohol has been known for many years as a cause of various diseases and conditions, including: mental and behavioral disorders, gastrointestinal conditions, cancers and cardiovascular diseases. This study is therefore aimed at investigating the histoarchitectural changes that occur in the cerebellum when flunitrazepam and alcohol cocktail are given to adult Wistar rats. Forty two (42) adult Wistar rats weighing 130-150 g were used for this study and were randomly assigned into six groups: 1, 2, 3, 4, 5 and 6 which had 7 rats each. Groups 1, 2, 3, 4, 5 and 6 received distilled water, 0.2ml (normal dose) of flunitrazepam drug, 1ml of 30% distilled alcohol, 0.1ml (low dose) of flunitrazepam drug, 0.4ml (high dose) of flunitrazepam drug and 0.2ml of flunitrazepam drug and 1ml of 30% distilled alcohol, respectively. Tissue processing and the open field test were carried out according to established methods. Normal cerebellar microstructure was observed in Group 2. Cerebellar oedema, atrophy of Purkinje cells and vascular congestion were observed in groups 3 and 4, while group 5 and 6 showed cerebellar oedema and Purkinje cell proliferation. Open field test parameters were analyzed using one-way ANOVA. $P<0.05$ was considered statistically significant. Findings revealed increased locomotory and exploratory activities in groups 2, 4, 5 and 6, while significantly ($P<0.05$) decreased exploratory/anxiety levels were observed in groups 5 and 6. The findings from this study showed that flunitrazepam and alcohol cocktail have deleterious effects on the histoarchitecture and behavioural parameters of the cerebellum of adult Wistar rats.

Keywords: Flunitrazepam, Alcohol, Cerebellum, Open field test, Wistar rats

Introduction
Flunitrazepam is a highly lipophilic benzodiazepine derivative used fundamentally as a hypnotic and sedative (1). It is a central nervous system depressant in a class of drugs called benzodiazepines. Benzodiazepines are sedatives/hypnotics used in the treatment of sleep and seizure disorders. They are also used as skeletal muscle relaxant (2). Swiss pharmaceutical company, Hoffman La Roche, Inc., first described and developed benzodiazepines (3).

Flunitrazepam is tasteless, and odorless (4). As soon as it is absorbed, flunitrazepam is distributed promptly to the body tissues from the plasma, thereby causing an acute clinical response to the drug that does not correlate with the elimination half-life, which is approximately 20 hours (5). The major contributing factors to its abuse are its rapid rate of absorption and high lipid solubility, which facilitates its speedy entry into the brain and results in a swift onset of effects (5). Sedative effects of the drug typically takes place within twenty (20) minutes and could last 6 - 8 hours, with accompanying psychomotor impairment that may possibly linger on for 12 hours (5). Flunitrazepam, however, is ten times more potent than valium, and it is commonly prescribed for anxiety and sleep disorders in Europe and Latin America (6).The accidental or deceitful ingestion of flunitrazepam is generally associated with cases of sexual assault, in which it is used to induce a rapid and profound state of unconsciousness and subsequent amnesia (7). This has led to the media dubbing flunitrazepam the “date rape drug” (8).

Ab initio, flunitrazepam was marketed as white tablets. This made their detection in drinks close to impossible. However, in response to reports implicating flunitrazepam in drug-facilitated sexual assaults, the manufacturer reformulated the drug by making its dissolution more difficult and generating a bright blue colour (9).

Alcohol (ethanol) is the ingredient found in beer, wine, and spirits which causes drunkenness. It is formed when yeast ferments. Chronic excessive alcohol consumption is a global healthcare problem of epidemic proportion (10).

Alcohol has been known to act as a stimulant, even at low doses, inducing feelings of euphoria and talkativeness (11). However, drinking too much alcohol at one session can lead to drowsiness, respiratory depression, coma or even death. Alcohol abuse is recognized worldwide as a major cause of mortality and morbidity (12). Alcohol is circulated through the water in the body. This way, most tissues such as the heart, brain, and muscles are exposed to the same concentration of alcohol as the blood. The exception is the liver, where exposure is greater because blood is received direct from the stomach and small bowel via the portal vein (13).

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Once absorbed, alcohol must be metabolized immediately, for two reasons: first, the body has no capacity to store alcohol, in contrast to the other energy-yielding macronutrients (protein, fat and carbohydrate), and second, alcohol has toxic properties. The liver is the principal site of alcohol metabolism, but some alcohol is also immediately metabolized in the stomach by alcohol dehydrogenase (the enzyme responsible for breaking down alcohol), found in the gastric mucosa (14). In healthy individuals, the majority of alcohol consumed is metabolised, and therefore removed from the blood at a constant rate; on average, about 6 g per hour (14). However, the rate at which alcohol is metabolised varies significantly between individuals and is affected by a number of factors which comprises drug intake, frequency (and usually quantity) of alcohol consumption, age, bodyweight and liver size, and some differences may be genetic (15).

Drunkenness impairs judgment, inhibitions and concentration, and in increasing amounts, leads to drowsiness and coma (12). Apart from being a drug of dependence, alcohol has been known for many years as a cause of some 60 different types of disease and condition, including injuries, mental and behavioral disorders, gastrointestinal conditions, cancers, cardiovascular diseases, immunological disorders, lung diseases, skeletal and muscular diseases, reproductive disorders and pre-natal harm, including an increased risk of prematurity and low birth weight (16, 17, 18, 19).

The term "cerebellum" is of Latin origin and means "little brain". Although it is only about one tenth the weight of the cerebrum, it contains about as many nerve cells (approximately 5 billion for each structure) (20). The cerebellum is the organ responsible for motor coordination, posture and balance. The cerebellum plays an important role in motor control and it may also be involved in cognitive functions such as attention and languages as well as in regulating fear and pleasure response (21).

Alcohol-related cerebellar degeneration is one of the commonest causes of acquired cerebellar ataxia (22). The pathophysiology remains unclear, but proposed mechanism includes excitotoxicity, dietary factors, oxidative stress, compromise energy production and cell death (23). Nerve cells in the cerebellum communicate with nerve cells in the cerebrum, brainstem, and spinal cord, including regions involved in cognitive functions, such as spatial and other sensory perception, problem solving, organization, and planning. Skilled performance of motor tasks involves timing, feedback from visual and sensory cues, coordination, and learned patterns or dynamics of movement that allow movements to be made quickly, smoothly and relatively effortlessly (24). These functions include both motor and cognitive processes. The cerebellum also participates in certain aspects of motor skill learning (25), control of reflexive actions (26) and tactile learning of complex figures (27).

When flunitrazepam is mixed with alcohol, another CNS depressant, blackout, stupor, respiratory depression and death are more likely to occur (2). Considering the increasing demand of flunitrazepam and alcohol cocktail, there is a need for determination of its effect on the cerebellum.

Materials and Methods

Materials

Experimental Animals

Forty-two (42) adult male Wistar rats weighing between 130g - 150g were used for this study. The rats were obtained from the Animal House of the Department of Anatomy, University of Benin, Benin city and housed in the same location for a period of 4 weeks (31 days), in plastic cages, at normal room temperature ($20^{o}C – 25^{o}C$).

Flunitrazepam and alcohol

The flunitrazepam used was manufactured by Sigma-Aldrich Chemicals Company, St. Louis, Missouri, United States while the alcohol was manufactured by Wuhan Biet Co., Ltd., Hubei, China.

Methods

Experimental Design

The animals were randomly assigned into six (6) groups: 1, 2, 3, 4, 5 and 6, comprised of seven (7) animals each (n=7). All groups received food and water *ad libitum*. After two (2) weeks of acclimatization, administration to test groups commenced via orogastric tube, as seen in the protocol below:

Group 1 (control group) were administered with distilled water.
Group 2 were administered with 0.2ml (normal dose) of flunitrazepam.
Group 3 were administered with 1ml of alcohol.
Group 4 were administered with 0.2ml of flunitrazepam and 1ml of alcohol.
Group 5 were administered with 0.1ml (low dose) of flunitrazepam.
Group 6 were administered with 0.4ml (high dose) of flunitrazepam.

Behavioral Study

The open field test was deployed to assess the animals' general locomotory activity and anxiety levels (28). These assessments were carried out after administration of the various treatment regimen.

Sample collection

After 31 days of administration, the rats were re-weighed to determine their final weight, then sacrificed via anesthesia. The brain tissues were harvested, weighed and promptly fixed in Bouins's fluid. Gross examination was carried out to investigate any physical or morphological changes associated with the organs harvested. The

harvested organs were placed in labeled universal bottles and fixed for 48 hours. Thereafter, the tissues were processed using automatic tissue processing schedule to minimize the introduction of artifacts (29). The tissues were dehydrated in alcohol, cleared in xylene and impregnated in paraffin wax. The tissue blocks were sectioned using rotary microtome at 5 micron per thickness, dewaxed in xylene and rehydrated in descending order before staining with hematoxylin and eosin, and then observed microscopically.

Data Analysis

Data generated for each group of animals were subjected to one way analysis of variance (ANOVA) using the statistical package SPSS windows version 25. Values were expressed as mean±SEM. Significant difference was considered at 95% confidence limit (P<.05).

Results

Figure 1 shows cerebellar weight of all experimental groups. There were no significant differences (P > 0.05) in the cerebellar weights when the experimental groups were compared with the control group.

Figure 2 shows cerebellosomatic indices of all the experimental groups. There were no significant differences (P > 0.05) in the cerebellosomatic indices when the experimental groups were compared with the control group.

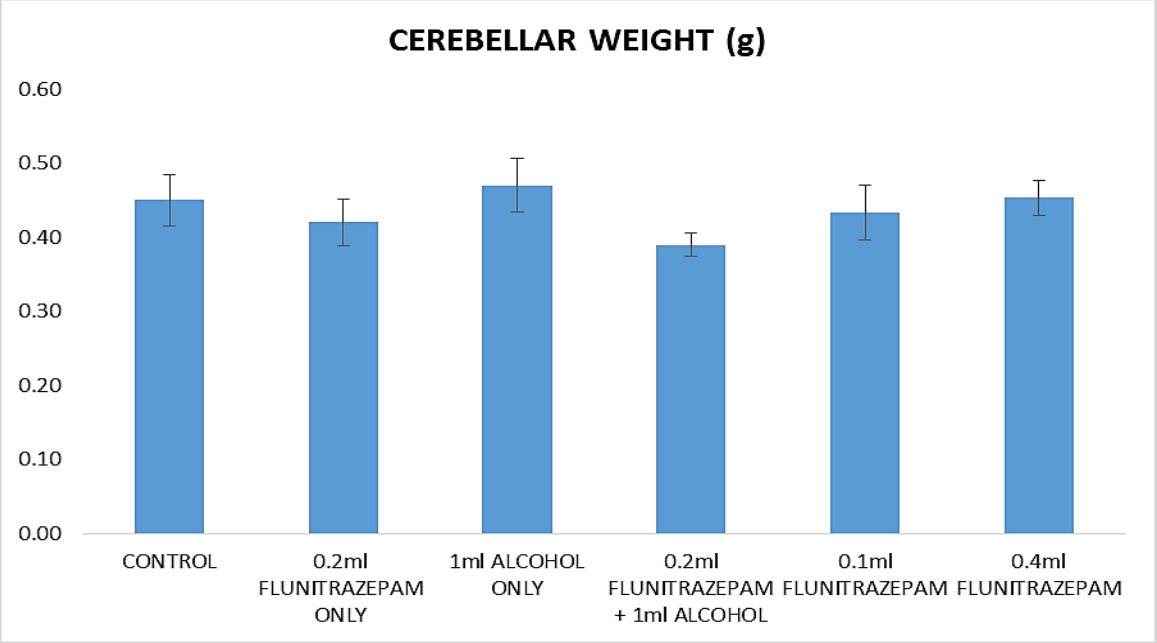

Fig. 1: Cerebellar weight of all the rats treated with various doses of flunitrazepam and alcohol mixture.

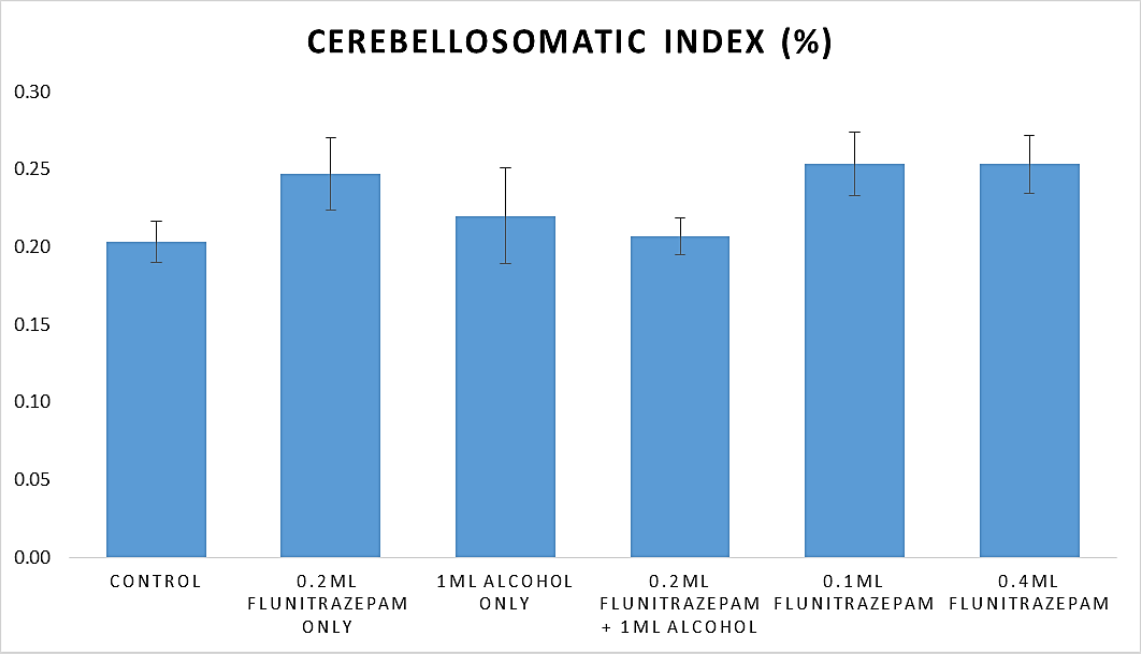

Fig. 2: Cerebellosomatic indices of all the rats treated with various doses of flunitrazepam and alcohol mixture.

Figure 3 shows the frequency of rearing observed in various groups. The increase observed in the frequency of rearing of the experimental animals was significant (P<0.5) in groups 2, 4, 5 and 6, when compared to the control group.
Figure 4 shows the frequency of line crossing observed in different groups. The increase observed in the frequency of line crossing of the experimental animals was significant (P<0.5) in groups 4 and 5, when compared to the control group.

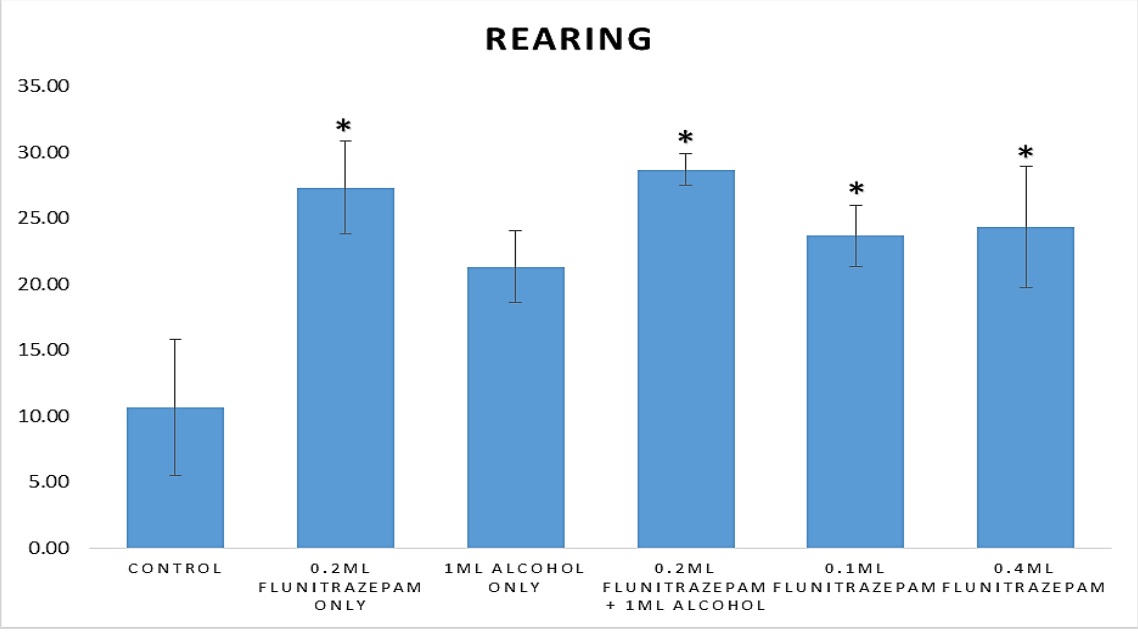

Fig. 3: Frequency of rearing of all the rats treated with various doses of flunitrazepam and alcohol mixture. *significantly different from the control group

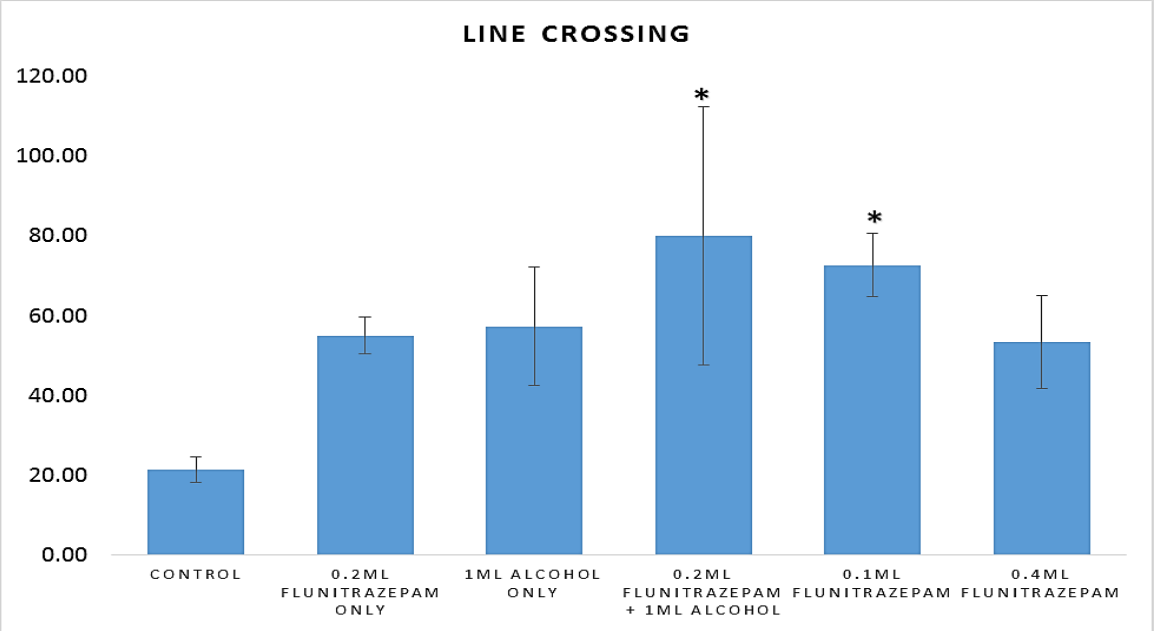

Fig. 4: Frequency of line crossing of all the rats treated with various doses of flunitrazepam and alcohol mixture.
*significantly different from the control group

Figure 5 shows the frequency of grooming observed in different groups. The increase and decrease observed in the frequency of grooming of the experimental animals was significant (P<0.5) in groups 5 and 6 only, when compared to the control group.
Figure 6 Shows the frequency of freezing observed in different groups. The decrease observed in the frequency of freezing of the experimental animals was significant (P<0.5) in groups 4 and 5, when compared to the control group.

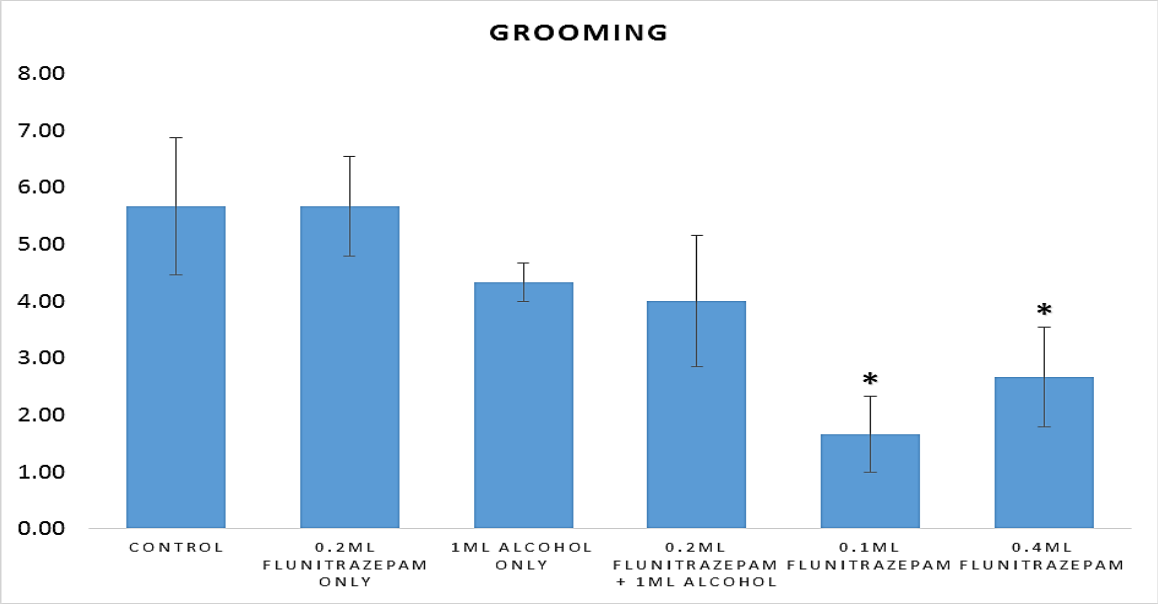

Fig. 5: Frequency of grooming of all the rats treated with various doses of flunitrazepam and alcohol mixture.
*significantly different from the control group

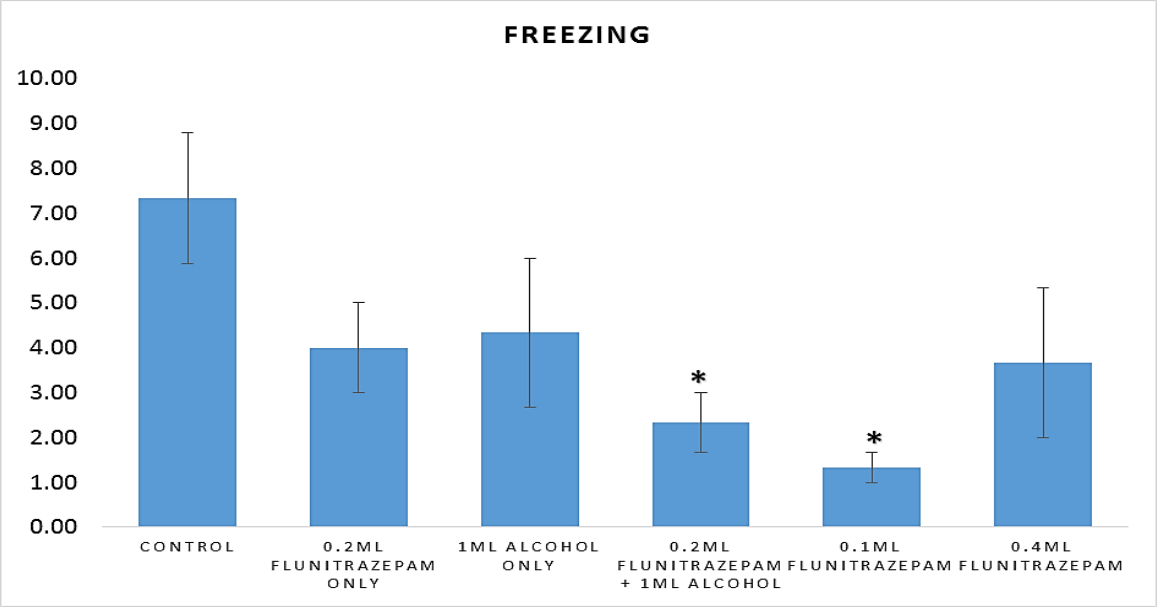

Fig. 6: Frequency of freezing of all the rats treated with various doses of flunitrazepam and alcohol mixture. *significantly different from the control group

Figure 7 Shows the frequency of centre square entry observed in different groups. The differences observed in the frequency of centre square entry of the experimental animals was insignificant (P>0.5) across all the experimental groups.

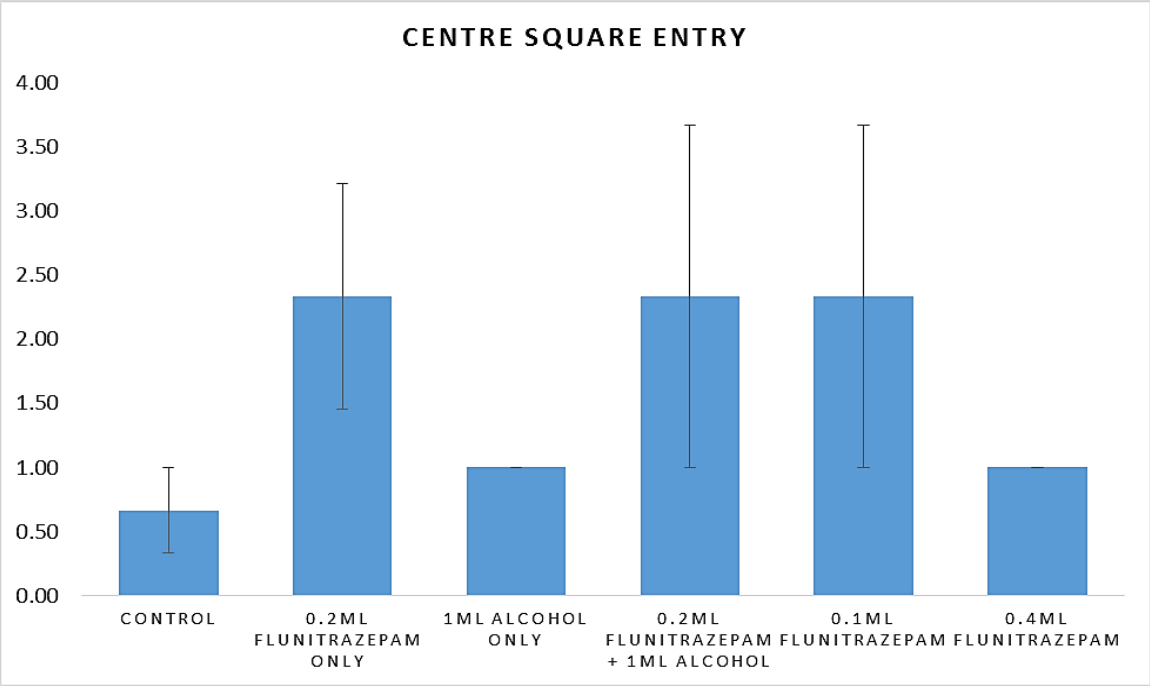

Fig. 7: Frequency of centre square entry of all the rats treated with various doses of flunitrazepam and alcohol mixture.

Plate 1 (Group 1) shows (control) rat cerebellum showing normal molecular layer, Purkinje layer and granular layer. In **Plate 2**, rat cerebellum given 0.2ml flunitrazepam (medium dose) only, for 4 weeks shows vascular congestion and normal Purkinje cell architecture.

Histopathological analysis

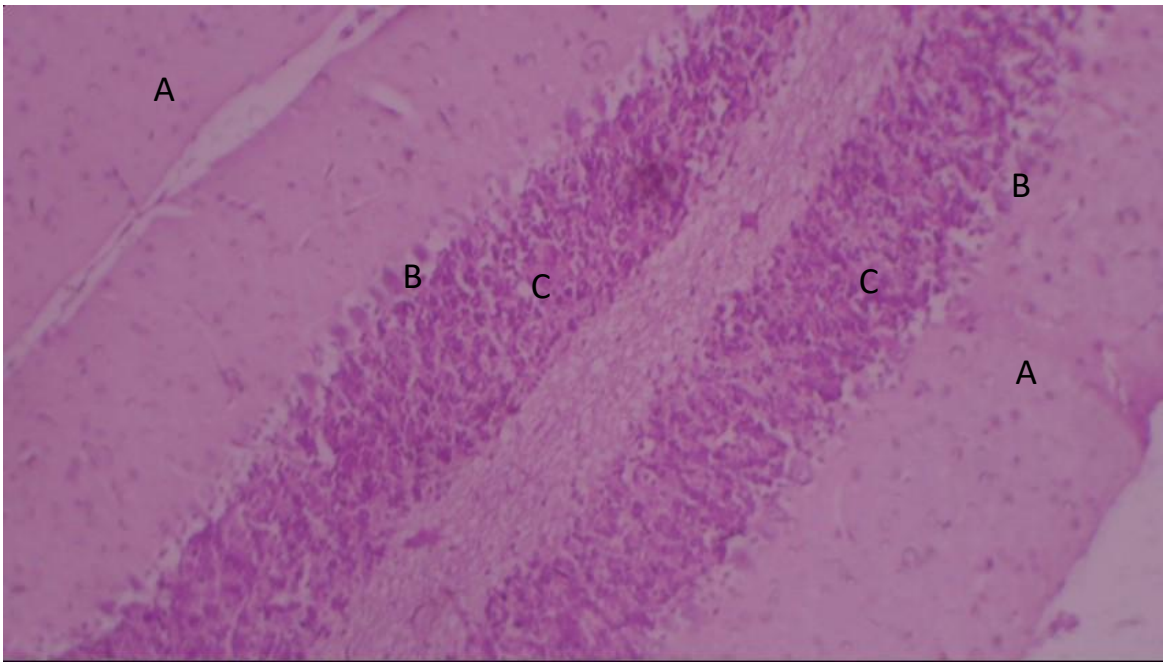

Plate 1: Control Rat cerebellum composed of A: molecular layer, B: Purkinje layer and C: granular layer (H&E x 100)

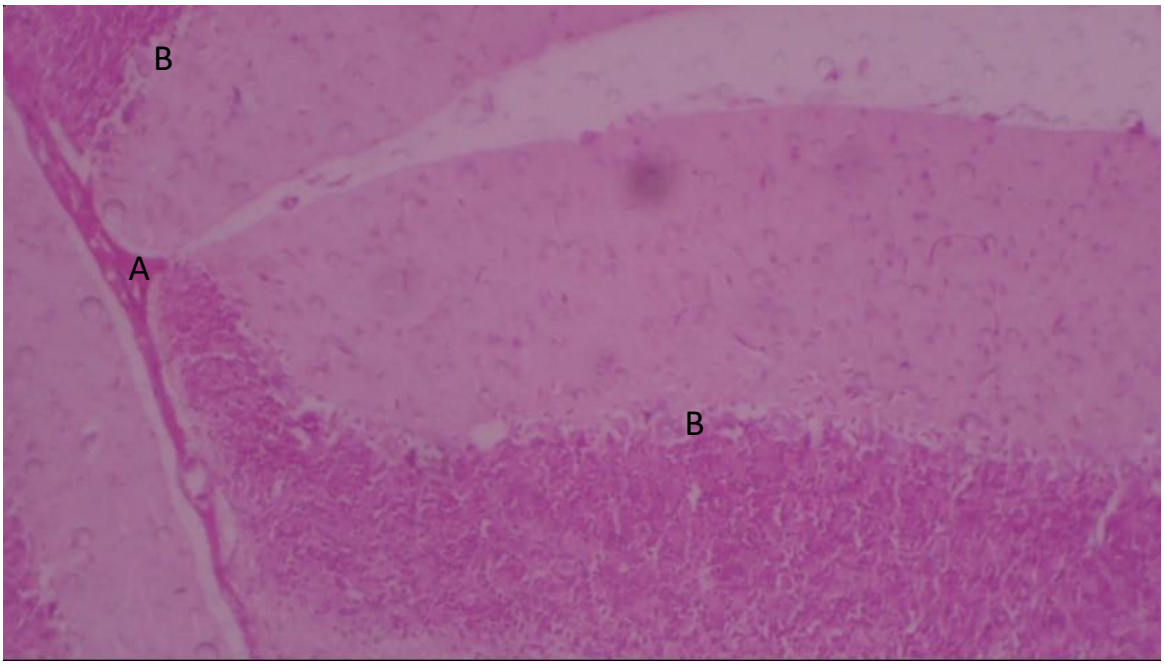

Plate 2: Rat cerebellum given 0.2ml flunitrazepam only for 4 weeks showing A: vascular congestion, and B: normal Purkinje cell architecture (H&E x 100)

However, the cerebellum of rats treated with 1.0ml of alcohol in Group 3, as shown in **Plate 3** shows moderate vascular congestion and Purkinje cell atrophy. Severe Purkinje cell atrophy, cerebellar oedema and severe vascular congestion were seen in **Plate 4** after the rats were given alcohol and flunitrazepam (0.2ml).

In **Plate 5**, rats given 0.1ml flunitrazepam (low dose) only shows moderate cerebellar oedema, mild Purkinje cell degeneration and molecular layer vacuolation, while **Plate 6** shows rats given 0.4ml flunitrazepam (high dose) displaying moderate cerebellar oedema and marked Purkinje cell degeneration.

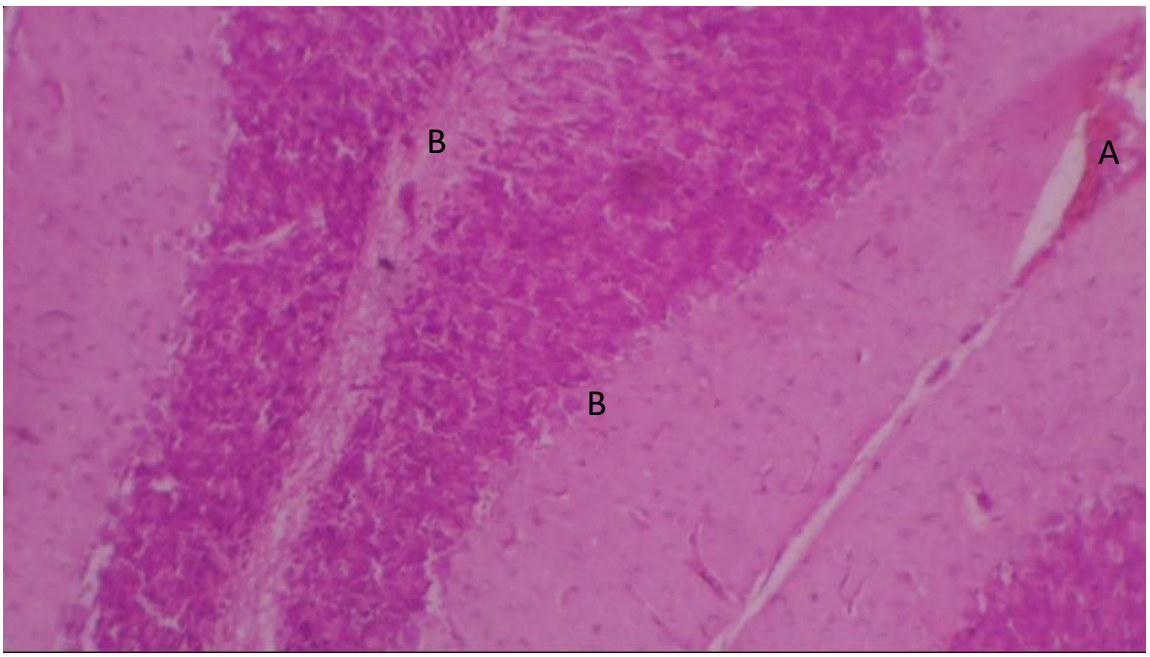

Plate 3: Rat cerebellum given 1.0ml alcohol only for 4 weeks showing A: severe vascular congestion, B: Purkinje cell atrophy (H&E x 100)

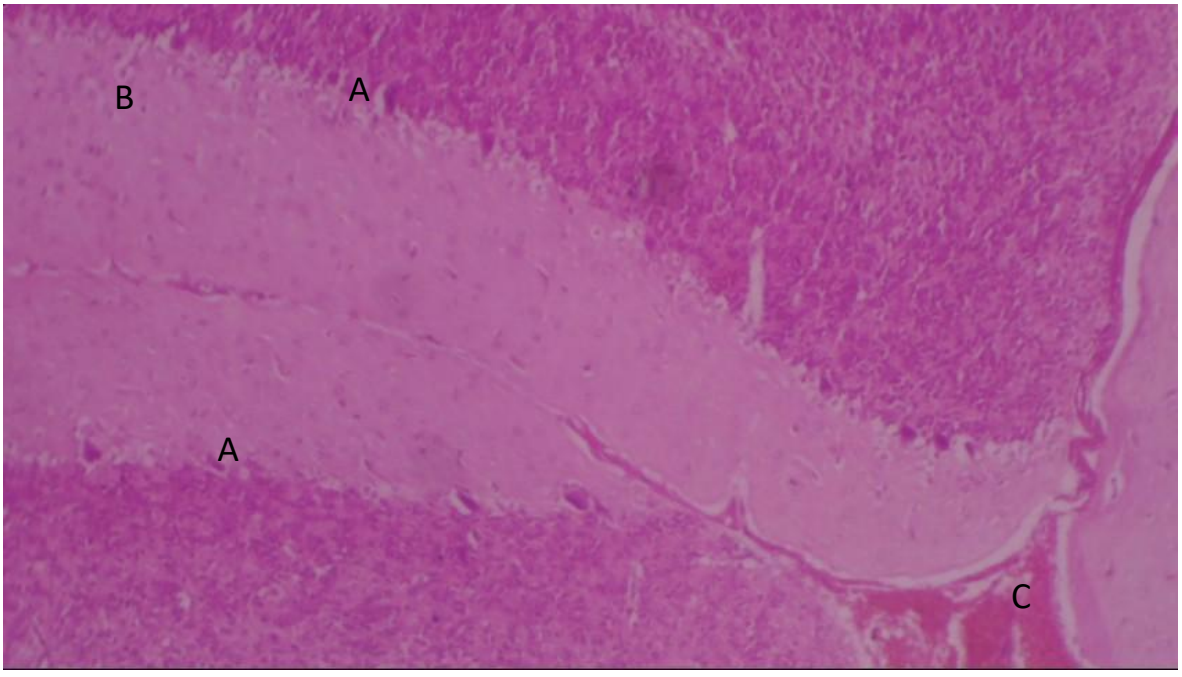

Plate 4: Rat cerebellum given alcohol and flunitrazepam (0.2ml) for 4 weeks showing A: severe Purkinje cell atrophy, B: cerebellar oedema and C: severe vascular congestion (H&E x 100)

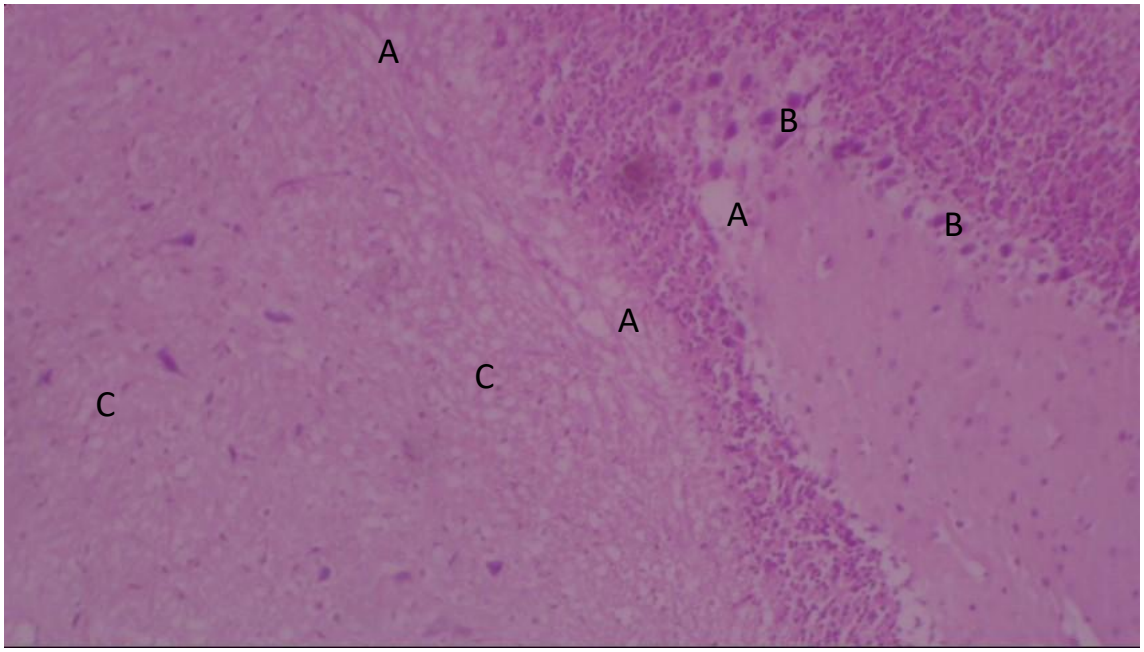

Plate 5: Rat cerebellum given 0.1ml flunitrazepam (low dose) only for 4 weeks showing A: moderate cerebellar oedema, B: marked Purkinje cell degeneration C: molecular layer vacuolation (H&E x 100)

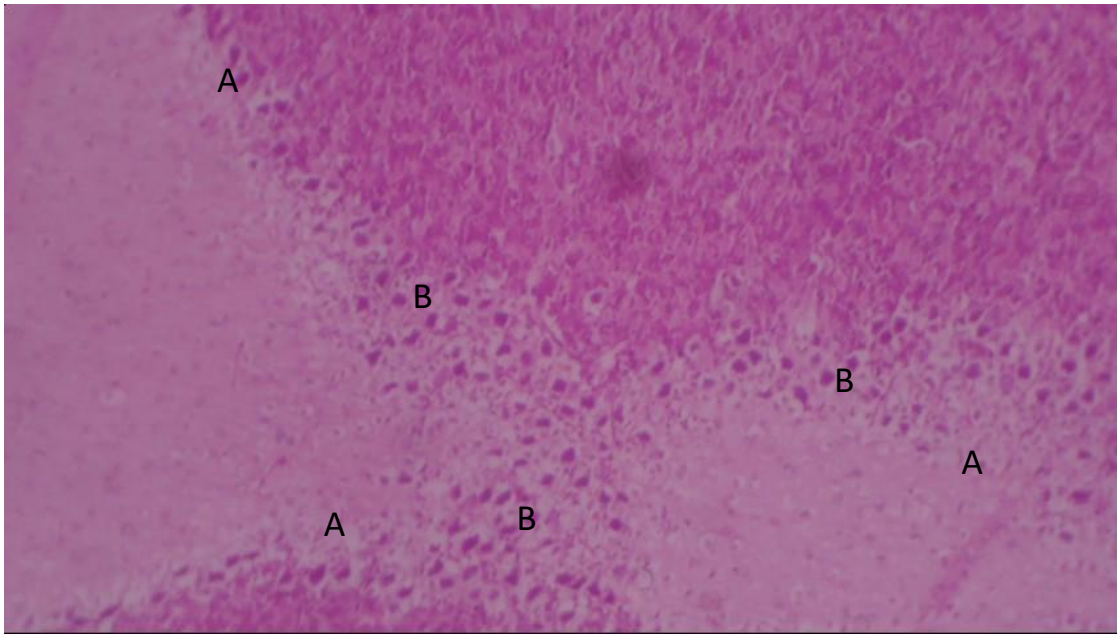

Plate 6: Rat cerebellum given 0.4ml flunitrazepam (high dose) only for 4 weeks showing A: moderate cerebellar oedema and, B: marked Purkinje cell degeneration (H&E x 100)

Discussion

The results (H & E reactions) of Group 3 which were given alcohol only and Group 4 which were given both flunitrazepam and alcohol cocktail showed degeneration/atrophy of Purkinje cells, cerebellar oedema and severe vascular congestion. The Purkinje cells, as part of the cerebellum, are involved in motor coordination and

posture control. As a result of this, loss of Purkinje cells lead to a moderate ataxia. In addition, the degeneration of Purkinje cells is accompanied by the loss of cerebellar granule neurons and some thalamic neurons (30). Degeneration of cerebellar Purkinje cells also affects large perineuronal net bearing deep cerebellar nuclei neurons (31). Atrophy of Purkinje cells and the poor development of the surviving ones led to a decrease in inhibitory function of its fibres which consequently resulted in a decrease in motor coordination in the cerebellar cortex (32). The results obtained suggests that the distortion of the Purkinje cells could be associated with functional changes that may be detrimental to the health of the rats.

Cerebellar degeneration is common in alcoholics (22). Researchers have studied cerebellar damage in the brains of alcoholics during postmortem examination. The most consistently reported structural damage in the cerebellum of alcoholics is tissue volume loss in the anterior superior vermis (33). Tissue volume loss in this area is due especially to either shrinkage or atrophy of Purkinje cells (34, 33, 35).

Cerebellar oedema refers to the neuropathological description of cell loss, atrophy, gliosis, etc. (36). It is usually based on the subjective impression of changes of cell density one gets from a microscopic study (36). This condition, accompanied with cerebellar infarction, can lead to sudden respiratory arrest due to increased intracranial pressure in the posterior fossa (37, 38). Vascular congestion was also observed in this study (compromised blood flow), which when severe may increase hydrostatic pressure and subsequently lead to cerebellar herniation (39), which causes compression of the midbrain and medulla resulting in states of decreasing consciousness, impaired upward gaze and lower cranial nerve palsies, dilated or fixed pupils and respiratory irregularity (40).

A study on the amnesic action of intravenous flunitrazepam showed that it caused amnesia without effect on the level of consciousness. Also, the effect of flunitrazepam were harmful to coordination (41).

The use of flunitrazepam in combination with alcohol is of particular concern as both drugs interactions as central nervous system depressants potentiate each other's toxicity (42).

Patchy vascular intimal erosion/ulceration indicates complicated atherosclerosis which is the most common vascular disease and is a major cause for stroke and vascular dementia (43).

The open field test has also been used widely to assess anxiety and locomotor performance
(44, 45, 46). The most important outcome of open field test is movement coordination, which is greatly influenced by motor output and exploratory drive (47). In this study, the increase observed in the frequency of rearing of the experimental animals was significant ($P<0.5$) in groups 2, 4, 5 and 6, when compared to the control group. This shows that all the groups that were given flunitrazepam had a significant increase in locomotion and exploration /or a lower level of anxiety. The number of line crosses and the frequency of rearing are usually used as measures of locomotor activity, but are also measures of exploration and anxiety (48). Increased grooming behaviour has been related to fear or increased emotional response (49). It has also been associated with conflict or frustration in different species (50). The increase and decrease observed in the frequency of grooming of the experimental animals was significant ($P<0.5$) in groups 5 and group 6 only, when compared to the control group. Grooming behaviour is a displacement response and is expected to be displayed in a novel environment. Therefore, grooming behaviour should decrease with repeated exposure to the testing apparatus (51).

Anxiety can be referred to as behavioral, psychological and physiological state induced in animals and humans by stress resembled conditions, characterized by feeling fear or dread. Fear is a motivational state stimulated by specific stimuli that result in escape and defensive behavior (52). Considering that the cerebellum coordinates muscular activities of the body, it is not far-fetched that alteration to its microstructure may have resulted in the observed motor deficiencies during the open field test.

The results obtained in this study following the administration of flunitrazepam and alcohol cocktail showed that there is a compromising effect on both the histomorphology of cerebellum and by extension, neurobehaviour of the adult Wistar rats. Since the use of alcohol in combination with flunitrazepam has become one of the consumed drugs among youths for various reasons, it has become imperative to educate the general public about the impending damage that such practices could result in.

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