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Research Article

Nigerian Psychoactive Alcoholic Herbal Mixture Impacts Behavioural Performance and Caused Brain Biochemical and Histopathological Alterations in Mice

Ajayi A.M.^{1*}, Arasi R.O.₁, Ben-Azu B._{1,2}, Godson J.C.₁, Umukoro S.₁ & Aderibigbe A.O.₁

¹Neuropharmacology unit, Department of Pharmacology & Therapeutics, University of Ibadan, Oyo-State, Nigeria.

² Department of Pharmacology, PAMO University of Medical Sciences, Nigeria

ABSTRACT

Psychoactive alcoholic herbal mixture is popularly consumed because of the belief on its stimulant and curative effects. This study investigated the neurobehavioral, biochemical and histopathological consequences following its single and repeated co-administration with ethanol to mice. Mice were assessed for sensorimotor, anxiety and memory functions following acute and repeated administration of alcohol herbal mixture (AHM). Alterations in brain acetylcholinesterase, nitrite, reduced glutathione (GSH) and malondialdehyde levels were assessed in striatum, prefrontal cortex (PFC) and hippocampus. Neuronal cells counts were determined in the prefrontal cortex and hippocampal tissues. In the acute study, AHM significantly impaired locomotor activity and motor coordination in mice. Repeated administration of AHM and AHM combined with ethanol caused significant impairment of locomotor and motor coordination, increased anxiety-like behavior and impaired memory in mice. Acetylcholinesterase activity was significantly increased in the PFC while nitrite level was elevated in the striatum and PFC. There was significant elevation of malondialdehyde and depletion of GSH in all brain regions as well as reduced neuronal cell counts in the PFC and hippocampus. This study showed evidences of behavioral perturbation and brain biochemical changes in mice, hence repeated consumption of alcoholic herbal mixture might produce substance-attributable harm and quicken neurodegenerative diseases in humans.

Keywords: *Alcohol herbal beverage, psychoactive, behavior, brain, damage*

*Author for correspondence: Email: yomexj@yahoo.com; Tel: +234 810 386 1380

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INTRODUCTION

Alcohol is one of the most widely used recreational drugs in the world and is a drug that is easily abused. There is an increasing risks of mortality and morbidity associated with harmful alcohol use. Globally, harmful alcohol use is responsible for about 3.3 million deaths and it's a causal factor in more than 200 disease and injury conditions. Overall, 5.1 % of the global burden of disease and injury is attributable to alcohol (WHO, 2009). The increase in alcohol related problems in Low and middle income countries (LAMICs) is alarming. The pattern of alcohol consumption in developing countries is more harmful since it interacts with malnutrition, unsafe housing, stressful lifestyles and poor living conditions (Cook *et al.*, 2014). Approximately 1.9 million alcohol-related cancer cases are estimated in developed countries whereas in LAMICs, 2.8 million alcohol-related cancer cases are estimated (Lee and Hashibe, 2014).

Alcohol has been implicated in a large proportion of road crashes especially in Low-middle income countries where buses, mini-buses and motorcycle taxis are the predominant means of transportation. (Ogden and Moskowitz, 2004). Alcohol remains the dominant drug causing impairment of driving performance, and when combined with other psychoactive drugs, increase collision risk. (Ogden and Moskowitz, 2004). Early life harmful alcohol use goes beyond health consequences to later life significant social and economic losses to individual due to injuries (Romano *et al.*, 2014).

Historically, alcoholic beverages such as beers, wines, and spirits are used for recreational purposes. When used at low doses for recreational purposes, alcoholic drink causes euphoria, reduced anxiety, and sociability. However, long-term use can lead to alcohol abuse, physical dependence and alcoholism (Crocq, 2007). Although some studies have shown beneficial effects of moderate alcohol consumption on the risk for cardiac diseases, other studies had shown increased risks

for liver cirrhosis, neuromuscular disorders, and cancers (Snopek *et al.*, 2018). There is also a link between harmful alcohol use and a range of mental and behavioural disorders, and other non-communicable conditions (Rehm *et al.*, 2010; Parry *et al.*, 2011).

In Nigeria, alcohol misuse is in the form of alcoholic herbal mixtures known as “paraga” in the South Western states. It is one of the most easily accessible forms of medicinal herbs combined with alcohol that is abused (Kehinde and Adegoke, 2012). The alcoholic herbal mixture (*Paraga*) is an alcoholic herbal drink commonly taken by commercial drivers and motorcyclists at the motor parks in Nigeria. The prevalence use of non-commercial alcohol either alone or together with commercial alcohol (beers, wines, spirit and gins) is reported to be about 57.6% among tanker drivers in Lagos, Nigeria (Makanjuola *et al.*, 2014). *Paraga* as an herbal mixture has variable ingredients and indeterminate alcohol content. *Paraga* is used as stimulants by the commercial drivers and motorcyclists. They also believe that *paraga* has curative effects on an extraordinary range of ailments (Kehinde and Olusegun, 2012).

In a survey in South Western Nigeria by Kehinde and Olusegun (2012), *paraga* as an unregistered product with no formal recipes and sold in or near motor parks, and alcohol by volume content in twenty eight (28) samples ranged from 1.20% to 20.84% alcohols. Alcoholic beverages are globally categorized into beers (3-8% alcohol), wines (8-12% alcohol) and spirits (40-50% alcohol). Thus, more than two-thirds of the *paraga* samples had ethanol strength that was either equal to or stronger than beers. The reports also mentioned the herbal ingredients to include Lime, eru (*Xylopi aethiopica*), aayu (garlic, *Allium sativum*), iyere (African black pepper, *Piper guineense*), pineapple (*Ananas comosus*), ginger (*Zingiber officinale*), onion leaves (*Allium cepa* or *Allium ascabricum*), and orogbo (bitter kola, *Garcinia kola*). Although the herbal extract of these ingredients singly are not known to have deleterious effect on neurobehavioral function, but little is known about their combined effect with alcohol. However, drinking too much of alcohol on a single occasion or over time can have a serious health implications on the brain, heart, liver, pancreas and immune system. The long term effects of alcohol include: blackouts, memory loss, liver disease, thiamine deficiency (O’Keefe, et.al, 2014).

In Nigeria, there are several online newspaper reports of untowards effects ascribed to the use of alcohol herbal mixture. For example, “man dies of local gin ‘paraga’ in Lagos” (<http://dailypost.ng/2015/07/15/>), “paraga craze: the beat goes on despite Ode-irele tragedy” (<http://thenationonlineng.net/paraga-craze>), “paraga consumers applaud its efficacy-in spite of doctors warning” (<http://encomium.ng/paraga>). Importantly, the perception of harmful effect of *paraga* was reported to be below 40% among drivers in the survey conducted by Kehinde and Olusegun (2012). Although there were reports on alcoholic beverages such as beers (Gasbarrini *et al.*, 1998), and wines (Micallef *et al.*, 2007; Covas *et al.*, 2010; Snopek *et al.*, 2018), however, to the best of our knowledge, no scientific evidence exist on the substance-attributable harm on the use of the alcoholic herbal mixtures. Pharmacological investigations and reporting of alcohol-related harm is greatly needed as such information

generates valuable data to health policy makers for policy development and legislation. Hence, we aimed to investigate the neurobehavioral and biochemical effects of single and repeated administration of alcoholic herbal mixture (*paraga*) in mice.

MATERIALS AND METHODS

Experimental animal care and handling: Male Swiss mice (20 – 25g) were obtained from the Central animal house of the University of Ibadan, and acclimated in the Department of Pharmacology & Therapeutics experimental animal facility. The animals were housed in polycarbonates animal cages, in groups of 8 – 10 mice, with wood shaven as beds and fed with standard pellet diet (Vital feeds®, Jos, Nigeria) and water *ad libitum*. The room lighting was maintained under environmental light and dark cycle, temperature fluctuation of 28-33 °C. The animal handling and care was carried out following protocols for animal handling and experiments in the Department of Pharmacology & Therapeutics, with strict compliance to the National Institutes of Health “Principle of Laboratory Animal Care” (NIH Publication No. 85-23).

Herbal and ethanol content of the Alcoholic Herbal Mixture (AHM): The alcoholic herbal mixture (AHM) otherwise known as *paraga* was obtained from commercial outlets at the popular Sango Motor Park located on the Oyo-Ibadan expressway, Ibadan, Oyo state, Nigeria. The vendor explained that the alcohol herbal mixture is made up of ‘abeere seed’ (*Hunteria umbellata*), ‘oko esin’ (*Setaria megaphylla*), ‘eeru’ (*Xylopi aethiopica*) and other undisclosed constituents. The mixture was filtered to remove the herbs and the filtrate was stored in the refrigerator at 4°C. The ethanol content analysis of the alcohol herbal mixture was determined using gas chromatography (CLARUS 500GC). The ethanol content was found to be 66.4%. Based on preliminary dosing studies, fresh dilution of *paraga* filtrate was administered intraperitoneally at 2.5 mL/kg, 5 mL/kg and 10 mL/kg to give corresponding 15.6%, 33.2% and 66.4% of ethanol, respectively. Ethanol (2 g/kg, 20%) was prepared by adding 26.8 mL of 95% ethanol (BDH, England) to 73.2 mL of normal saline (Coleman *et al.*, 2008). Fresh dilutions were prepared each day in normal saline before intraperitoneal administration to mice. The control group was treated with normal saline.

Assessment of single administration of AHM in mice: Thirty (36) mice were assigned to six different groups (n = 6) and treated intraperitoneally. The group one (control group) was given (10 mL/kg of normal saline). Groups 2-4 received AHM at 2.5 mL/kg, 5 mL/kg and 10 mL/kg, respectively. Group 5 was given ethanol (2 g/kg) while group 6 was given 2.5 mL/kg of AHM mixed with 20% ethanol. Neurobehavioral effect of treatments in different groups were assessed for any behavioral alterations at 1, 3, 5 and 24 h after treatment using an open field activity cage (Ugo Basile, Italy) to assess locomotory activity in mice and walk beam balance apparatus to assess the effects of motor coordination at 3rd hour after treatment.

Repeated administration of AHM to mice: Thirty two (32) mice were assigned to four different treatment groups of (n = 8). Group 1 which is the control group received (10 mL/kg of normal saline), group 2 received AHM (2.5 mL/kg), group 3 received ethanol (2 g/kg) and group 4 received a cocktail of AHM (2.5 mL/kg) + ethanol (2 g/kg). The animals were treated every 6 days for 28 days, and the 7th, 14th, 21st, and 28th, days were drug free days. Behavioural assessment in open field and walk beam apparatus were performed on drug free days during the light cycle. Assessment of anxiety-like behaviour in the elevated plus maze and memory function were performed on days 28th and 29th, respectively.

Assessment of locomotory activity in activity cage: Assessment of treatment effect on locomotory activity was performed in Ugo Basile activity cage (Model 7401, Comerio, Italy). The number of times the animals breaks the horizontal beam (locomotor activity) or vertical beam break (rearing activity) was counted for each 5 min duration by the activity meter. Each animal was used only once with the activity cage cleaned with 70% ethanol after each assessment to remove olfactory cue from previous animal to the other. The time of the experiment was kept constant (9.00 a.m-1.00 pm) daily to avoid change in biological rhythm.

Assessment of motor function on walk beam apparatus: The beam apparatus consists of 1 meter beams with a flat surface of 12 mm width resting 50 cm above the table top on two poles. The animals were trained before the test. On training days, mice are placed at one end of a beam and the time required to cross to the other end (100 cm away) was noted. On the test day, the animals were subjected to neurobehavioral impairment test 3 hours after treatment. Each animal was subjected to walk for 2 min. on the beam walk apparatus. Time to cross each beam, number of foot slips and total distance travelled by mice on the beam balance were recorded. In genetically or pharmacologically manipulated animals, the slips and falls may become more frequent and can be quantified. Impaired mice may cling onto the side of the beam, which increases their time to cross (Luong, et. al., 2011).

Test for Anxiety-like behavior on elevated plus Maze: The EPM apparatus is made of wood and consisted of two open arms (45 cm x 5 cm) and two closed arms (45 cm length x 5 cm width x 45 cm height) elevated 50 cm above the floor. Mice were gently placed the centre area on the platform facing the open arm. An entry with all feet put into one arm is defined as an entry in this experiment. At the start of the session the mouse was placed at the center of the maze with its head facing an open arm and allowed to explore the maze for five minutes. During this test period, the following measurements were recorded: the number entries in open and closed arm, time spent in open and closed arms and the exploratory behaviors (total number of arm entries). 70% ethanol was used to clean the maze after each animal to prevent odour bias. The results were expressed as mean ratio of time spent in open arms to total time spent in both open and closed arms, (percentage of time spent in open arms); mean ratio of entries into open arms to total entries into both open and close arm

entries (percentage of number of entries) and number of entries of open arms. The index of open arms avoidance [OIAA] was determined (Trullas and Skolnick, 1993) i.e .IOAA= 100-(%Time spent in open arms + % entries into open arm.)/2

Assessment of memory function in Y-maze test: Memory function test was performed using the Y-maze apparatus on mice sub-acute treated with alcohol herbal mixture and ethanol after 28 days of treatment. The Y-maze apparatus is an instrument adapted for assessing the spontaneous alternation as a measure of spatial working memory. The Y-maze is a wooden apparatus composed of three equally spaced arms (33 x 11 x 12 cm) symmetrically separated at 120°. On day 29 of treatment, memory function test using Y-maze was determined. Each mouse was placed in compartment A and was allowed to move alternately between the other two arms. The movement between the arms are manually recorded by a blind observer. An alternation is defined if the mouse entered into all three arms on consecutive manner. Ethanol 70% was used to clean the Y-maze at interval. The percentage of alternations was expressed as the ratio of the total alternations/(total arm entries-2) (Hughes, 2004).

Biochemical assay of brain acetylcholinesterase and oxidative stress markers: Brains were harvested from ether anaesthetized mice after perfusion with cold sodium phosphate buffer (0.1M, pH 7.4). The brain were dissected into region of interest (striatum, pre frontal cortex and hippocampal) and were individually homogenised in iced cold phosphate buffer, centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant of the brain region was aliquot and stored at -20 °C for later determination of acetylcholinesterase, nitrite, reduced glutathione, and malondialdehyde levels. The brain homogenates supernatant were used for determination of acetylcholinesterase enzyme activity using acetylthiocholine as substrate (Ellman, 1961). The level of reduced glutathione was measured using the Ellman's reagent as described by Jollow *et al.*, 1974. Nitrites was estimated in brain region using the Griess reagent (Green *et al.*, 1982). The malondialdehyde level as an index of lipid peroxidation in the brain region was determined using the thiobarbituric acid reactant substance (Nagababu *et al.*, 2010).

Histopathological assessment of mice brain: The brain tissues were fixed in 10% buffered formalin, the fixed tissues were dissected longitudinally, placed in embedding cassettes, embedded in paraffin, and then cut into 4µm section. Brain slices were stained with hematoxylin and eosin (H & E) for determination of histopathological changes in the prefrontal cortex and hippocampus. Histological photomicrographs were captured using Olympus binocular research microscope (Olympus, New Jersey, USA) which was connected to a Leica ICC50 E Digital Camera (Germany) and a computer interface (MagnaFire). The population of healthy neuronal cells were also determined according to the method described by Woeffler *et al.*, 2014.

Data analysis: All data were presented as mean ± standard error of mean (SEM) and statistical significance was taken for

$p < 0.05$. Behavioral observation in the open field, walk beam were analysed using two-way analysis of variance followed by Bonferroni post hoc test. All other data were analyzed using one-way analysis of variance (ANOVA), significant main effects were further analyzed by Newman-Keuls *post hoc* test for multiple comparison. Analysis was done in GraphPad Prism® software version 5.01 (GraphPad Software, Inc. La Jolla, CA 92037 USA).

RESULTS

Effect of acute administration of Alcoholic Herbal Mixture (AHM) and Ethanol (EtOH) on locomotor activity in mice

Acute administration of alcoholic herbal mixture AHM (2.5, 5 and 10 mL/kg) caused a dose dependent reduction on locomotor activity (Fig 1A-B). There was a significant locomotor impairment as determined by two-way, repeated measures ANOVA (treatment: $F_{5, 116} = 34.65, p < 0.0001$, time: $F_{3, 116} = 20.19, p < 0.0001$), and a significant interaction effect (interaction time x treatment: $F_{15, 116} = 3.12, p = 0.0003$, Fig 1A). The locomotor impairment which was observed from 1st -5th h was restored 24 h post treatment (Fig 1A). Analysis of the locomotor activity vs time area under the curve (Fig 1B) showed that the impairment of locomotor activity was significant ($p < 0.05$). Co-administration of AHM and ethanol caused a significant ($p < 0.05$) enhancement of locomotor activity impairment when compared with animals that received EtOH (2 g/kg) alone.

Intraperitoneal administration of AHM (2.5, 5 and 10 mL/kg), EtOH (2 g/kg) and AHM (2.5 mL/kg) + EtOH (2 g/kg) to mice showed significant rearing activity impairment in mice (Fig 1C). This effect was significant as determined by two-way, repeated measures ANOVA (treatment: $F_{5, 108} =$

$33.47, p < 0.0001$, time: $F_{3, 108} = 13.65, p < 0.0001$), and a significant interaction effect (interaction time x treatment: $F_{15, 108} = 3.17, p = 0.0003$, Fig 1C). Conversely, EtOH (2 g/kg) showed a gradual increase in rearing activity from 1-5 h post treatment. There was evidence of recovery from treatment at the 24th h measurement. Co-administration of AHM and ethanol caused a significant reversal of the effects of ethanol alone (Fig 1D).

Effect of acute administration of Alcoholic Herbal Mixture and ethanol on motor co-ordination in mice

The observed effect of acute administration of alcoholic herbal mixture and ethanol showed a significant impairment of motor coordination on the walk beam test. Statistical analysis as determined by one-way ANOVA showed significant dose dependent increase on beam crossing time ($F_{5, 18} = 4.85, p = 0.0055$, Fig 2A), dose dependent increase on number of foot slips ($F_{5, 22} = 3.57, p = 0.0162$, Fig 2B), and dose dependent decrease on beam total distance travelled ($F_{15, 28} = 5.030, p = 0.0021$, Fig 2C).

Effect of sub-acute administration of Alcoholic Herbal Mixture and ethanol on locomotor Activity.

Repeated administration of AHM (2.5 mL/kg) and EtOH (2 g/kg) a time-dependent reduction on locomotor activity in mice (Fig 3A). A significant ($p < 0.05$) effect was observed in animals receiving EtOH (2 g/kg) or AHM (2.5 mL/kg) + EtOH (2 g/kg) after 14 days of treatment. These effects however, waned off on day 28. Similarly, mice showed a time-dependent reduction in rearing activity which was significant only in mice receiving AHM (2.5 mL/kg) + EtOH (2 g/kg) on days 14 and 28 of measurement (Fig 3B).

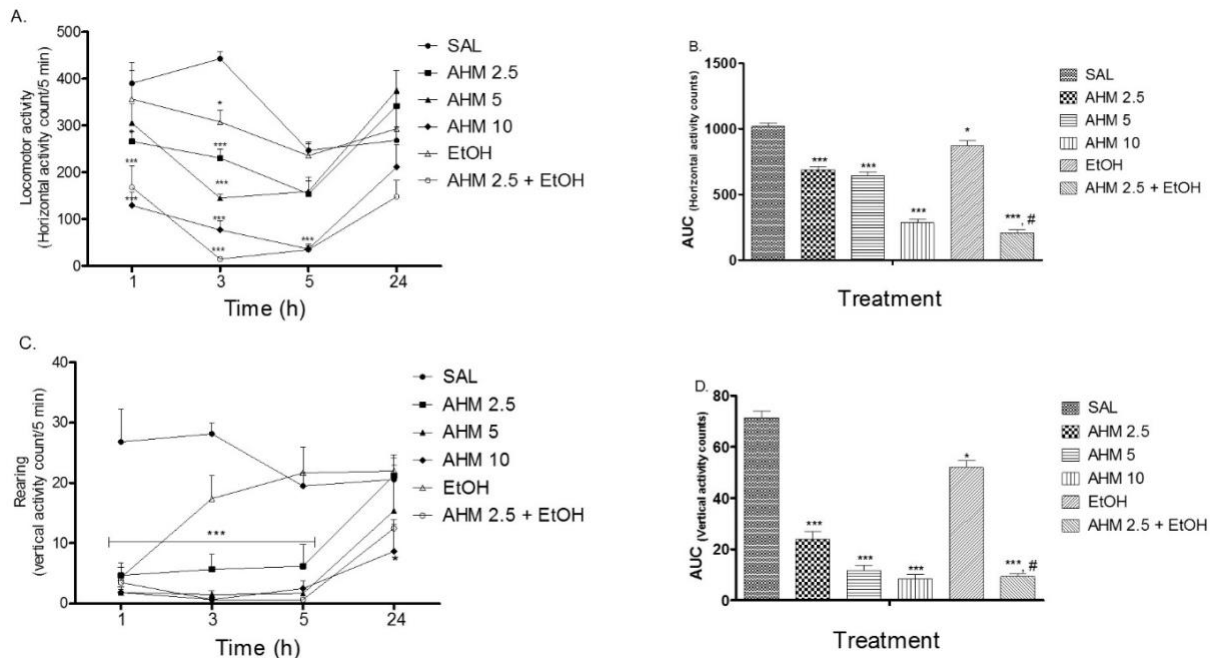


Figure 1: Effect of acute administration of alcohol herbal mixture and ethanol on locomotory activity in the open field test. (A) Horizontal activity count, (B) Area under the curve of horizontal activity counts (C) Vertical activity count and (D) Area under the curve of vertical counts. Values represents Mean ± SEM (n=6). * $p < 0.05$, *** $p < 0.001$ vs SAL; # $p < 0.05$ vs EtOH using 2-way ANOVA (Bonferroni test) or 1-way ANOVA (Newmann Keuls test). SAL- Saline, AHM –Alcoholic herbal mixture, EtOH – Ethanol.

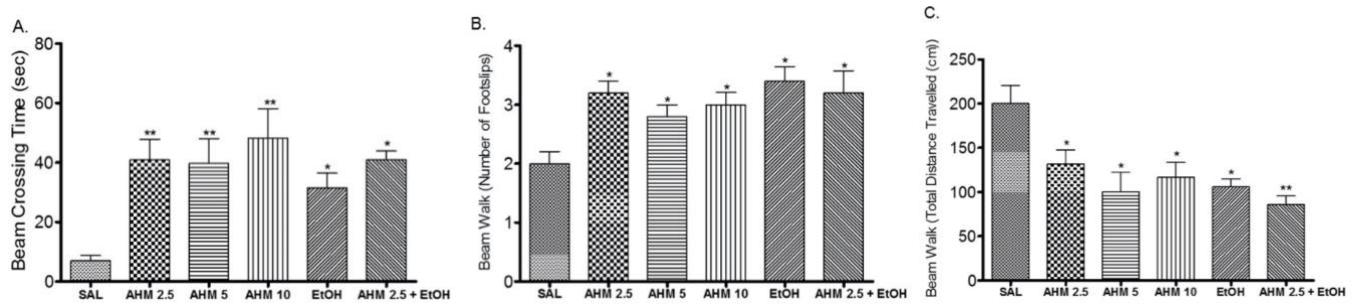


Figure 2: Effect of acute administration of alcoholic herbal mixture and ethanol on motor performance in the beam walk test. (A) Beam crossing time, (B) Number of footslips and (C) Total distance travelled. Values represents Mean \pm SEM (n=6). * p < 0.05, ** p < 0.01 vs SAL using 1-way ANOVA followed by Newman-Keuls multiple comparison post hoc test. SAL- Saline, AHM –Alcoholic herbal mixture, EtOH – Ethanol.

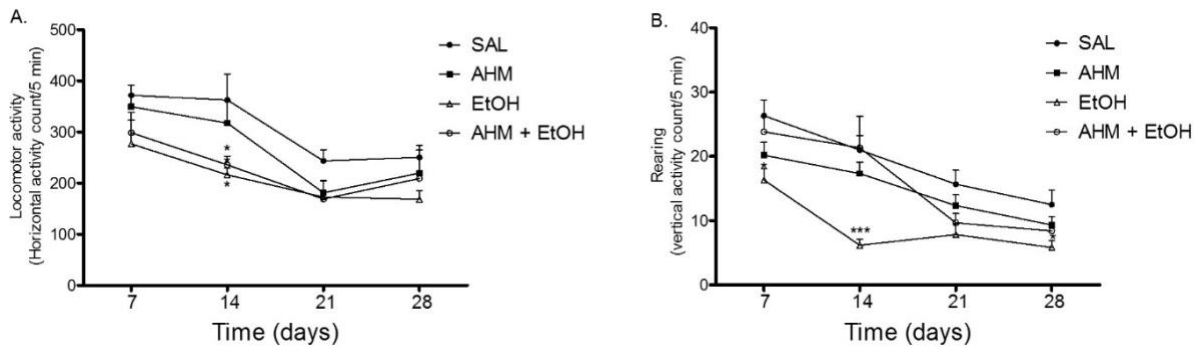


Figure 3: Effect of sub-acute administration of alcoholic herbal mixture and ethanol on locomotory activity in the open field test. (A) Locomotor activity and (B) Rearing. Values represents Mean \pm SEM (n=6). * p < 0.05, ** p < 0.01, *** p < 0.001 vs SAL using 2-way ANOVA (Bonferroni test). SAL- Saline, AHM –Alcoholic herbal mixture, EtOH – Ethanol.

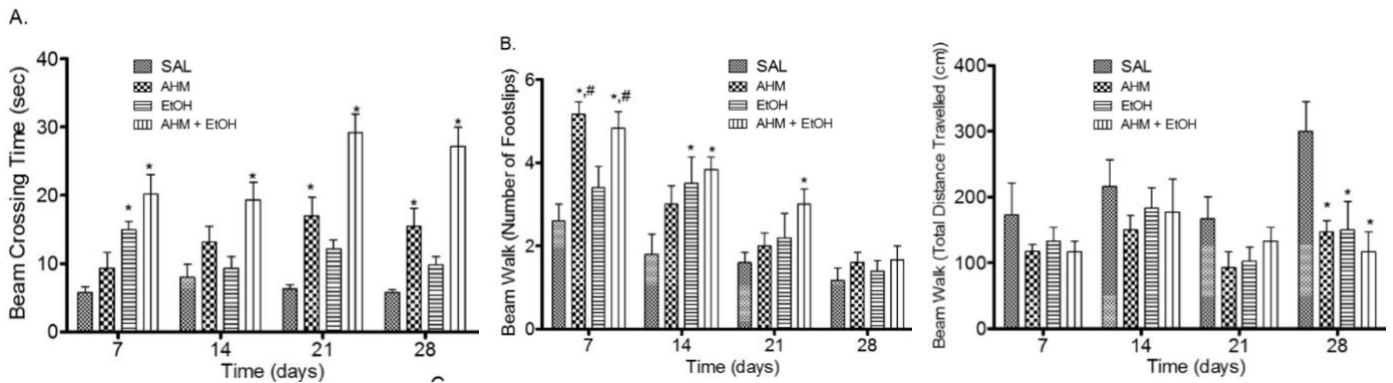


Figure 4: Effect of sub-acute administration of alcoholic herbal mixture and ethanol on motor performance in the beam walk test. (A) Beam crossing time, (B) Number of footslips and (C) Total distance travelled. Values represents Mean \pm SEM (n=6). * p < 0.05, ** p < 0.01 vs SAL, # p < 0.05 vs EtOH using two – way analysis of variance (ANOVA) followed by Bonferroni post hoc test. SAL- Saline, AHM –Alcoholic herbal mixture, EtOH – Ethanol.

Effect of sub-acute administration of Alcoholic Herbal Mixture and ethanol on motor co-ordination in mice.

The observed effect of sub-acute administration of alcoholic herbal mixture and ethanol showed a significant impairment of motor coordination on the walk beam test. The beam crossing time (Fig 4A) was significantly increased in mice that received AHM 2.5 mL/kg (days 21 and 28), EtOH 2 g/kg (only on day 7), and AHM 2.5 mL/kg + EtOH 2 g/kg (days 7, 14, 21 and 28) when compared to saline-treated animals. Following the first six days of repeated administration, animals receiving

AHM (2.5 mL/kg) and AHM (2.5 mL/kg) + EtOH (2 g/kg) showed a significant (p < 0.05) increase in number of foot slips when compared to mice receiving EtOH (2 g/kg) alone (Fig 4B). There was a significant (p < 0.05) decrease in the total distance travelled (Fig 4C) after 28 days of repeated treatments with AHM (2.5 mL/kg), EtOH (2 g/kg) and AHM (2.5 mL/kg) plus EtOH (2 g/kg) when compared to saline treated mice.

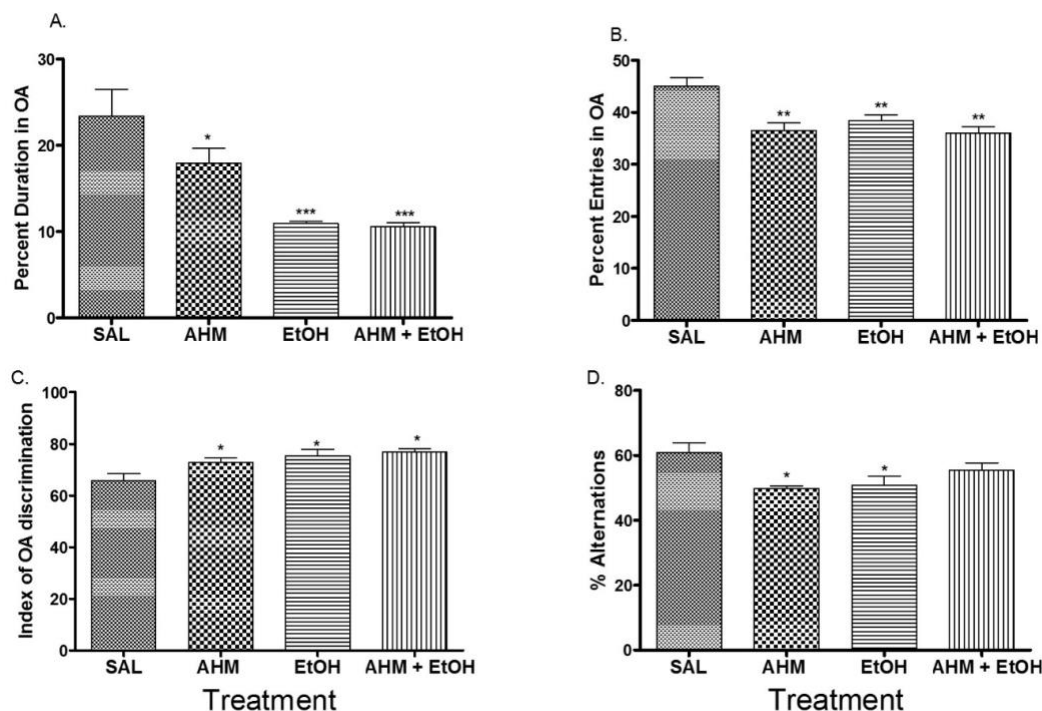


Figure 5: Effect of sub-acute administration of alcohol herbal mixture and ethanol on mice in the elevated plus maze (EPM) and Y-maze tests. (A) Percent duration in open arm (OA), (B) percent number of entries in the open arm and (C) Index of open arm (OA) discrimination, and (D) Percentage alternation in Y-maze. Values represents Mean \pm SEM (n=6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs SAL using one – way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison post hoc test. SAL- Saline, AHM –Alcoholic herbal mixture, EtOH – Ethanol.

Effect of sub-acute administration of alcoholic herbal mixture and ethanol on anxiety-like behaviour and memory in mice: There was significant anxiogenic effect when mice were sub-acutely administered with AHM (2.5 mL/kg), EtOH (2 g/kg) and AHM + EtOH as measured on the elevated plus maze platform. Statistical analysis as determined by one–way ANOVA showed significant effect on percentage duration in open arm ($F_{3,23}= 11.70$, $p < 0.0001$, Fig 5A), percentage entries in open arm ($F_{3,23}=8.711$, $p = 0.0007$, Fig 5B), and index of open arm discrimination ($F_{3,23}=5.384$, $p = 0.0070$, Fig 5C). The observed effect of sub-acute administration of alcoholic herbal mixture and ethanol showed a significant impairment of working memory on the Y-maze test. Repeated administration of AHM (2.5 mL/kg) and EtOH (2 g/kg) but not the co-administration caused a significant ($p < 0.05$) deficits in working memory when compared to SAL group (Fig 5D).

Effect of sub-acute administration of alcoholic herbal mixture and ethanol on brain acetylcholinesterase and oxidative stress markers.: Assessment of brain acetylcholinesterase activity in treated animals showed differential effects in the three brain regions of striatum, prefrontal cortex and hippocampus (Fig 6A). A significant ($p < 0.05$) increase in brain acetylcholinesterase activity in animals treated with AHM (2.5 mL/kg), EtOH (2 g/kg), and AHM + EtOH was observed in the prefrontal cortex when compared to saline-treated animals. No significant effect was observed in the striatum but a loss in acetylcholinesterase activity was observed in the hippocampus. Reduced

glutathione (GSH) was significantly depleted in all brain regions in mice treated with AHM (2.5 mL/kg), EtOH (2 g/kg), and AHM + EtOH when compared to saline-treated mice (Fig 6B). Nitrite levels in brain tissue was significantly elevated in the striatum and prefrontal cortex of animals treated with AHM (2.5 mL/kg), EtOH (2 g/kg), and AHM + EtOH when compared with saline-injected animals (Fig 6C). AHM (2.5 mL/kg) consistently increase nitrite levels in the three brain regions of striatum, prefrontal cortex and hippocampus. Malondialdehyde (MDA), a marker of lipid peroxidation in brain tissues was shown to be significantly elevated in the three brain regions of mice treated with AHM (2.5 mL/kg), EtOH (2 g/kg), and AHM + EtOH when compared to saline-treated animals (Fig 6D).

Effect of sub-acute administration of alcoholic herbal mixture and ethanol on histopathological alterations in the prefrontal cortex and hippocampus

Fig. 7A shows the photomicrographs of H&E staining of the prefrontal cortex in mice. Histopathological changes were observed in mice treated with AHM, EtOH and AHM + EtOH with increased number of pyknotic cells (black arrow) and decreased number of viable cells when compared to saline treated mice (white arrow). Quantification of the population of viable neuron showed that AHM, EtOH and AHM + EtOH significantly reduced the mean neuronal cell counts when compared to saline treated animals (Fig. 7B).

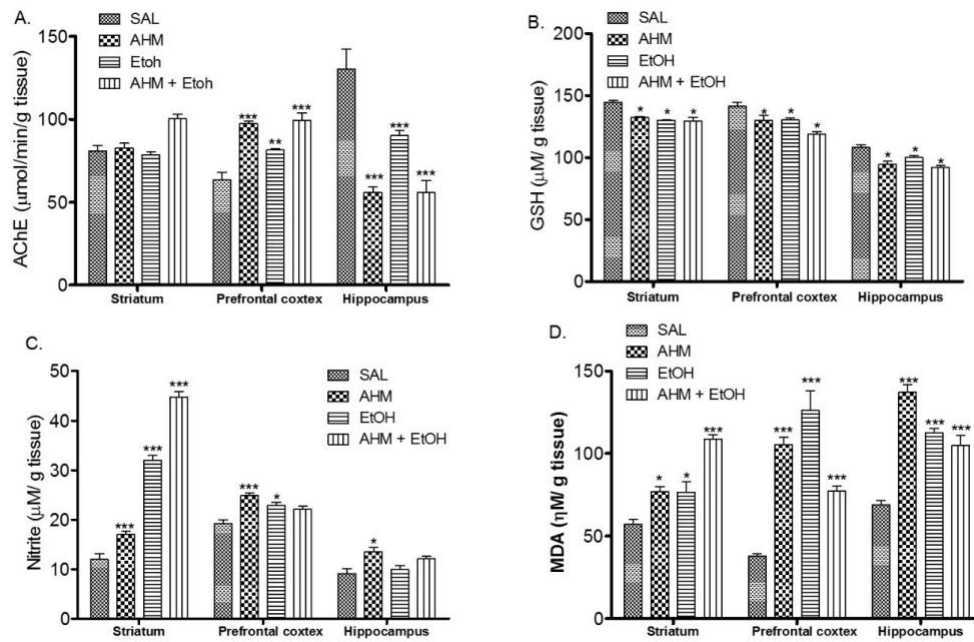


Figure 6: Effect of sub-acute administration of alcohol herbal mixture and ethanol on (A) brain acetylcholinesterase, (B) reduced glutathione (GSH), (C) nitrite, and (D) malondialdehyde (MDA). The results are Mean \pm SEM. * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs SAL using one – way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison post hoc test. SAL- Saline, AHM –Alcoholic herbal mixture, EtOH – Ethanol.

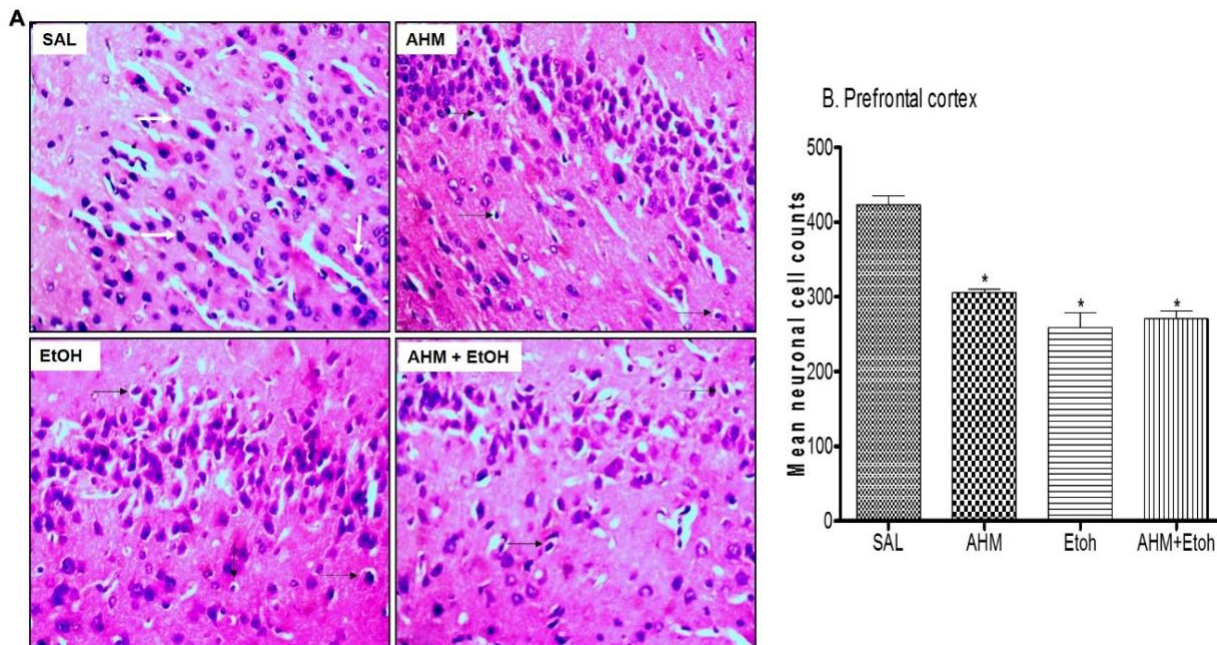


Figure 7: Effect of sub-acute administration of alcohol herbal mixture and ethanol on histopathological changes in the prefrontal cortex (A) Photomicrographs (H&E staining, mag x 400) and (B) Mean neuronal cell counts. The results are Mean \pm SEM. * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs SAL using one – way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison post hoc test. SAL- Saline, AHM –Alcoholic herbal mixture, EtOH – Ethanol.

As shown in Figure 8A, hippocampal section of CA2 region of saline-treated mice revealed the pyramidal neurons are normal and showing intense staining of nuclei and laminar arrangement of the neurons (white arrow). However, in the mice treated with AHM and AHM + EtOH, there was increase number of pyknotic cells (black arrow), reduction in cell

volume with less staining intensity and laminar distortion (Fig. 8A). Furthermore, AHM and AHM + EtOH significantly ($p < 0.05$) reduced mean neuronal cell count in the hippocampus (Fig. 8B)

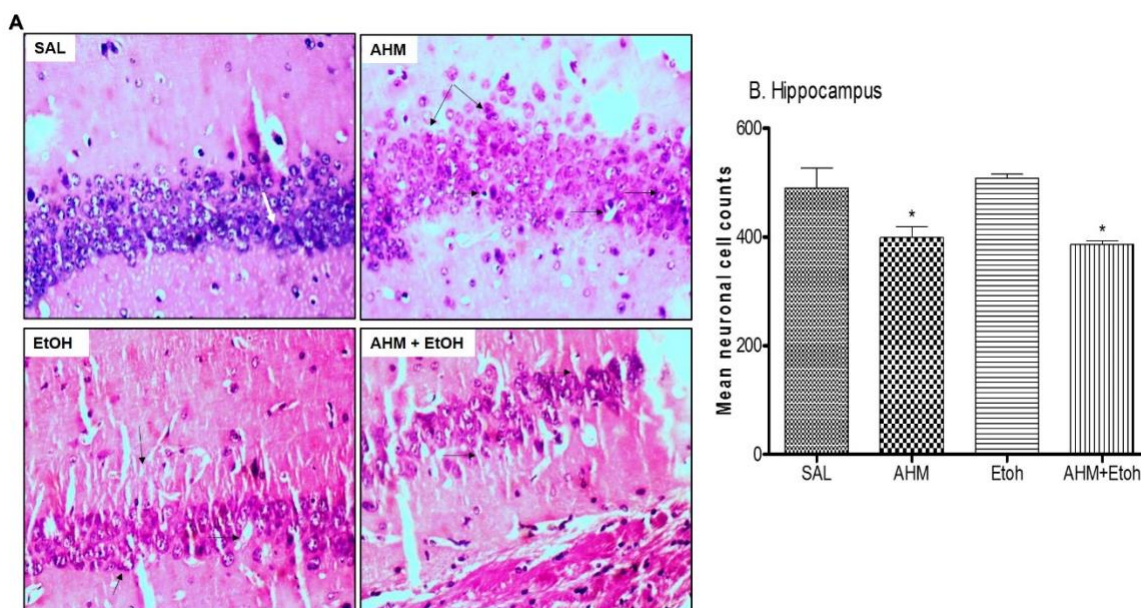


Figure 8: Effect of sub-acute administration of alcohol herbal mixture and ethanol on histopathological changes in the hippocampus (A) Photomicrographs (H&E staining, mag x 400) and (B) Mean neuronal cell counts. The results are Mean \pm SEM. * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs SAL using one – way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison post hoc test. SAL- Saline, AHM –Alcoholic herbal mixture, EtOH – Ethanol.

DISCUSSION

Here, our results showed that alcohol herbal mixture, ethanol and the combination caused neurobehavioral and biochemical perturbations in mice. The results showed enhanced reduction in locomotor activity and impaired motor coordination in mice following acute and sub-acute administrations of alcoholic herbal mixture combined with ethanol.

Alcohol is generally accepted as a sedative drug. However, it has been shown to possess both stimulatory and depressant effects on locomotor activity in a variety of rodents, and these are age, dose, and time-related effects (Fukushiro *et al.*, 2012). In our study, AHM alone and when combined with ethanol, showed a profound dose-dependent reduction on locomotion. The acute ethanol effect revealed a mild decrease in the sensorimotor function compared with the least dose of AHM. These observations indicate that AHM contains other phytochemicals with depressant activity on the central nervous system beyond the effect of ethanol. Furthermore, it also indicates that AHM contains higher ethanol content since literatures have shown that large doses of ethanol are known to decrease the locomotion of rats and mice (Crabbe *et al.*, 1982). In some recent studies, combination of ethanol with energy drinks containing caffeine have shown significant reduction on locomotor activities in mice (Robins *et al.*, 2016; Curran and Marczynski, 2017). In the present study, we have recorded reduced locomotor activity in mice repeatedly administered with AHM, ethanol and the combination. It is important to note the effect on locomotor and motor activities were observed on drug free days. At this time points, animals exhibited no perceptible withdrawal symptoms on the locomotor activity if compared to the increased locomotor activity noted at 24 h in the acute study.

Acute administration of alcoholic herbal mixture alone or combined with ethanol reduce rearing activity in mice. This effect was masked in the sub-acute treatment. Induced rearing behavior in mice is a measure of central nervous system excitation or explorative behaviour. Drug that stimulate the central nervous system increase rearing behavior while those that depress the central nervous system inhibit rearing behavior. The induced rearing behavior response is regulated by multiple neurotransmitter system such as the gamma amino butyric acid (GABA), dopamine, glutamate (Walting and Keith, 1998).

The walk beam balance test is used for assessing motor strength and coordination. Motor incoordination in mice is often considered as an index of neurotoxicity produced by drugs acting as depressants of the central nervous system (Crabbe *et al.*, 2003; Luong, *et al.*, 2011). Animals treated acutely and repeatedly with AHM alone and combined with ethanol showed a marked increase in walk beam parameters indicating an impairment in motor coordination. In fact, similar to our results, a protocol that used binge drinking episodes revealed an increased number of foot slips on the beam-walking test (Fernandes *et al.*, 2018). Also, the combination of nicotine and ethanol have shown impairment in motor performance in rats (McDaid *et al.*, 2016).

Anxiety is a state of excessive fear. It is characterized by motor tension, sympathetic hyperactivity, apprehension and vigilance syndromes (Ruiz, 2000). Anxiety interfere with intelligence, psychomotor function and memory (Vytal *et al.*, 2013). Alcohol is generally believed to exhibit anxiolytic effect. Individuals who suffer from symptoms of anxiety often turn to alcohol abuser because of its potential anxiolytic effect. However, anxiety is also one of the significant consequence of alcohol withdrawal (Hall and Zador, 1997). In our study, sub-

acute administration AHM and when combined with ethanol significantly caused anxiety-like behaviors in mice. Although studies have shown that acute administration of ethanol produces anxiolytic-like behavior in rodents in the elevated plus maze (Correa *et al.*, 2008), however, anxiety-like behaviors have been shown in rodents following chronic exposure (Kliethermes, 2005) or during alcohol withdrawal from moderate to high doses of alcohol (Rasmussen *et al.*, 2001).

In this study, AHM and ethanol but not the combination showed memory impairing effect in the Y-maze. The cognitive deficit effect of alcohol is well characterized in both acute and chronic alcohol consumption. Disruption of spatial learning and memory by repeated dosing with ethanol has been previously reported (Melchior *et al.*, 1993; Sircar *et al.*, 2009). In chronic alcohol abuse, one of the common neuropsychological sequelae is the impairment of learning and memory including spatial memory dysfunction (Bowden and McCarter, 1993). These signs have been reported in abusers of alcohol herbal mixtures in Nigeria (Chikere and Mayowa, 2011; Kehinde and Olusegun, 2012).

Alcohol intake has been associated with behavioural dysfunction in learning and memory structures of the brain. The hippocampus and prefrontal cortex which are associated with executive function are implicated in alcohol-induced neuronal toxicity. However, differential sensitivity of prefrontal cortex to the hippocampus to alcohol has been reported (Fowler *et al.*, 2014). Cholinergic innervation in the forebrain is altered by binge ethanol in adolescent rats leading to selective functional deficits in the prefrontal cortex (Fernandez and Savage, 2017). This study showed a modulation of acetylcholinesterase activity in the different brain regions of the striatum, prefrontal cortex and hippocampus. Acetylcholinesterase (AChE) is one of the most crucial enzymes associated with nerve response and function, and it is a biomarker of toxicity of the nervous system (Lionetto *et al.*, 2013). Estimation of acetylcholinesterase activity in the brain and erythrocytes is measure of acetylcholine activity in the brain and peripheral nerves.

A significant increase in the acetylcholinesterase activity in the brain prefrontal cortex of animals treated with AHM and combined with ethanol was demonstrated in our study. Alcohol was shown to impact the typical development of the prefrontal cortex by way of persistent alterations in frontal cortical volume, myelination, neuroinflammation and neurodegeneration (Fowler *et al.*, 2014). The prefrontal cortex is an essential area of the cortex responsible for working memory and higher cognitive functions and is modulated by acetylcholine. AHM and ethanol by enhancing acetylcholinesterase activity in the prefrontal cortex inhibits acetylcholine's effect on working memory.

Repeated administration of alcohol herbal mixture and ethanol elicits a significant increase in nitrite, malondialdehyde and depleted glutathione in different brain regions of the striatum, prefrontal cortex and hippocampus. Alcohol abuse has been associated with oxidative stress at the cellular level, resulting in oxidative damage to DNA and other biomolecules (Cunha-Oliveira *et al.*, 2008). Also, alcohol-induced oxidative stress affects many physiological processes, such as memory, motor function and cognitive abilities

(Fowler *et al.*, 2014). The metabolism of ethanol through acetaldehyde to acetate is linked with the overproduction of reactive oxygen species that accentuate the oxidative stress of cells (Hernandez *et al.*, 2016). The degree of lipid peroxidation can be measured by the level of 4-hydroxynonenal (HNE) or malondialdehyde and the increased production of these products have been related to decrease in neuronal viability (Chen *et al.*, 1997; Das and Vasudevan, 2007). Ethanol-induced lipoperoxidation by oxidative stress also decrease the intracellular reduced glutathione (Ramezani *et al.*, 2012; Fernandes *et al.*, 2018).

AHM showed depletion of glutathione and increased malondialdehyde in all brain regions, and was consistent with effect of ethanol (Montoliu *et al.*, 1994; Bailey *et al.*, 2001). Herbs were thought to have good antioxidant properties, however the higher ethanol content in AHM overwhelms the antioxidant effects of the herbal mixture. Alcohol-induced oxidative stress generates DNA damage, which is responsible for neuronal apoptosis and neuronal dysfunction often associated with different neurological pathologies (Ramezani *et al.*, 2012).

AHM treatment resulted in a significant histopathological alterations in the prefrontal cortex and hippocampus. These parts of the brain are known to play important roles in locomotory functions and learning processes (Sarkaki *et al.*, 2014). Increased populations of pyknotic nuclei is an indication of loss of viable neurons in the prefrontal cortex and hippocampal regions of the mice brain. Pyknotic nucleus have been identified as a form of necrotic neuronal death seen in adult rats exposed to ethanol (Obernier *et al.*, 2002). Damage to the prefrontal cortex affects executive functions including the working memory, impulse control and decision making. The loss of these functions is suspected to contribute to the development of addiction (Bechara *et al.*, 1994; Fowler *et al.*, 2014). Earlier studies have shown that chronic ethanol exposure have resulted in neuronal cell loss in the hippocampus, which was associated with oxidative stress-induced neurotoxicity (Lukoyanov *et al.*, 2000).

In conclusion, this study has provided evidence of behavioral perturbations such as anxiety and poor working memory and brain biochemical changes including increased level of oxidative stress biomarkers. Thus, consumption of alcoholic herbal mixture (*paraga*) might produce substance-attributable harm and quicken neurodegenerative diseases in humans.

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