THE SYNERGISTIC EFFECT OF *Curcuma longa* AND *Rosmarinus officinalis* ON SOME BIOCHEMICAL MARKERS IN THE BRAIN OF ALCOHOL- TREATED RATS

ABSTRACT

Background: Neurological disorders remain a global health challenge. Chronic alcohol consumption induces damage to the brain that can cause various forms of dementia. An abundance of acetaldehyde is produced by excessive alcohol consumption and accumulates in the body to induce oxidative stress, apoptosis, and inflammation in neuronal cells, which results in brain damage leading to memory loss. This study is aimed at investigating the effect of mixed ethanol extract of *Curcuma longa* rhizome and *Rosmarinus officinalis* leaf on oxidative stress and proinflammatory markers in the brain of alcohol- treated male Wistar rats. **Methods:** A total of forty-three adult male Wistar rats were used for this study. 18 rats were used for determination of median lethal dose (LD_{50}) of the mixed extract while 25 rats were randomly divided into 5 groups of 5 rats each. Group 1 (control) received distilled water, group 2 was treated with 600mg/kg bwt of mixed extract only and group 3 was administered 15 mg/kg of 40% ethanol only. Group 4 received 300 mg/kg mixed extract along with 15 mg/kg of 40% ethanol while group 5 received 600 mg/kg of mixed extract along with15 mg/kg of 40% ethanol. Treatments were given orally once daily for 21 days. Twenty-four hours after the administration of the last treatment, brain tissues were removed from the animals for biochemical analysis. **Results:** LD_{50} up to 5000 mg/kg body weight of mixed extract did not show any fatality of animals Administration of ethanol significantly ($p < 0.05$) reduced the activities of brain superoxide dismutase, Catalase, glutathione, and glutathione peroxidase while the level of malonaldehyde, Interleukin- 1β and tumor necrosis factor-α were significantly ($p<0.05$) increased when compared with control group. The histological results also showed significant neuron insult such as marked degeneration and necrosis of the pyramidal cells of the hippocampus. Co-administration with 300 or 600 mg/kg mixed extract significantly and dose-dependently prevented all biochemical alterations and histological lesions in rats chronically administered alcohol. **Conclusion:** Moreover, alcohol enhanced acetylcholinesterase activity in the brain, but this was significantly inhibited by co administration with the mixed extract. Therefore, the mixed extract may play role in the prevention of alcohol-related dementia.

Key words: Alcohol, dementia, *Curcuma longa, Rosmarinus officinalis,* Oxidative stress and proinflammatory markers

1. INTRODUCTION

Alzheimer's disease is the most common form of dementia, accounting for 60% to 80% of dementia cases, according to the Alzheimer's Association [1]. Currently, more than 57 million people live with dementia worldwide, and this number is expected to increase to more than 152 million by 2050 [2]. Among the several kinds of dementia, substance-induced persisting dementia, which may be due to drug abuse, medication, or toxin exposure is on the increase [3].

A number of toxins have been reported to cause dementia, but the principal one in most societies is alcohol (ethanol). As classified by the World Health Organization (WHO), alcohol use is the third-highest health risk factor in developed countries and the greatest risk factor in developing countries [4].

Overindulging in alcohol has been shown to have detrimental consequences on both short- and long-term health, including the possibility of developing dementia or Alzheimer's disease due to brain damage [5-9]. Alcoholic dementia is a disorder characterized by multiple cognitive deficits that include memory impairment associated with one or more cognitive disturbances [10]. Studies have showed that even modest amounts of alcohol can accelerate brain atrophy, which is the loss of brain cells, and increase the number of amyloid plaques, which are the accumulation of toxic proteins in Alzheimer's disease [11].

Oxidative stress is fundamental to the etiology of many diseases including Alcoholic dementia [12]. Alcohol generates reactive oxygen species (ROS) whether it is consumed acutely or chronically [13]. Studies have showed that in Alcoholic dementia, alcohol utilizes oxidative stress as one of the main tools to wreak havoc in the central nervous system [14]. Alcohol can easily cross the cell membranes, including the blood-brain barrier. Primarily, alcohol is metabolized by alcohol dehydrogenase (ADH) and Cytochrome P450 (CYP450) in the liver [15]**.** The brain is devoid of ADH [12], hence alcohol metabolism in brain is predominantly facilitated by CYP450 subtype 2E1 (CYP2E1) [16]. CYP2E1 is localized in various cellular sites, including the plasma membrane, endoplasmic reticulum (ER), Golgi apparatus and highly expressed in the mitochondria. By-products of alcohol metabolism by CYP2E1 are acetaldehyde and reactive oxygen species (ROS), like radical superoxide anion (O2-) and hydrogen peroxide [13,16].

Aside from CYP2E1, lipopolysaccharides (LPS), a serum bacterial endotoxin also was associated with alcohol consumption and oxidative stress. LPS-induced oxidative stress promotes ROS production by activating nicotinamide adenine dinucleotide phosphate (NADH) oxidase (NOX). Oxidative stress refers to an imbalance of the redox system characterized by excessive level of free radicals and impaired antioxidant system. Prolonged alcohol drinking worsens oxidative stress affecting the whole cell, or specific cellular constituents including proteins, lipids, and DNA [17]. The brain is one of the most metabolically active organs and thus, it is profoundly susceptible to oxidative stress, owing to its high demand for oxygen consumption and low levels of endogenous antioxidants to eliminate free radicals [18]. Excessive ethanol exposure in animals also induces inflammatory mediators and brain damage via activation of the innate immune receptor, toll-like receptor 4 (TLR4), in glial cells [19], and inflammation through TLRs further drives ROS production and mitochondrial damage [20]. Reduced acetylcholine levels have been implicated in AD pathology [21]. Acetylcholinesterase, the predominant cholinesterase in the brain, hydrolyzes acetylcholine to choline and acetate, thereby terminating the effect of this neurotransmitter at cholinergic synapses. Therefore, acetylcholinesterase is the target of cholinesterase inhibitors used for addressing the cholinergic deficit in AD patients.

Several chemical agents are generally used in AD treatment such as Aniracetam, Oxiracetam, Pramiracetam, Piracetam and Choline esterase inhibitors like Donepezil, but the side effects of these agents have made their applicability limited [22]. Herbal remedies are traditionally used all over the world to enhance poor memory and related ailments [23,24]. The increasing demand for herbal remedies worldwide is because herbal compounds have lesser or no side effects than any other chemical compound. Several medicinal plants such as *Curcuma longa* (*C. longa*) and *Rosmarinus officinalis* (*R. officinalis*) and their extracts have shown nootropic properties or memory-enhancing properties by suitable character of their medicinal components [25].

R. officinalis (Rosemary) a member of the mint family Lamiaceae, is a fragrant evergreen herb native to the Mediterranean. It is used as a culinary condiment, to make bodily perfumes, and for its potential health benefits. Since the beginning of time, the herb has been praised for its healing abilities. In the past, people have used *R. officinalis* to relieve muscle discomfort, stimulate blood flow into the brain, enhance memory and focus, strengthen the immune and circulatory systems, and encourage hair development [26]. Furthermore**,** the anticancer prevention, antiaging, anti-inflammatory and antioxidant properties, antiulcer effect of *R. officinalis* leaf have also been documented [27]. *C. longa* (Turmeric), sometimes called Indian saffron or the golden spice, is a tall plant that grows in Asia and Central America. *C. longa* is the spice that gives curry its yellow color. The spice may be the most effective nutritional supplement in existence, it has been used in India for thousands of years as both a spice and medicinal herb. In recent years, science has begun to support traditional claims that turmeric contains bioactive compounds with medicinal properties. *C. longa* and its major compound of interest, curcumin, has been extensively studied to treat a variety of diseases and ailments. The ability of *C. longa* rhizome to reduce inflammation, increase antioxidant capacity, increase brain-derived neurotrophic factor, decrease the risk of heart disease, prevent cancer, and help treat Alzheimer's disease have also been documented [28-30]. In addition to helping combat chronic diseases linked to aging and delaying aging, *C. longa* rhizome protective effect against depression has also been demonstrated [26].

Multiple studies in rodents and humans have shown that *curcumin* crosses the blood brain barrier. However, its main drawback is the low bioavailability due to poor solubility, low absorption, rapid metabolism, and rapid excretion [31,32]. This obstacle may be overcome through the use of combination treatments with other extracts that improve bioavailability or hinder additional pathways [33]. Synergistic interactions between the compounds of medicinal plant extracts are a vital part of their therapeutic efficacy [34,35]. Levine et al. [36] and Mohamed et al. [37] have demonstrated that plants such as *R. officinalis* that have potent anti-inflammatory and antioxidant effect can positively support the action of Curcuma longa due to a greater increase in intracellular curcumin accumulation when the combination treatment was used**.** However, whether this synergic interaction will be effective in neurological disorders in animal model has not been tested to the best of our knowledge. If no new treatment methods are created to slow down, stop, or prevent AD, the predicted total healthcare expenses for AD treatment in 2021 will rise to more than $1.1 trillion by 2050 due to an aging population. The aim of this study is to determine the synergic effect of mixed extract of *C. longa* rhizome and *R. officinalis* leaf on some biochemical markers in alcohol- induced oxidative damage in the brain.

2. MATERIALS AND METHOD

2.1 Plant materials

Plants materials including *C. longa* rhizome and *R. officinalis* leaf were obtained from a popular vegetable market at Obigbo, Oyibo LGA, Rivers State, Nigeria. The identity of the plants was confirmed by a plant taxonomist at the Department of Plant Science and Biotechnology, Faculty of Science**,** Rivers State University. Voucher specimens were deposited in the herbarium of the Department of Plant Science and Biotechnology, Rivers State University.

Figure 1. *Rosmarinus officinalis* leaf and *Curcuma longa* rhizome

2.2 Preparation of extracts

For the preparation of extracts, the method used by Ijioma *et al*. [38] was adopted. Briefly, each air-dried sample was pulverized using a manual blender. Two hundred grams of the powdered sample was macerated in 1.5 liters of 96% ethanol within 48 hours and filtered, first with a clean handkerchief and then a filter paper. The filtrate so obtained was concentrated in a hot air oven at 40^{o}C to obtain the extract of *C. longa* which weighed 6.79 g and represented a 3.40 percentage extract yield. For *R. officinalis*, same procedures were followed but 9.35 g extract was obtained, representing an extract yield of 4.68%. A strong congealed jelly-like residue for *C. longa* and a powder-like residue for *R. officinalis*. The extracts so obtained were preserved in a refrigerator until use. A 1:1 ratio of *C. longa* and *R. officinalis* was used for this study.

2.3 Experimental Animals

For this study, forty-three (43) male Wistar rats weighting between 140-160g obtained from the animal house of the Department of Biochemistry, University of Port Harcourt and transferred to the animal house of the Department of Biochemistry, Rivers State University, Port Harcourt, Nigeria. The rats were initially acclimatized for a period of 2 weeks after their purchase. They were housed in wooden cages placed in a well-ventilated rat house. Rats were provided with rat pellets and unlimited supply of water. All the animals received humane care according to the

criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Science and published by the National Institute of Health. The experimental protocol was permitted by the Institutional Animal Care and Use Committee of Rivers State University.

2.4 Acute toxicity evaluation

Acute toxicity tests on the extracts were carried out in accordance with the new Lore's method used by Orieke et al. [39]. For each extract, 2 phases of tests were involved. In the first phase, 9 rats assigned to 3 groups (A, B and C) of 3 rats each were administered via the oral route, 10, 100 and 1000 mg/kg of the extract respectively. When no mortality was observed across the groups after 24 hours, the study proceeded to the second phase. In the second phase, another set of 9 rats also assigned to 3 groups as mentioned earlier were treated with 1600, 2900 and 5000 mg/kg of extract respectively. With no mortality still observed, 5000 mg/kg was repeated on a last set of 3 rats. These rats were thereafter observed within 24 hours and a further 7 days. Lorke's formula was employed in the final determination of the LD_{50} values of the extracts.

2.5 Experimental Design

The twenty-five (25) male Wistar rats were randomly allocated to five groups consisting of five rats each. See Table 1 for treatment approach

Table 1. Experimental and treatment design

Group	No. of Animals	Administered Dose
1	5	control: distilled water (vehicle)
2	5	600mg/kg body weight of mixed extract of *R. officinalis* leaf and *C. longa* rhizome
3	5	15ml/kg of 40% ethanol
4	5	300mg/kg of body weight of mixed extract + 40% ethanol
5	5	600mg/kg of body weight of mixed extract + 40% ethanol

The dose of ethanol used in this study is well documented to induce neuro toxicity and oxidative damage in rats (Hamid *et al*., 2018), while the 300mg or 600mg/kg of mixed extract of *C. longa* rhizome and *R. officinalis* leaf was extrapolated from acute toxicity result (LD_{50} >5000mg/kg).

2.6 Sample collection for biochemical analysis

Treatment was done by the oral route and lasted for twenty-one (21) days. On the last day of drug administration, the animals were fasted overnight, weighed and three (3) animals from each group were sacrificed via cervical dislocation. Whole brain organs were excised, weighed and placed in a clean sample bottle containing phosphate buffer solution for the preparation of

homogenates. This is to prevent the breakdown of the antioxidant enzymes in the organ. Portions of the brain samples were also preserved in 10% formalin for histopathological examination.

2.7 Preparation of Brain Homogenates

One gram of the brain tissue was homogenized in 5 ml of 0.5 M phosphate buffer (pH 7.4) solution using a laboratory mortar and pestle. The homogenized sample was then centrifuged (Model No. Yp-502N) at 10,000 *g* for 20 min at 4 °C. The clear supernatant from the brain homogenate was then transferred into a clean plane bottle and labeled appropriately. The supernatants from brain homogenate were used for biochemical analysis.

2.8Antioxidant enzymes ad oxidative stress biomarkers

The activities of superoxide dismutase (E.C.1.15.1.1.) and Catalase (CAT, E.C.1.11.1.6.) glutathione were measured by using the sensitive rat SOD Enzyme-linked Immunosorbent Assay (ELISA) kit KT-60703 (Kamiya Biomedical Company) and MBS701713 (MyBiosource inc, Company, Southern California, USA) respectively, with the aid of ELISA kit Microplate Reader according to the manufacturer's protocol. Reduced glutathione (GSH) content of brain tissues homogenate were determined using a high sensitivity and excellent specificity rat glutathione enzyme-linked immunosorbent assay (ELISA) Kit MBS724319 (MyBiosource.com Company, San Diego, CA. USA) while the Malondialdehyde (MDA) level of cardiac tissues homogenate was measured as index of lipid peroxidation using high specificity ELISA kit MBS9389391 (My BioSource Inc., Company, San Diego, CA. USA) and presented as activity units per mg of protein (units/mg proteins).

2.9 Proinflammatory markers

Two proinflammatory cytokines, interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), were examined in the supernatant using commercially accessible enzyme-linked immunosorbent assay (ELISA) kits subsequent to the instructions described by the manufacturer (CUSABIO, Biotechnology Company Sweden). The obtained values were presented in pg/mL.

Protein quantification

Total Protein in the cardiac tissue was quantified using Randox kit Biuret method as described by Tietz [40] with bovine serum albumin as the standard and expressed as mg protein/ml.

2.10. Acetylcholinesterase (AChE) inhibition assay

The AChE inhibitory activities of the Lamiaceae extracts and their phenolic acid constituents were determined using modified Ellman's colorimetric method described by Conforti et al. [41]. In brief, 1900 µL of 50 mM Tris-HCl buffer (pH 8.0), 40 µL of 0.02 U/mL AChE and 20 µL of tested solution (0.25–1 mg/mL) were mixed and pre-incubated for 30 min at 4 °C. The reaction was then initiated with the addition of 20 µL of 10 mM DTNB and 20 µL of 12 mM ATChI. AChE activity was determined spectrophotometrically through measuring the change in ultraviolet absorbance of an assay solution at 412 nm over a period of 10 min at 25 °C. Physostigmine (0.03–1 µg/mL) was used as positive control. The experiment was run in triplicate. Percentage enzyme inhibition was calculated by comparing the enzymatic activity

with, and without inhibitor. The result of AChE inhibition was calculated using the following formula and then, IC_{50} values were determined for each sample.

$$\%AChE\ inhibition = \frac{1 - (OD_{sample} - OD_{control})}{OD_{blank} - OD_{control})}\ x\ 100$$

2.11 Histological examination

Tissue samples from the brain were fixed in 10% formalin, embedded in paraffin wax. The obtained tissue sections were collected on glass slide, deparaffinized in xylene, hydrated in descending series of ethyl and stained by hematoxylin stain dehydrated with ethyl alcohol. The slides were viewed under a light microscope (Nikon Ci-S, type 104c), and selected images were captured using moticam 2.0 digital camera attached to a Toshiba computer screen computer.

2.12 Statistical analysis

All experimental data were expressed as mean ± standard deviation (SD) for five animals in each group, with the aid of Microsoft Excel 2016 (Roselle, Ill., USA). Significant differences were analyzed using one-way ANOVA with Tukey's multiple comparison tests. The results obtained were considered as statistically significant if $p<0.05$.

3. RESULTS

Results of acute toxicity evaluation of the mixed ethanol extract of *Curcuma longa* rhizome and *Rosmarinus officinalis* leaf

No mortality was observed in all stages of acute toxicity tests carried out on both extracts, even at 5000 mg/kg oral dose level. Toxicity signs like aggression, agitation, calmness, display of writhing reflexes, convulsions, roughness of hairs were also not observed across the groups. The animals instead remained active and stable throughout the test period of 24 hours (Table 2) and a further 7 days (Table 3). Therefore, the oral LD_{50} of mixed ethanol extract of *C. longa* rhizome and *R. officinalis* leaf in Wistar rat was greater than 5000 mg/kg.

Table 2: Phase 1 LD_{50} results

Group	Dose (mg/kg)	No. of death	Observation
1	10	0/3	Animals were active and physically stable. Signs of toxicity like agitations, roughness of hairs, depression, writhing reflexes and death were absent.
2	100	0/3	Animals were active and physically stable. Signs of toxicity like agitations, roughness of hairs, depression, writhing reflexes and death were absent.
3	1000	0/3	Animals were active and physically stable. Signs of toxicity like agitations, roughness of hairs, depression, writhing reflexes and death were absent.

Table 3: Phase 2 LD_{50} results

Group	Dose (mg/kg)	No. of death	Observation
1	1600	0/3	Animals were active and physically stable. Signs of toxicity like agitations, roughness of hairs, depression, writhing reflexes and death were absent.
2	2900	0/3	Animals were calm and physically inactive for about 25 minutes but regained physical activity thereafter. Signs of toxicity like agitations, roughness of hairs, depression, writhing reflexes and death were absent.
3	5000	0/3	Animals were calm and physically inactive for about 2 hours, but regained physical activity thereafter. Signs of toxicity like agitations, roughness of hairs, depression, writhing reflexes and death were absent.

LD_{50} > 5000 mg/kg body weight

Effect of treatment with mixed ethanol extract of *Curcuma longa* rhizome and *Rosmarinus officinalis* leaf on relative brain weight

There was no significant difference in brain weight (relative to body weight) in all tested groups when compared to the control (Figure 2).

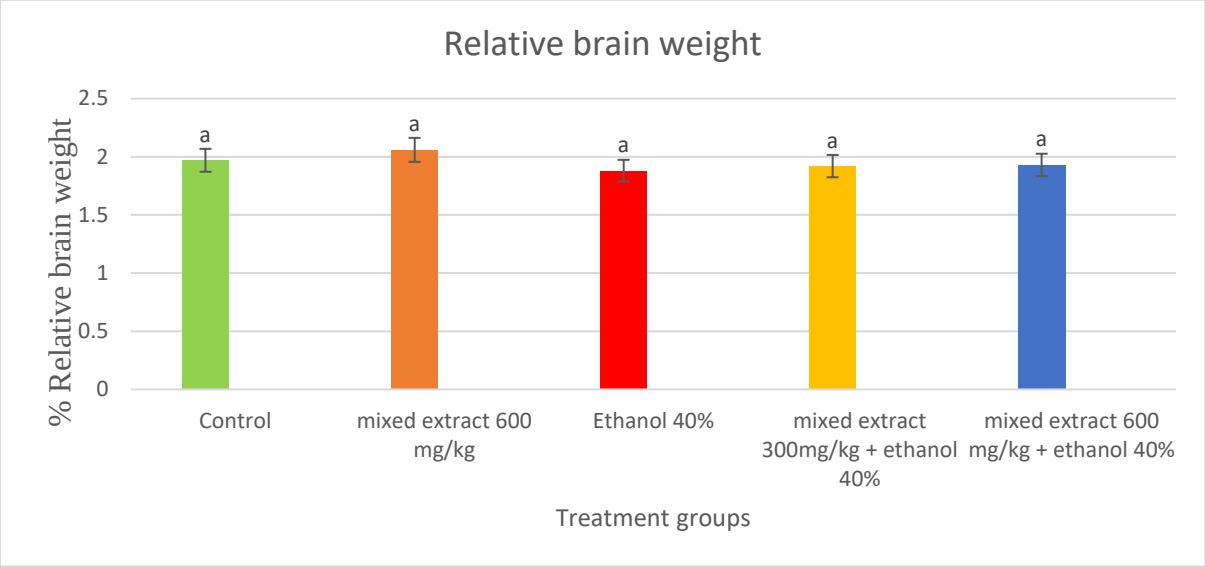


Figure 2: Effect of mixed ethanol extract of *Curcuma longa* rhizome and *Rosmarinus officinalis* leaf on relative organ weight in alcohol – induced oxidative damage in adult male Wistar rats. Values are presented as mean ± standard deviation (n = 5). Bars with different letter superscripts are significantly different ($p < 0.05$).

Effect of treatment with mixed ethanol extract of *Curcuma longa* rhizome and *Rosmarinus officinalis* leaf on brain homogenate oxidative stress and antioxidant enzymes activities in alcohol- induced oxidative damage

Alcohol administration caused a significant decrease ($p>0.05$) in brain tissue activity of SOD, catalase, GPx and GSH in the group which was only fed alcohol when compared to the control. Treatment with graded doses of mixed extract caused significant ($p>0.05$) increase in SOD, catalase, GPx and GSH level in alcohol fed groups when compared to the control (Figure 1-6). Elevated level of MDA was observed in the group which was only fed alcohol but treatment with mixed extract significantly reduced the MDA levels level in alcohol fed groups when compared to the control (Figure 7).

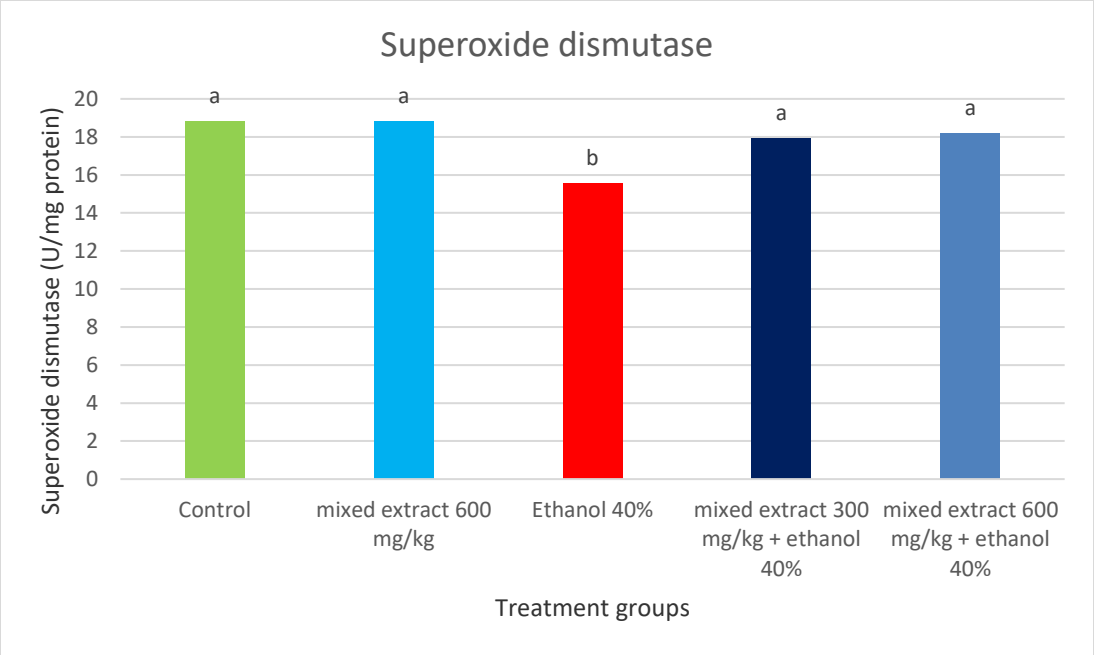


Figure 3: Effect of mixed ethanol extract of *Curcuma longa* rhizome and *Rosmarinus officinalis* leaf on brain homogenate superoxide dismutase activity in alcohol – induced oxidative damage in adult male Wistar rats. Values are presented as mean ± standard deviation (n = 5). Bars with different letter superscripts are significantly different ($p < 0.05$).

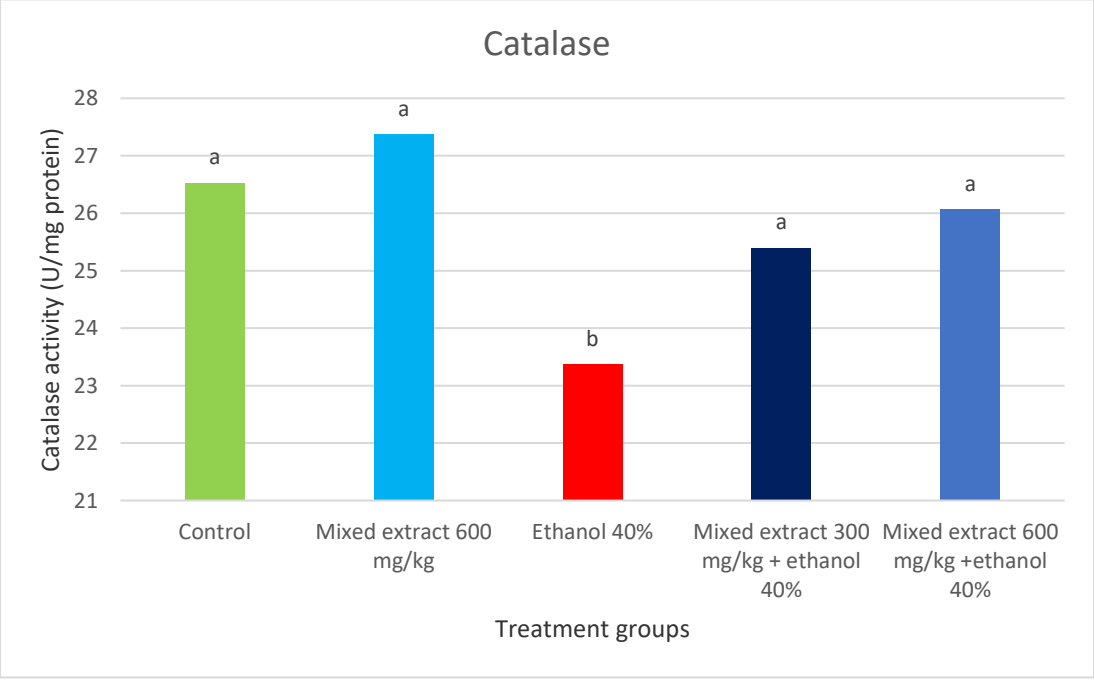


Figure 4: Effect of mixed ethanol extract of *Curcuma longa* rhizome and *Rosmarinus officinalis* leaf on brain homogenate catalase activity in alcohol – induced oxidative damage in adult male Wistar rats. Values are presented as mean ± standard deviation (n = 5). Bars with different letter superscripts are significantly different ($p < 0.05$).

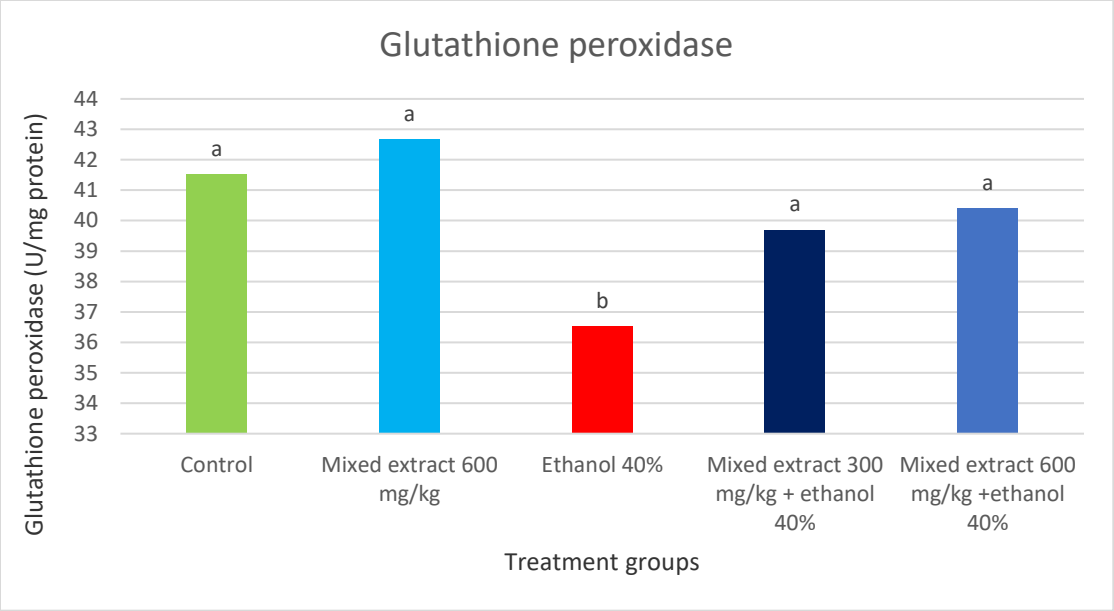


Figure 5: Effect of mixed ethanol extract of *Curcuma longa* rhizome and *Rosmarinus officinalis* leaf on brain homogenate glutathione peroxidase activity in alcohol – induced oxidative damage in adult male Wistar rats. Values are presented as mean ± standard deviation (n = 5). Bars with different letter superscripts are significantly different ($p < 0.05$).

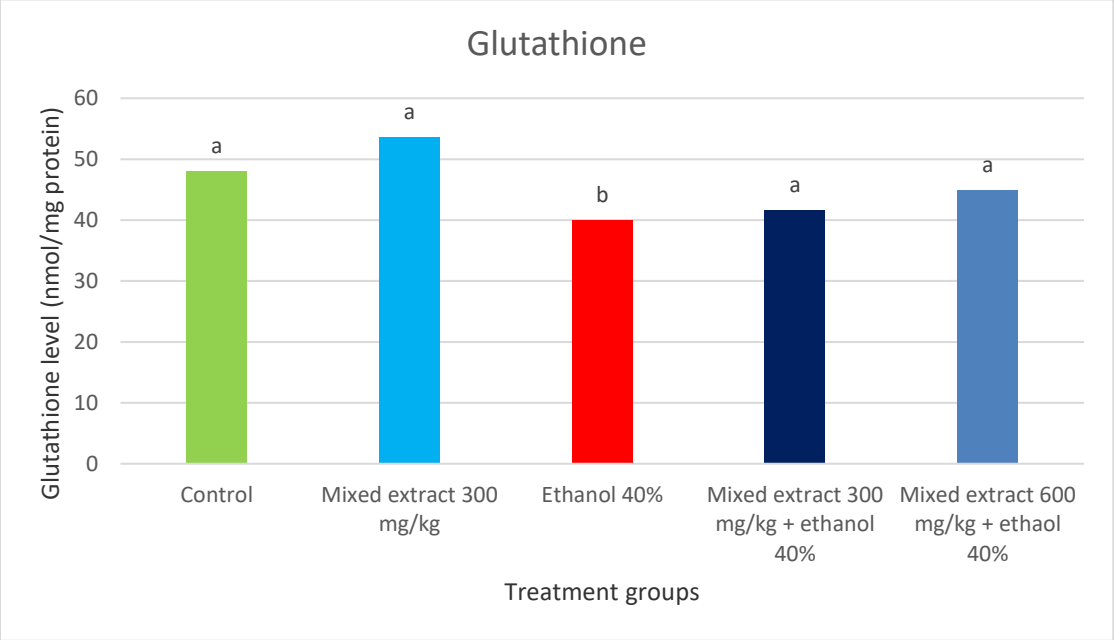


Figure 6: Effect of mixed ethanol extract of *Curcuma longa* rhizome and *Rosmarinus officinalis* leaf on brain homogenate glutathione level in in alcohol – induced oxidative damage in adult male Wistar rats. Values are presented as mean ± standard deviation (n = 5). Bars with different letter superscripts are significantly different ($p < 0.05$).

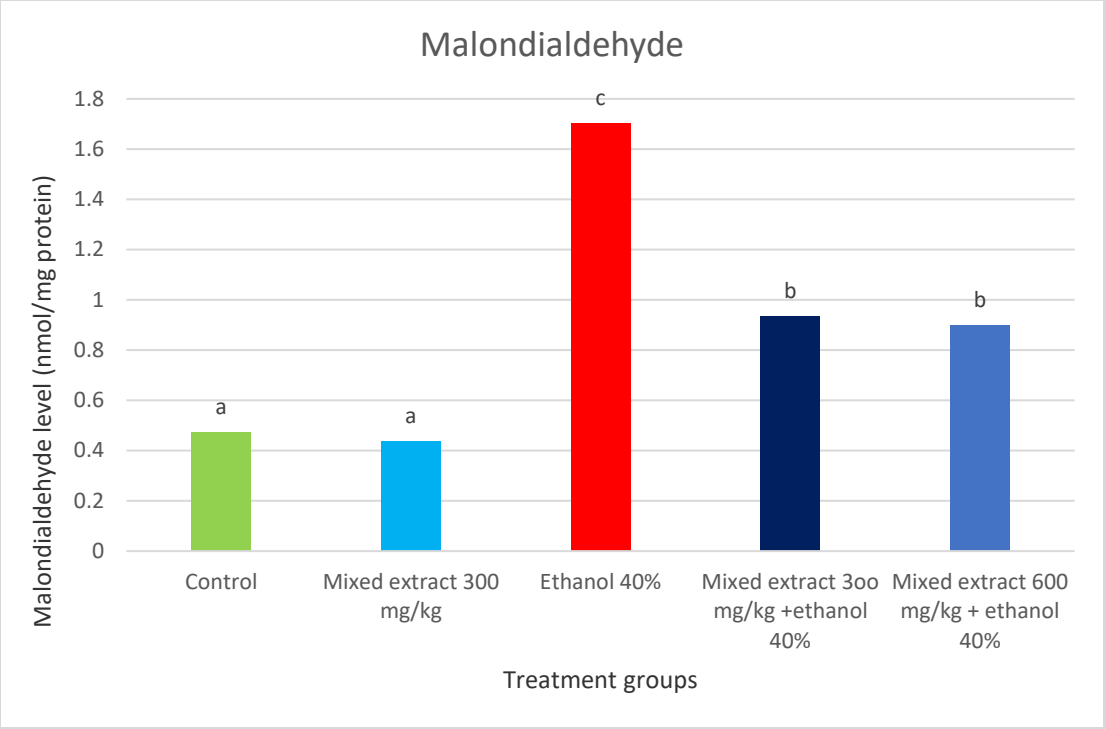


Figure 7: Effect of mixed ethanol extract of *Curcuma longa* rhizome and *Rosmarinus officinalis* leaf on brain homogenate malondialdehyde level in in alcohol – induced oxidative damage in adult male Wistar rats. Values are presented as mean ± standard deviation (n = 5). Bars with different letter superscripts are significantly different ($p < 0.05$).

3.3 Effect of treatment with mixed ethanol extract of *Curcuma longa* rhizome and *Rosmarinus officinalis* leaf on brain homogenate pro-inflammatory markers in alcohol-induced oxidative stress

The result shows a significant ($p>0.05$) increase in TNF-α and IL-1β levels in group 3 rats that was administered alcohol only when compared to control. TNF-α and IL-1β levels were significantly ($p>0.05$) reduced in group 4 and 5 rats treated with mixed extract when compared to the control (Figure 8 and 9).

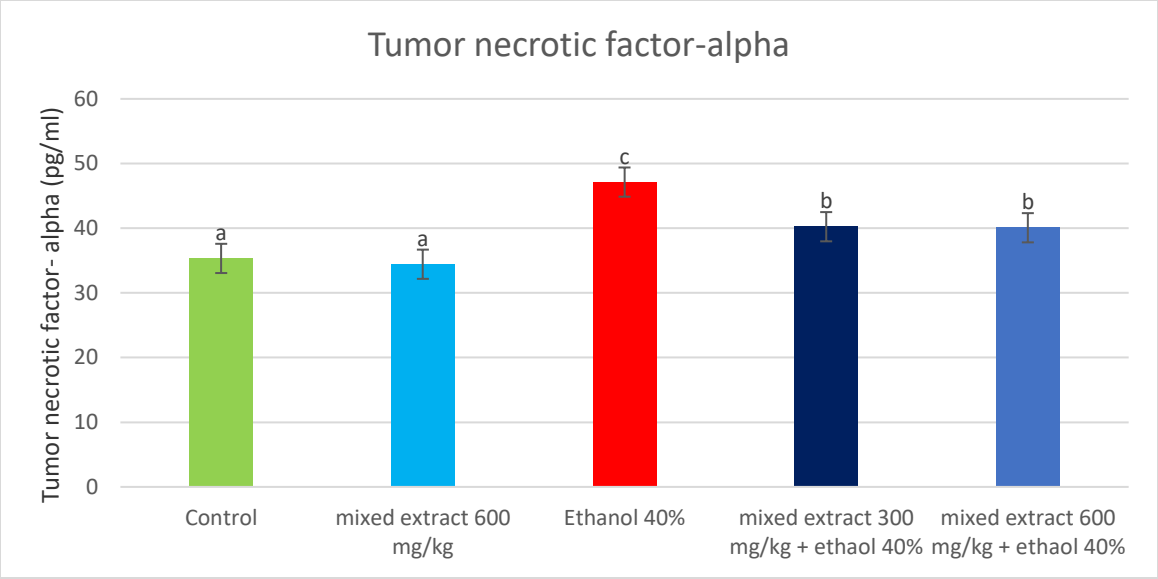


Figure 8: Effect of mixed ethanol extract of *Curcuma longa* rhizome and *Rosmarinus officinalis* leaf on brain homogenate tumor necrotic factor- alpha (TNF-α) level in in alcohol – induced oxidative damage in adult male Wistar rats. Values are presented as mean ± standard deviation (n = 5). Bars with different letter superscripts are significantly different ($p < 0.05$).

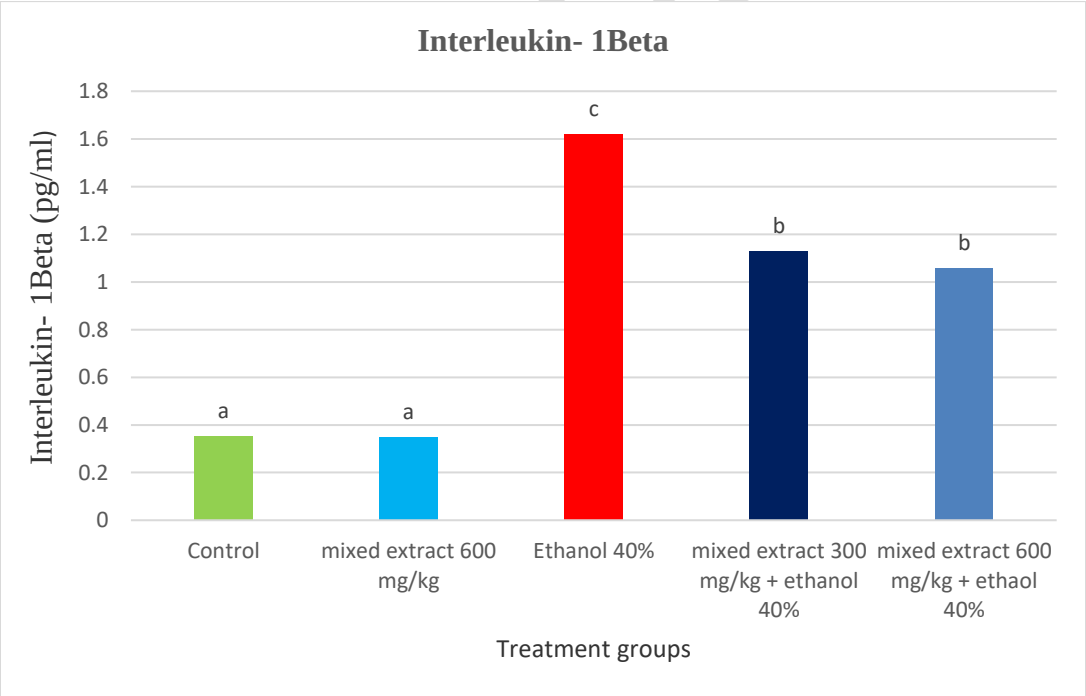


Figure 9: Effect of mixed ethanol extract of *Curcuma longa* rhizome and *Rosmarinus officinalis* leaf on brain homogenate interleukin-1beta (IL-1β) level in in alcohol – induced oxidative damage in adult male Wistar rats. Values are presented as mean ± standard deviation (n = 5). Bars with different letter superscripts are significantly different ($p < 0.05$).

3.4 Effect of treatment with mixed ethanol extract of *Curcuma longa* rhizome and *Rosmarinus officinalis* leaf on brain tissue acetylcholinesterase activity in alcohol- induced oxidative stress

The result shows a significant ($p>0.05$) increase in acetylcholinesterase activity in group 3 rats that was administered alcohol only when compared to control. Acetylcholinesterase activity was significantly ($p>0.05$) reduced in group 4 and 5 rats treated with mixed extract when compared to the control (Figure 10).

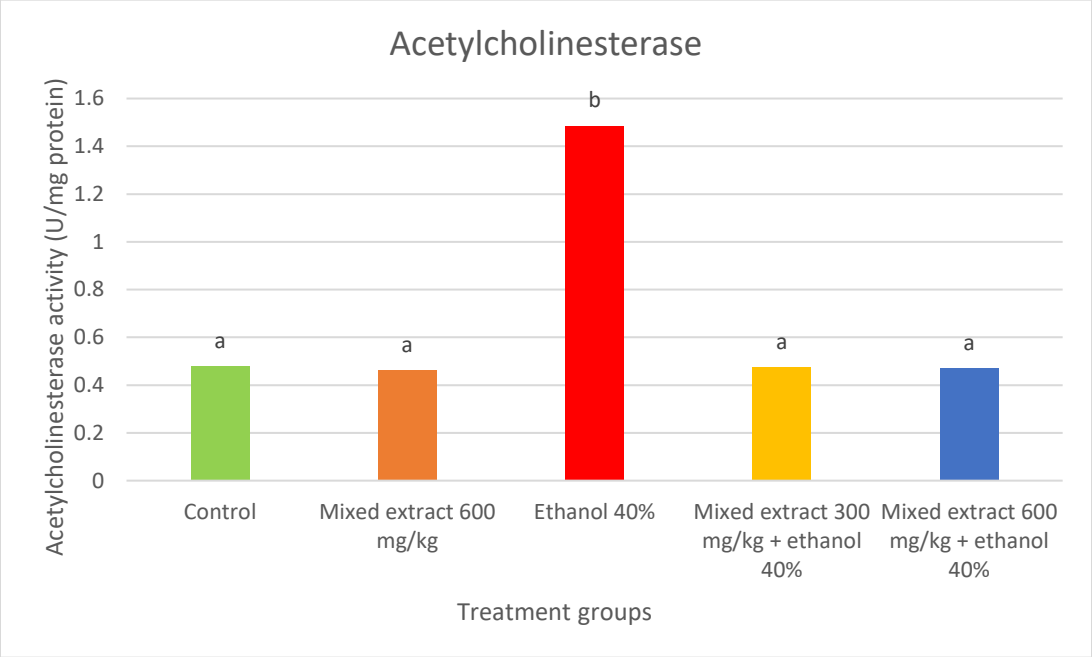
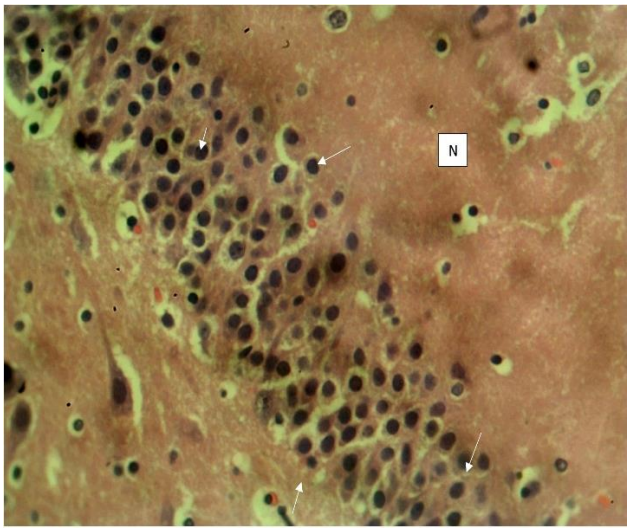


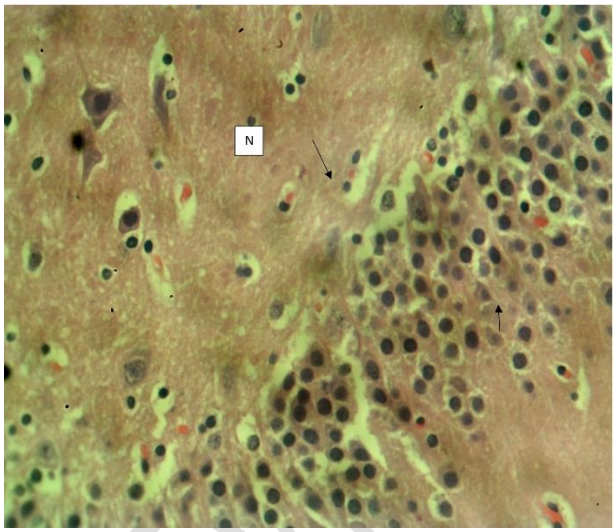
Figure 10: Effect of mixed ethanol extract of *Curcuma longa* rhizome and *Rosmarinus officinalis* leaf on brain homogenate Acetylcholinesterase activity in alcohol – induced brain damage in adult male Wistar rats. Values are presented as mean ± standard deviation (n = 5). Bars with different letter superscripts are significantly different ($p < 0.05$).

3.5 Effect of treatment with mixed ethanol extract of *Curcuma longa* and *Rosmarinus officinalis* on alcohol-Induced Histopathological Changes

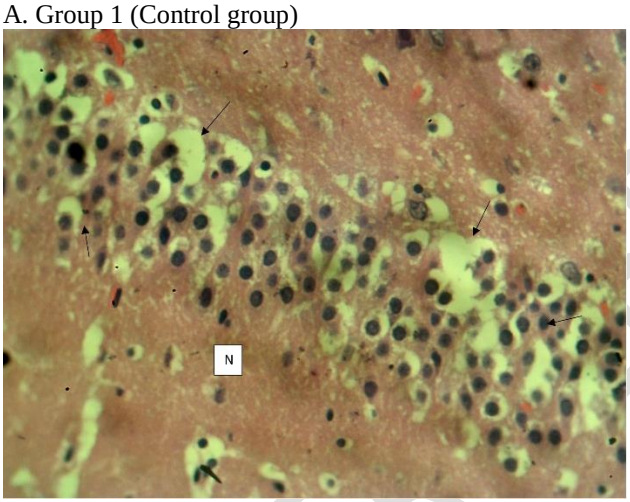
Figure 11 presents the photomicrographs of the brain sections stained with H & E. The control group showed normal architectures of neurocytes of hippocampus and cerebral cortex in the control group (Figure 11A). The alcohol group showed marked degeneration and necrosis of the pyramidal cells of the hippocampus (Figure 11C). The affected cells showed vacuolation of the cytoplasm with pyknosis of the nuclei (black arrow). The mixed extract group showed normal architectures of neurocytes of hippocampus and cerebral cortex in the brain of the rats. The mixed extract plus alcohol groups restored neuro damage characterized by mild degeneration and necrosis of the pyramidal cells of the hippocampus and normal encephalic histoarchitecture of cerebral cortex (Figure 11D and 11E).



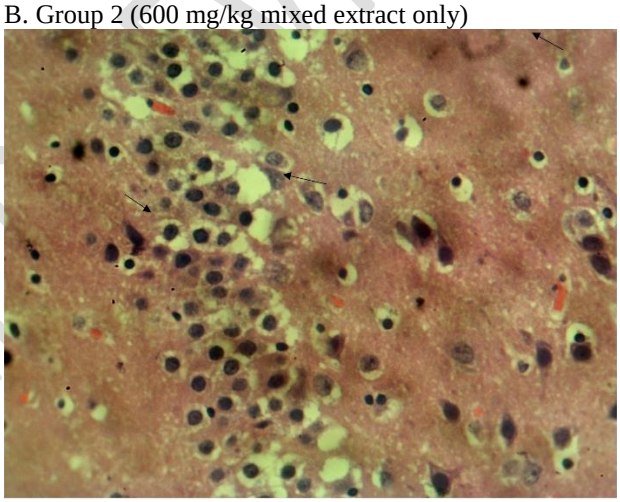
A. Group 1 (Control group)



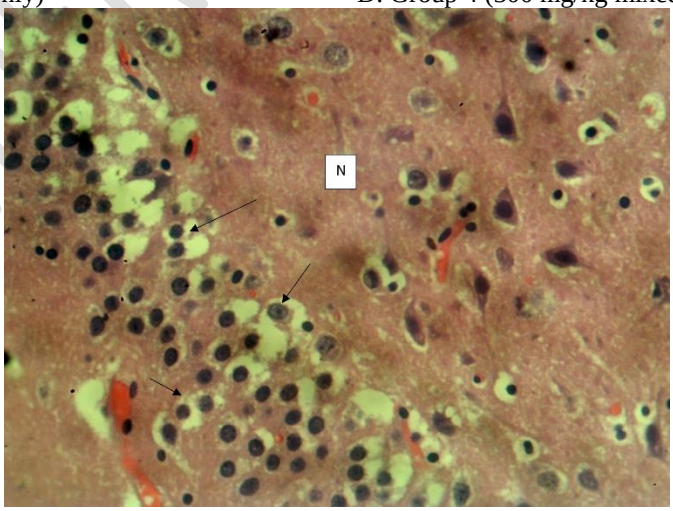
B. Group 2 (600 mg/kg mixed extract only)



C. Group 3 (40% ethanol only)



D. Group 4 (300 mg/kg mixed extract + ethanol)



E. Group 5 (600 mg/kg+ ethanol)

Figure 11. Photomicrograph of the Brain: (A) Group 1 (control): Normal histoarchitecture observed with intact pyramidal cells (black arrow), neuronal cell bodies (white arrow), and

neuropil (N). (B) Group 2 (600 mg/kg mixed extract): Normal histoarchitecture similar to control, with normal pyramidal cells and neuropil. (C) Group 3 (40% ethanol): Marked degeneration and necrosis of pyramidal cells in the hippocampus, with cytoplasmic vacuolation and pyknosis (black arrow); cerebral cortex remained normal. (D) Group 4 (300 mg/kg mixed extract + ethanol): Mild degeneration and necrosis of pyramidal cells, with cytoplasmic vacuolation and pyknosis; normal cerebral cortex. (E) Group 5 (600 mg/kg mixed extract + ethanol): Similar mild degeneration and necrosis as Group 4; normal cerebral cortex. **Magnification:** H&E ×400.

4.0 DISCUSSION

There is alarming increase of alcohol abuse in society today. As classified by the World Health Organization (WHO), alcohol use is the third-highest health risk factor in developed countries and the greatest risk factor in developing countries [4]. Overindulging in alcohol has been shown to have detrimental consequences on both short- and long-term health, including the possibility of developing dementia or Alzheimer's disease due to brain damage [5-9]. If no new treatment methods are created to slow down, stop, or prevent AD, the predicted total healthcare expenses for AD treatment in 2021 will rise to more than $1.1 trillion by 2050 due to an aging population [1].

Polyherbal compositions improve the therapeutic effect and lower the concentrations of individual herbs, hence lowering side effects [42]. This study is aimed at investigating the synergic effect of mixed ethanol extract of *C. longa* rhizome (rich in curcuminoids) and *R. officinalis* leaf (rich in Carnosic acid) some biochemical parameters in the brain of alcohol-treated male Wistar rats.

Acute toxicity studies of mixed extract of *C. longa* and *R. officinalis* showed an estimated LD_{50} greater than 5000mg/kg, which is an indication of an excellent safety profile and remote risk of acute intoxication.

Result obtained for oxidative stress markers showed that there was a significant decrease in the activities of SOD, CAT, GPx and GSH in group 3 rats treated with alcohol (ethanol) when compared to control group. The toxic effect of alcohol administration leads to a large population of unquenched free radicals leading to the state of oxidative stress. This is evidence in inhibition in the activities of antioxidants enzymes, SOD, CAT, GPx and GSH in the brain of rats. This result agrees with previous report of Owoade et al. [43] that acute and chronic alcohol consumption decreased antioxidants enzymes and GSH level in the brain Endogenous antioxidants such as SOD, GPx, and GSH are important for ROS elimination. SOD inactivates radical O_{2-} and forms H_2O_2. In the GPx reaction, GSH serves as an electron donor for H_2O_2 that subsequently removes ROS [18]. Reactive oxygen species induce oxidative stress in cells and potentially damage the neurons. Prolonged oxidative stress in the brain causes neuronal dysfunction and cell death, which could lead to neurodegeneration [7]. The brain is one of the most metabolically active organs and thus, it is profoundly susceptible to oxidative stress, owing to its high demand for oxygen consumption and low levels of endogenous antioxidants to eliminate free radicals [18]. However, co- administration of the combined extract with graded

dose of 40% alcohol, significantly increased the antioxidants levels in a dose dependent manner. This result agrees with previous report that Rosemary and turmeric are excellent antioxidant [28].

A large amount of polyunsaturated fatty acids found in all the biological membranes is susceptible to peroxidative attacks by oxidants resulting in lipid peroxidation. Lipid peroxidation is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. it is used as a marker of oxidant-induced tissue injury. Administration of alcohol for 21 days significantly increased the level of MDA in group 3 rats when compared with control group, indicating Oxidative stress. The brain is abundant with phospholipids that contain a high amount of polyunsaturated fatty acids (PUFA) and therefore, is susceptible to lipid peroxidation [18]. Malondialdehyde is one of the final products of lipid peroxidation. This finding agrees with the works of Omolola et al. [44] and Joshi et al. [45] who reported that ethanol metabolism, which increases aldehydic load through increased acetaldehyde accumulation, causes oxidative stress and lipid peroxidation not only in the liver but also in the brain. However, administration of the combined extract, reduced the MDA significantly. LPS-induced oxidative stress promotes ROS production by activating nicotinamide adenine dinucleotide phosphate (NADH) oxidase (NOX). turmeric has been reported to be a potent inhibitor of NOX [46,47], thereby preventing oxidative stress as evidence in our results.

The inflammation cascade has a vital role in the pathogenesis of neurodegenerative diseases, including Alzheimer's disease [48]. IL-1β and TNF-α are important proinflammatory cytokines that that contribute to immune dysfunction and mediate inflammation of the tissues and organ injury. From figure 1 and 2, the result shows that intoxication of rats with alcohol markedly elevated TNF-α and IL-1β in the brain tissues, suggesting that alcohol preferentially affects macrophage functions and stimulates the development of brain injury. This result agrees with the reports of Garcia-Suastegui et al. [16] and Tiwari and Chopra [49] that besides lipid peroxidation, alcohol-induced oxidative stress also causes protein and DNA damage, mitochondrial dysfunction, increased cytokine production and eventually neuronal cell death. However, co- administration with the mixed extract significantly decreased brain tissue level of IL-1β and TNF-α. In the presence of ROS, there is an increase production of these cytokines and in turn, signaling accentuates oxidative stress [50].

Repeated daily administration of 15 mg/kg of alcohol for 21 days resulted to marked degeneration and necrosis of the pyramidal cells of the hippocampus exhibiting vacuolation of the cytoplasm with pyknotic nuclei when compared to control group. These results point to the pyramidal cell body as primary targets of alcohol neurotoxicity. The neuron damage score revealed in the histopathological examination is in accordance with the biochemical changes earlier observed in alcohol treated group. Our results are compatible with the previous studies reporting that neurodegeneration due to ethanol exposure can occur through apoptotic or necrotic mechanisms [51]. This finding agrees with the report of Halder et al. [52] that in Alzheimer's disease, the function of the hippocampus is destroyed as the neurons become degenerated. Hamid et al. [15] in their study have also demonstrated that although alcohol is metabolized mainly in the liver, it also causes toxic effects to the brain**.** However, the neuron protective effect

of mixed extract on alcohol-induced oxidative damage was further confirmed by the histological findings. Repeated cotreatments with 300 mg/kg or 600 mg/kg for 21 days significantly improved the alcohol-induced histopathological alterations with mild necrosis of the pyramidal cells observed in the hippocampus. This result has demonstrated that *C. longa* and *R. officinalis* work synergically to reduce the alcohol-induced histopathological alterations in the brain.

The hippocampus is the center of all the memory and cognitive functions of the brain [52]. This mechanism involves the interaction between various neurotransmitters in the brain [53]. Acetylcholine (ACh) is the most important neurotransmitter involved in memory and cognitive functions apart from several neurodegenerative pathogenicity [54]. Acetylcholinesterase modulates proper levels of acetylcholine by breaking acetylcholine into choline (the building block of acetylcholine). Estimation of this enzyme's activity is an important parameter to assess the central cholinergic function. Repeated daily administration of 15 mg/kg of alcohol for 21 days significantly increased the activity of acetylcholinesterase. Similar findings were declared in a previous study by Choi et al. [55] which revealed that excessive alcohol consumption increased acetylcholinesterase activity in the brain. Studies have shown that the excessive activity of acetylcholinesterase results in the deficiency of acetylcholine, leading to memory impairment [56,57]. Co-administration with the mixed extract inhibited the over expression of acetylcholinesterase induced by chronic treatment with alcohol. Ozarowsk et al. [58] reported that the memory enhancing effect of rosemary extract (200 mg/kg, p.o.) in the scopolamine-induced dementia model of AD has also been shown to be linked with direct effect on acetylcholine esterase activity. This result agrees with the works of Tiwari and Chopra [49] who have demonstrated effectiveness of *curcumin* in preventing cognitive deficits associated with chronic alcohol consumption. Banerjee et al. [23] also reported that *Bacopa monnieri* anti-acetylcholine esterase activity may be responsible for its positive effect on cognition. Additionally, numerous studies have linked cholinergic enzymes to the development of amyloid plaques [59]. As a result, inhibiting acetylcholine esterase causes an increase in acetylcholine in the brain, which reduces plaque formation [60]. These results are in accordance with Levine et al. [36] and Mohamed et al. [37] who reported that, combination of *R. officinalis* and *C. longa* is more effective than *C. longa* extract alone in improving the outcome due to a greater increase in intracellular curcumin accumulation when the combination treatment was used.

5.0 CONCLUSION

This result suggests that treatment with mixed extract of *C. longa* rhizome and *R. officinalis* leaf inhibited oxidative stress, inflammation, histological alteration and increased acetylcholinesterase activity in the rat brain which could be associated with the plethora of phytoconstituents present there-in. The co-administration of both *C. longa* rhizome and *R. officinalis* leaf together enhanced their neuroprotective activity indicating a synergistic activity between both natural products Therefore, mixed extract of *C. longa* rhizome and *R. officinalis* leaf may be considered as a potential therapeutic agent against alcohol-induced oxidative damage in brain tissues.

Consent

It is not applicable.

Ethical approval

Animal Ethic committee approval has been collected and preserved by the author(s).

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