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Research Article

Toxicity Effects of *Ganoderma lucidum* ethanol extract on Cognition and Anxiogenic-like behavior is dose-dependent in Swiss mice

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Abstract

Ganoderma lucidum as one of the folk medicinal mushrooms has been used for the prevention and treatment of diverse disorders including neurasthenia, dizziness, anorexia, hypertension, deficiency fatigue, and arthritis, but its CNS-related safety is scanty and undefined. This research was designed to evaluate the toxicity effects of *Ganoderma lucidum* ethanol extract (EEGL) on cognitive and anxiogenic-like behaviours in Swiss mice. Forty Swiss mice (n = 5/group) of both sexes were treated orally with distilled water (10 mL/kg; vehicle), and graded doses of EEGL (100, 200, and 400 mg/kg, respectively) for 30 days, during which neurobehavioral data from Morris water maze, light/dark box, and open field test were obtained. Thereafter, neurochemical and histological assessments were evaluated. The EEGL-treated groups showed a significant reduction in spatial cognitive performance in males without remarkable difference in females except at high dose (400 mg/kg). A high dose of EEGL induced anxiety-like behaviours by significantly increasing time spent in the dark compartment and decreasing the number of transitions in the light/dark box, as well as increasing time spent in the outer zone of the open field apparatus. Furthermore, EEGL at higher doses (≥ 200 mg/kg) disrupted neurochemicals significantly by decreasing glutathione, superoxide dismutase and catalase activities while remarkably increasing malondialdehyde, nitric oxide, acetylcholinesterase, tumor necrosis factor-alpha, and interleukin-6 levels. Consequently, exposure to a high dose of EEGL resulted in mild hippocampal alterations in males, while the prefrontal cortex appeared normal. Taken together, EEGL shows potential neurotoxic effects at doses greater than 100 mg/kg for 30 days, while lesser doses might be beneficial.

Key Words: *Ganoderma lucidum*, Cognition, Anxiogenic, Neurotoxic, Cholinergic, Oxidative stress

INTRODUCTION

Mental disorders such as cognitive impairment, depression, and anxiety manifestations are the most common health challenges ravaging the worldwide population, especially the middle-aged and the aged, affecting their cognitive ability, regulation of their emotions, or behaviors (Mirza *et al*., 2017; Kulak-Bejda *et al*., 2021). According to the Institute of Health Metrics and Evaluation (IHME), 1 in every 8 people, or 970 million people around the world constituting about 10.7% of the world population live with a mental disorder (IHME, 2022). This will likely increase mainly because of demographic changes coupled with preventable physical conditions such as lack of mental health awareness, low effective treatment coverage, as well as the stigma and discrimination experienced (WHO, 2019). From research findings, it is becoming clear that many of these mental conditions are caused by a combination of psychological, environmental, and biological factors. However, no exact cause of most of the mental disorders is known (Fakhoury, 2015; WHO, 2019).

Learning and memory unarguably are fundamentals behind understanding the nitty-gritty of cognitive processes and functions, which, to a larger extent, are tools that provide useful insight into identifying neurobiological components and neuropsychological processes in the memory domain (Brem *et al*., 2013; Brown *et al*., 2020). These mental processes serve a critical function in allowing organisms to alter their behavioral performance when exposed to changing and/or challenging environmental conditions (Kredlow *et al*., 2018; Savarimuthu and Ponniah, 2023). Anxiety symptoms characterized by a perceived unavoidable or uncontrollable feeling of fear and anxiety likely result to physical and cognitive symptoms ranging from restlessness, irritability,

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chest pain, easy fatigue, increased heart rate, and abdominal pain (DeMartin *et al*., 2019). Varieties of other symptoms on individualization are also common and capable of affecting 30% of adults at some point in their lives (Alfaro *et al*., 2022). Anxiety-related behaviors and other mental sicknesses such as cognitive deficits have been reported to be triggered by neuroinflammatory response (which may be mediated by the production of secondary messengers, reactive oxygen species (ROS), cytokines, and chemokines) and free radical toxicity in the brain (Bauer *et al*., 2014). These may greatly affect the normal physiological, physical, and mental coordination and functioning of the central nervous system (CNS) (Xu *et al*., 2020; Guignet *et al*., 2020).

Mushrooms, owing to their diverse health benefits and reported bioactive components and pharmacological properties, are important bioresources that are widely spread in nature and consumed by humans (Odeyemi *et al*., 2014). *Ganoderma lucidum* (GL), a common medicinal mushroom, is used in traditional folklore in most parts of Asia but is also found in tropical regions of Europe, America, and West Africa (Siwulski *et al*., 2015). It is acclaimed to be effectively utilized in folkloric medicine to promote longevity, improve health, and enhance vitality miraculously (Tello *et al*., 2013). This is achieved via managing various ailments ranging from diabetes, anorexia, hepatitis, hypertension, deficiency fatigue, arthritis, and neoplasia, as well as aiding easy child delivery (Tello *et al*., 2013; Wang *et al*., 2014; Okigbo and Obanubi, 2020). The pharmacological activities of GL, such as antioxidant, anti-inflammatory, anti-depressive-like, anticancer, immunomodulatory, hypolipidaemic, and cytotoxicity, have been reported in literature. These activities might be as a result of the interplay of its major bioactive components of polysaccharides, triterpenoids, and lectins (Choi *et al*., 2014; You *et al*., 2017; Liang *et al*., 2019; Zeng *et al*., 2019). Interestingly, analytical HPLC-DAD fingerprinting techniques has revealed the presence of 16 compounds in GL; 13 lanostane steroids, adenosine, uracil, and gentisic acid (Ha *et al*., 2015).

Recently, *Ganoderma lucidum* has demonstrated usefulness to improve CNS-related illnesses (Nascimento *et al*., 2020; Mi *et al*., 2022; Ji *et al*., 2022; Ezurike *et al*., 2023). However, these reports have not been largely established on its continuous use at high doses of the mushroom extract, which may likely present symptoms of delayed neurotoxic debilitations or not. It is interesting to note that this mushroom is increasingly being consumed both in developed and developing countries, due to its nutritional, medicinal, therapeutic benefits, and the use of the mushroom as a health promoter in traditional medicine (Pinya *et al*., 2019). In addition to its reported pharmacological properties, it is of paramount importance to provide more useful information on its efficacy, safety, potency, and CNS effects via cognitive and non-cognitive processes/symptoms, so as to validate the continuous use of GL both in short and long-term administration. Therefore, the present study was designed to evaluate the toxicity effects of *Ganoderma lucidum* ethanol extract (EEGL) on cognitive and anxiety-related behaviors in male and female Swiss mice.

MATERIALS AND METHODS

Animals: Male and female Swiss mice with an average weight of 20 ± 2 g were procured from the breeding unit of the Central Animal House, University of Ibadan, Ibadan, and used for the study. Standard polystyrene cages were used to accommodate the animals while standard environmental conditions were maintained with a photoperiodic cycle of 12-hour light/dark and a temperature of 25 ± 1° C. They were allowed to acclimatize to the laboratory conditions for 7 days while being fed with standard pelletized mice feed (ACE® Capsfeed Limited, Olaogun, Nigeria) and allowed unrestricted access to clean water *ad libitum*. All the experimental protocols in the study were executed in line with the National Research Council's update of the NIH Guide for the Care and Use of Laboratory Animals (NIH, 2011), while ethical approval was gotten from the University of Ibadan Animal Care and Use Research Ethics Committee (U1-ACUREC), Ibadan, Nigeria, with the reference number: UI-ACUREC/080-0821/16.

Mushroom material collection, identification, and extraction: The fruiting body of the mushroom sample collected from the environs of the University of Ibadan that had already been identified and authenticated via molecular biology techniques (Ascension number –KF998092.1) was used for the study (Abiodun *et al*., 2022; Ezurike *et al*., 2023). Seven hundred grams (700 g) of the freeze-dried mushroom materials were coarsely pulverized using an electric blender and extracted with 2.5 L of 70% (v/v) ethanol by maceration at room temperature for 72 hours with intermittent shaking. Thereafter, the filtrate was collected via absorbent cotton wool and Whatman Grade 1 filter paper, concentrated, and evaporated to dryness with a rotary evaporator at 40° C and low pressure to obtain the yield (30 g) of the crude ethanol extract of *Ganoderma lucidum*. The dried ethanol extract was then stored at 4° C until it was needed for the experiment.

Experimental Design: After 7 days of acclimatization to standard laboratory conditions, 40 Swiss mice were assigned randomly into four groups (A-D, n = 10 (5 females and 5 males)/group) and accommodated in different polystyrene cages. Following the experimental protocols described in OECD Guidelines 407 (OECD 2008) with slight modification, animals in group A were given distilled water (10 mL/kg) as vehicle, while group B, C, and D received orally administered doses of EEGL (100, 200, or 400 mg/kg; respectively) every day for 30 days. These administered dose selections were based on a recent study (Ezurike *et al*., 2023) as well as the median lethal dose (LD_{50}) results (≥ 3200 mg/kg) obtained from previous studies (Kim *et al*., 1986; Shamaki *et al*., 2017; Ezurike *et al*., 2023) which considered EEGL to be relatively safe on a single administration of a high dose. On the last week of treatment (day 23 - day 29), between 8.30 am and 12.00 pm in a very quiet room, animals were exposed to batteries of neurobehavioral evaluations and functions in Morris water maze (MWM), light and dark box (LDB), and open field test (OFT) one hour after oral administration of EEGL. Behavioral performances were captured and recorded by a video camera mounted directly above the suited apparatus' as relevant parameters were tracked and analyzed using the ANY-maze (version 7.0) video tracking system software manufactured by Stoelting Co. (WoodDale, IL, USA) (Omeiza *et al*., 2021). On day 30, animals were fasted for 3-4 hours prior to euthanization under ketamine/diazepam (50/2.5 mg/kg) anaesthesia. The whole brain tissues were rapidly excised from the animals, weighed and placed on ice, after which they were processed for neurochemical, and histological preparations.

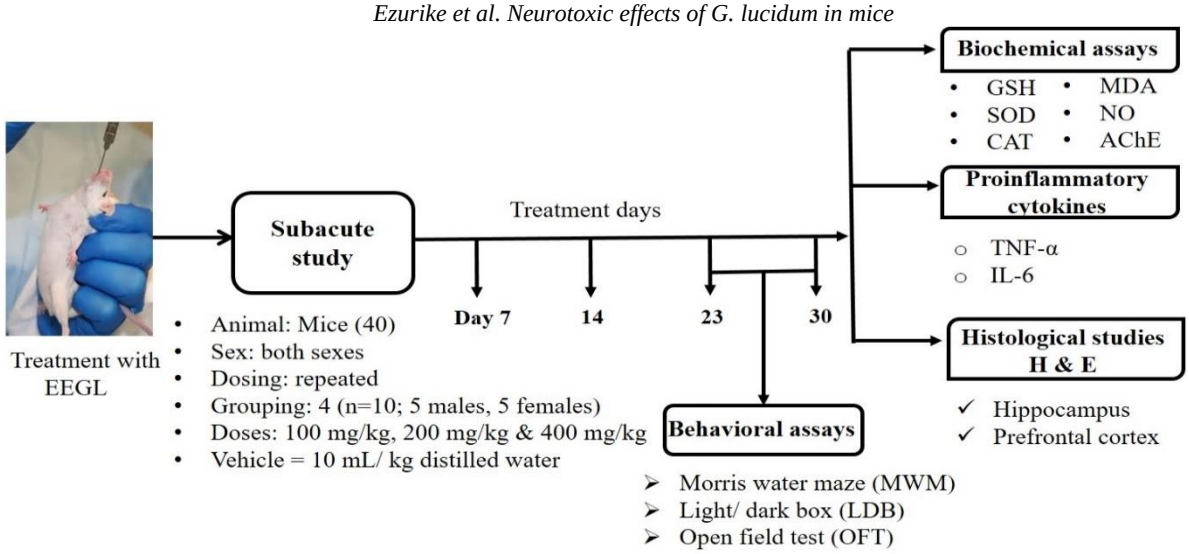

Figure 1: Design schedule of the experimental procedure/timeline

Neurobehavioral evaluations

Morris water navigation task (MWM) – Test for cognitive behavior (learning and memory): The Morris water maze assesses spatial and non-spatial learning ability and reference memory in experimental rodents (Nunez, 2008). In this study, MWM was used to evaluate the cognitive ability of the Swiss mice. The water maze apparatus was made up of a circular pool of diameter 800 mm and height 370 mm, filled with opaque water (coloured with evaporated non-fat milk) about 250 mm deep at a controlled temperature of the pool kept at 25 ± 1° C. The pool was then divided into four zones namely; east, west, north, and south zones with a round escape platform of diameter 100 mm, submersed 10 mm below the opaque water surface, and conceptually placed at the center of the southeast zone. Prior to the spatial acquisition (training) trials, Swiss mice were allowed to freely explore the water maze pool habitually for 2 minutes without the hidden platform to acclimatize. At the commencement of the acquisition trials, each mouse was gently placed in the northwest zone facing the round edge of the pool to navigate, search and locate the hidden platform that served as the means of escape for the mouse within 60 seconds, the maximum time allotted for the search. Swiss mice that were able to locate the hidden platform within 60 seconds were allowed to remain on the platform to further explore the maze pool for another 15 seconds, after which they were towel-dried and returned back into their cages. Animals that were unable to locate the escape platform were gently guided to the platform. Escape latency, which is the time taken to locate the hidden platform diagonally opposite the platform zone, was used as a measure of spatial learning ability. The spatial acquisition trials were performed twice per day with a 30-minute inter-trial interval for four consecutive training days (Salomon *et al*., 2011; Omeiza *et al*., 2021). Animals were allowed to rest on the following day. Twenty-four hours after the rest day, three probe trials were performed with the same inter-trial interval of 30 minutes. On the first probe trial, Swiss mice were allowed to swim freely for 60 seconds so as to explore the platform zone habitually without the escape platform. Variable parameters such as the time spent in the platform zone (annulus time) and average proximity to the platform zone border were all recorded to measure retention, learning, and memory. In the last two trials performed, the hidden platform was replaced to its original training position in the maze pool. This was used to evaluate the cognitive performance by scoring each trial by the performance of individual animals on the maze in each group and across using the scale: 6 = direct; 5 = corrected; 4 = focused; 3 = accidental circling; 3 = chaining; 3 = circling; 2 = random; 1 = thigmotaxis; 1 = passivity; as reported in previous studies (Illouz *et al*., 2016; Higaki *et al*., 2018).

Light and Dark Box (LDB) – Test for anxiety-like behavior: The light and dark box is a commonly used rodent test for anxiety-like behavior, that is based on an avoidance conflict (anxiety responses) between the desire to explore novel areas and a repugnance to open spaces that are brightly lit (Miller, 2011). On day 23, Swiss mice were exposed to the LDB to explore in order to assess anxiogenic-like behavior. The LDB apparatus comprised a rectangular-shaped wooden box (600 mm x 450 mm x 400 mm) with an open doorway (70 mm x 70 mm) on the wall partition connecting the two equal compartments (dark and light). The dark compartment was painted black and had a black wooden covering over it, while the light compartment was painted white with no covering on it. At the start of the paradigm, individual mouse was placed gently at the center of the light compartment facing the wall of the compartment, opposite the doorway and allowed to explore the compartments for 5 minutes. After each test, the box was thoroughly cleaned with 60% ethanol and towel-dried before placing the next animal in it. This was recorded by a video camera (webcam) mounted overhead the apparatus. Parameters such as time spent in the dark/light compartments and number of transitions were analyzed using the ANY-maze video tracking software.

Open Field (OFT) – Test for anxiety-related behavior: On day 24, Swiss mice were subjected to the open-field test paradigm to further assess anxiety-like and exploratory activities (Gould *et al*., 2009). The open-field apparatus consisted of a wooden white box arena (400 mm x 400 mm x 350 mm) partitioned into 16 squares of equal area (100 mm x 100 mm). After 30 minutes of acclimatization in the arena, each of the experimental animals was then placed at the center of the open field box and allowed to explore the field freely for 5 minutes while being recorded by an overhead video camera (webcam). At the end of each test, the apparatus was thoroughly cleaned with 60% ethanol and allowed to dry. The

time spent by experimental mice in the centre zone (4 squares at the center) and corner zone (12 squares close to the wall) of the open field arena were captured and analyzed automatically using the ANY-maze TM (Stoelting, USA) video tracking software.

Brain tissue collection and preparation for neurochemical and histopathological evaluations: On day 30 after overnight postprandial exposure, Swiss mice in the respective groups were sacrificed under ketamine/diazepam (50/2.5 mg/kg) anesthesia and transcardiac perfusion with normal physiological saline and phosphate-buffered formalin. After that, the whole brain was rapidly excised from the animals, weighed, and processed for neurochemical and histological preparations respectively. For neurochemical preparations, the brain tissues placed on ice were homogenized in chilled 0.1 M phosphate buffer, pH 7.4 (1:10 w/v). The brain homogenates were thereafter centrifuged at 10,000 rpm for 10 min at 4° C, and the resulting supernatants were immediately aspirated and separated into various portions in fresh chilled plain tubes and stored at -80°C for spectrophotometric and ELISA neurochemical assays. The brain tissues for histopathological analysis were then fixed in 10% phosphate-buffered formalin for 72 hours and thereafter processed and stained with hematoxylin and eosin (H and E).

Neurochemical evaluations: The reduced glutathione (GSH) level in the brain was assessed using the method reported by Anderson (1985), while a marker of the level of lipid peroxidation, malondialdehyde (MDA), was measured according to the method described by Varshney and Kale (1990). The levels of superoxide dismutase (SOD) and catalase activities in the mice brain were evaluated using the method previously described by Misra and Fridovich, (1972) and Beer and Sizer, (1952) respectively. The brain nitrite level, a biomarker of nitrergic transmission and one of the two primary stable and non-volatile breakdown products of nitric oxide (NO) was evaluated using Griess reagent according to a previously described method (Green *et al*., 2004). The level of proinflammatory cytokines; tumor necrosis factor alpha (TNF-α) and Interleukin (IL)-6 in the brain supernatant were evaluated and quantified following the instructions of the manufacturer strictly, using the enzyme-linked immunosorbent assay kits (ELISA MAXTM Deluxe) from Biolegend (San Diego, USA) with Cat. No: 430904 and Cat. No: 431315 respectively. In addition, a marker of cholinergic transmission, acetylcholinesterase (AChE) activity, was estimated in the mouse brain using a modified method of Ellman *et al*., (1961).

Hematoxylin and eosin (H & E) staining and examination: The brains harvested from the animals for histological studies in each group were fixed in 10% phosphate-buffered formalin. The fixed tissues were dehydrated in ethanol and clarified in xylene. Thereafter, the fixed brain tissues were then processed, and embedded in paraffin while the transverse sections (5 µm thick) of the hippocampus (CA1, CA3) and prefrontal cortex of the brain tissue were obtained via microtomy and processed by routine method (hematoxylin and eosin staining) as described by Romanucci *et al.,* (2018). Photomicrographs (x 400) showing viable neuronal cells were taken using a suitable digital camera and Stream Basic imaging software attached to a binocular microscope (Olympus CH, Japan).

Statistical analyses: Data were analyzed using GraphPad Prism® 8.04 (GraphPad Software Incorporated, San Diego, CA). All values were presented as mean ± SEM. Comparisons among the groups were done by One-way analysis of variance (ANOVA), while that between the treatment group and day was performed using repeated measures of two-way ANOVA followed by post-hoc test (Dunnett's) for multiple comparisons, respectively. The level of significance was fixed at $p < 0.05$ for all statistical tests.

RESULTS

Effects of EEGL on cognitive behavior (learning and memory) in Swiss mice: During the training days in the Morris water navigation task as represented in Figure 2A, the escape latency (the time taken to search and locate the hidden platform) decreased substantially over the first three days of acquisition (24th, 25th and 26th day of treatment) and stabilized slightly at the fourth day of acquisition across all the groups of either sex, which suggested that all the Swiss mice had considerably improved in learning to locate the hidden platform. Repeated measures using two-way ANOVA (group vs day) showed that each of the treatment groups of either sex had a significant reducing effect on the escape latency. For either sex (male and female), the group ($F(3, 56) = 9.707$, $p < 0.0001$; $F(3, 56) = 25.40$, $p < 0.0001$) and the day ($F(3, 56) = 97.07$, $p < 0.0001$ and $F(3, 56) = 39.68$, $p < 0.0001$) effects on escape latency were both significant but the interaction between the group and day effects were considered insignificant ($F(9, 56) = 1.610$, $p = 0.1347$; $F(9, 56) = 1.363$, $p = 0.269$) when compared to their control groups. In addition, administration of graded (100, 200, and 400 mg/kg) doses of EEGL significantly decreased the escape latency during the course of acquisition in both the male (70.4%, 67.8%, and 56.3%) and female (74.8%, 54.19%, and 45.7%) experimental groups when compared with the control untreated group (77.6% and 79.2%). During the probe trial on the 29th day of administration, retention memory was assessed in all groups without the hidden platform in the pool. The probe trial 1 (probe I) revealed that the time spent in the platform zone decreased significantly in a dose-dependent manner in the experimental groups of both males ($F(3, 9) = 61.70$, $p = 0.0001$) and females ($F(3, 10) = 17.11$, $p = 0.0003$) when compared with their control groups (Figure 2B). Administration of EEGL in experimental groups of either sex (male and female) significantly increased the average proximity to the platform zone ($F(3, 12) = 6.276$, $p = 0.0083$; $F(3, 12) = 6.030$, $p = 0.0096$) in a dose-dependent manner when compared to the respective control groups (Figure 2C). The second (probe II) and the third probe trial (probe III) as depicted in Figure 2E were used to evaluate the cognitive score. Administration of EEGL (200 and 400 mg/kg) significantly reduced the cognitive score in male Swiss mice ($F(3, 13) = 13.76$, $p = 0.0003$). However, the reduction in cognitive score was not significant in females ($F(3, 15) = 2.747$, $p = 0.0795$) that received the various treatments except at 400 mg/kg (Figure 2D).

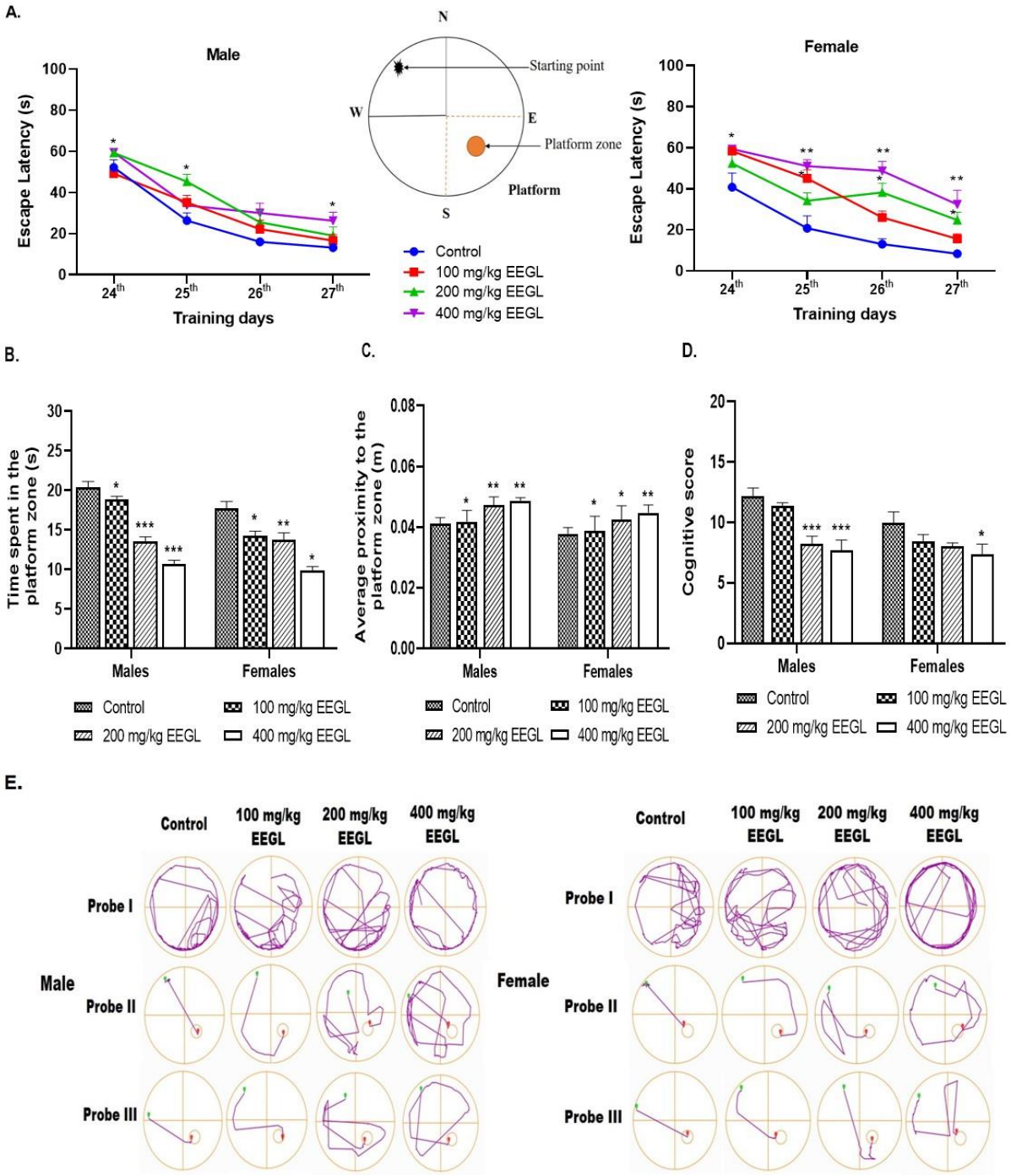

Figure 2: Illustrative results for the MWM showing the effects of EEGL on neurocognitive performance (Learning and memory) in male and female Swiss mice. A. Acquisition trial (training days 1-4) showing escape latencies at learning. B. Time spent in the platform zone. C. Average proximity to the platform zone. D. Cognitive score. E. Track maps depicting the swimming trajectories of Swiss mice during the probe trials (1, II & III). The value of each of the bars are expressed as mean ± SEM (Dunnett's Post hoc test for multiple comparisons after the analysis of variance), n = 5/group. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$ (significant difference) versus the control; MWM = Morris water maze; EEGL = Ethanol extract of *Ganoderma lucidum*; SEM = Standard error of mean; Control = distilled water; the red and green mark on the track maps (probe II & III) indicates the start and end points respectively in the MWM.

Effects of EEGL on anxiety-like behaviors in Swiss mice:
Open Field Test: The time spent in the corner zone of the open field (a measure of thigmotaxis vis-à-vis anxiety-like behavior) increased significantly in EEGL-treated (≥ 200 mg/kg) male and female Swiss mice (F (3, 15) = 10.31, p = 0.0006; F (3, 15) = 8.523, p = 0.0015) when compared to the control groups. Likewise, the time spent in the center zone (F (3, 15) = 10.23, p = 0.0006; F (3, 15) = 8.654, p = 0.0014) in comparison with their control groups (Figure 3A).

Light and Dark Box: Treatment with EEGL (200 and 400 mg/kg) showed anxiety-like symptoms in male Swiss mice as there was a significant reduction in time spent in the light compartment (F (3, 13) = 7.547, p = 0.0036) while the time spent in the dark compartment of the Light and dark Box was increased significantly. On the other hand, female Swiss mice experienced the same but the difference when compared with the control was not statistically significant (F (3, 15) = 3.012, p = 0.0631) except at 400 mg/kg dose (Figure 3B). The number of transitions which is an exploratory behavior was also affected as there was a significant reduction in the number

of transitions count in the EEGL treatment groups (200 and 400 mg/kg) of both males and females (F (3, 13) = 13.52, p = 0.0003; F (3, 15) = 15.36, p = 0.0001) when compared with the control untreated group as shown in Figure 3C.

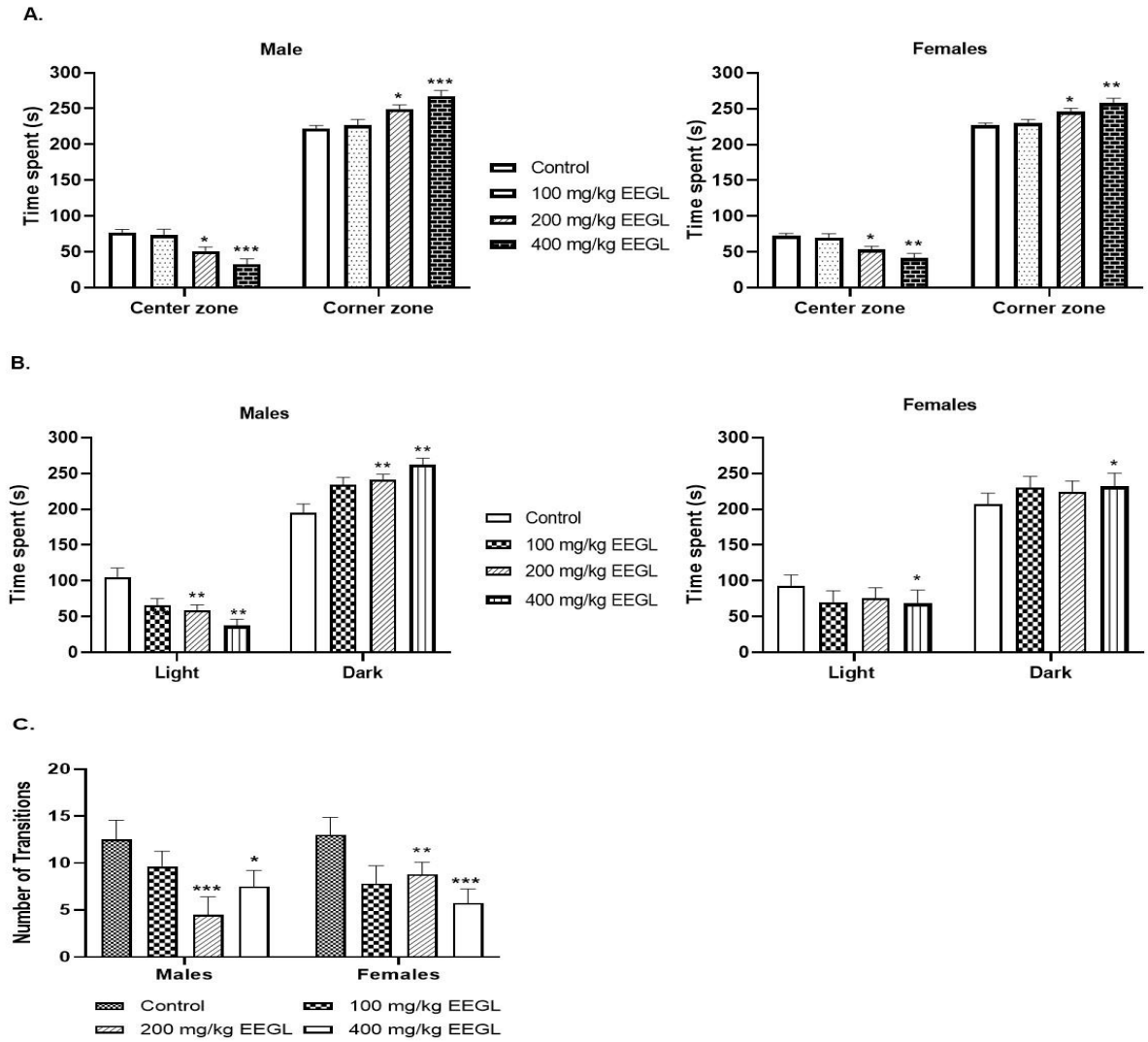

Figure 3: EEGL increases anxiogenic-related behavior in OFT and LDB paradigms. A. Time spent in the center/corner zones. B. Time spent in light/dark compartments. C. Number of transitions in the LDB. The value of each of the bars are expressed as mean ± SEM (Dunnett's Post hoc test for multiple comparisons after One-way ANOVA), n = 5/group. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$ (significant difference) versus the control; OFT = Open field test; LDB = Light and dark box; EEGL = Ethanol extract of *Ganoderma lucidum*; SEM = Standard error of mean; ANOVA = Analysis of variance; Control = distilled water

Neurochemical assessment of the effects of EEGL in Swiss mice: Nitro-oxidative stress status (MDA, SOD**, CAT, GSH, and** NO), proinflammatory cytokines (TNF-α and IL-6) and AChE activity were assessed to establish the effects of repeated administration of EEGL on neurochemical alterations in Swiss mice (Figure 4). Reduced Glutathione (GSH) activity level, an important antioxidant in the brain was significantly reduced in experimental groups that received EEGL (400 mg/kg) in either sex (male and female) (F (3, 6) = 19.22, p = 0.0018; F (3, 6) = 3.645, p = 0.0433) when compared with the control untreated group (Figure 4A). Malondialdehyde (MDA) level, one of the end products of lipid peroxidation in the brain increased significantly in experimental groups that received higher doses of EEGL (200 and 400 mg/kg) in either sex (male and female) (F (3, 6) = 8.543, p = 0.0138; F (3, 6) = 5.920, p = 0.0317) when compared with the control group (Figure 4B).

Superoxide dismutase (SOD) and Catalase (CAT) activities decreased significantly in either sex ((F (3, 6) = 7.430, p = 0.0191; F (3, 6) = 5.082, p = 0.0437) and (F (3, 6) = 6.907, p = 0.0169; F (3, 6) = 7.363, p = 0.0195) respectively that received EEGL (200 and 400 mg/kg) when compared with their respective control groups (Figure 4C & Figure 4D). Nitric oxide level was significantly elevated in male Swiss mice (F (3, 6) = 19.77, p = 0.0016) that received a high dose (400 mg/kg) of EEGL. However, the slight increase recorded in females was not significant (F (3, 6) = 1.214, p = 0.3825) at various dose levels of EEGL when compared with the control group (Figure 4E).

In addition, repeated administration of EEGL to experimental groups resulted in a dose-dependent increase in acetylcholinesterase activity significantly in both males and females (F (3, 6) = 45.28, p = 0.0078; F (3, 6) = 7.963, p = 0.0163) when compared with the control groups (Figure 4F).

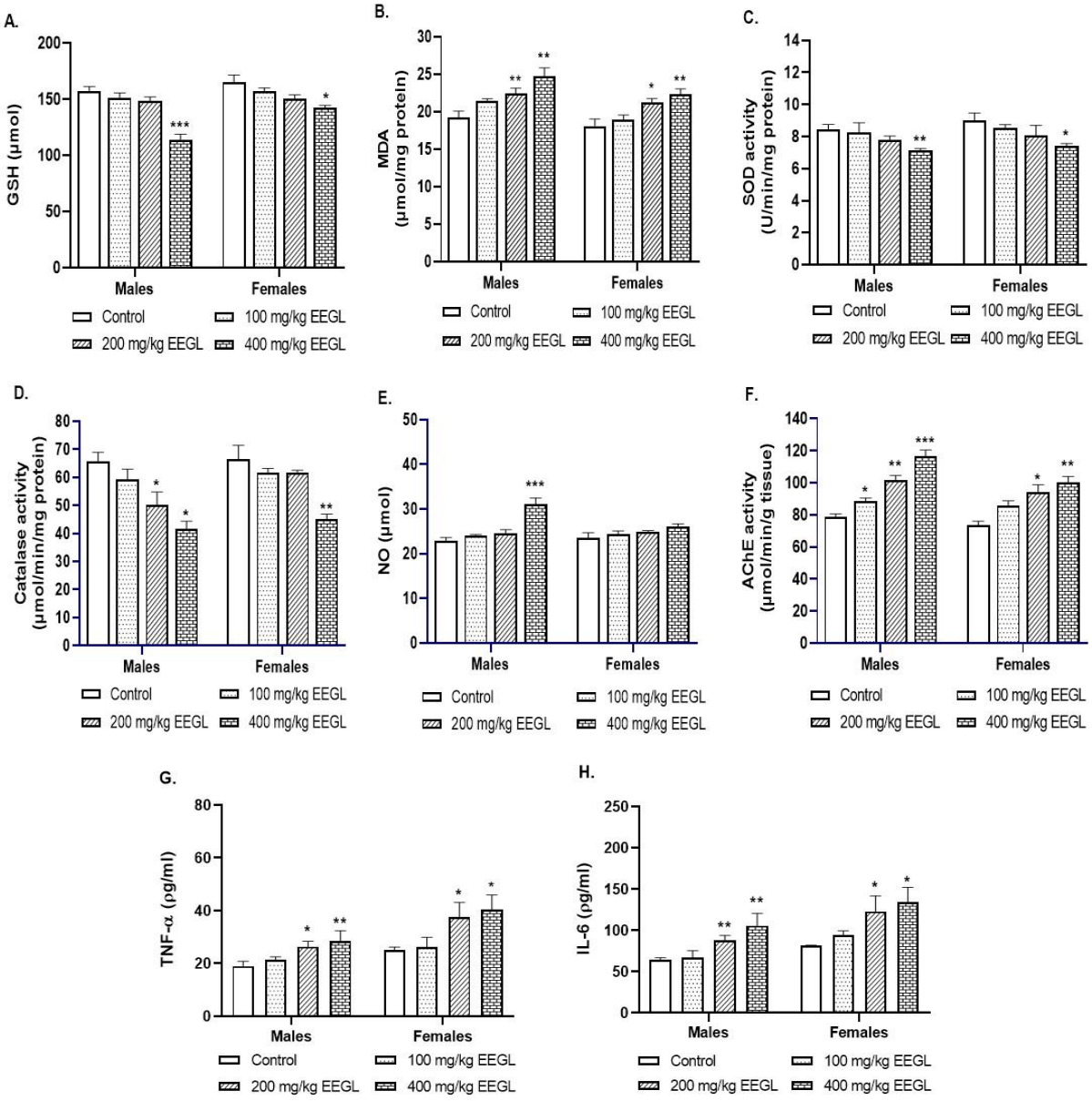

Figure 4: Representative effects of EEGL on neurochemical parameters in the brain of male and female Swiss mice. The following parameters were assessed: A. GSH. B. MDA. C. SOD. D. Catalase. E. NO. F. AChE. G. TNF-α. H. IL-6. The value of each of the bars are expressed as mean ± SEM (One-way ANOVA followed by Dunnett's Post hoc test), n = 3/group. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$ (significant difference) versus the control; GSH = Reduced glutathione; MDA = Malondialdehyde; SOD = Superoxide dismutase; NO = Nitric oxide; AChE = Acetylcholinesterase; TNF-α = Tumor necrosis factor-alpha; IL-6 = Interleukin-6; EEGL = Ethanol extract of *Ganoderma lucidum*; SEM = Standard error of mean; ANOVA = Analysis of variance; Control = distilled water

Furthermore, TNF-α levels significantly increased in male (F (3, 6) = 8.777, $p = 0.0130$) and female mice (F (3, 6) = 5.894, $p = 0.0320$) that received EEGL (200 and 400 mg/kg) in a dose-dependent manner when compared with their control groups (Figure 4G). Similarly, Interleukin (IL)-6 levels in mice brains were significantly elevated in males (F (3, 6) = 10.83, $p = 0.0078$) and females (F (3, 6) = 7.963, $p = 0.0163$) following repeated administration of EEGL (200 and 400 mg/kg) for 30 days when compared with their control groups (Figure 4H).

Effects of EEGL on histopathological morphology of the brain regions (Hippocampus, Prefrontal cortex)

Hippocampal region: The histopathology of the male hippocampal region exposed to graded doses of EEGL is presented in Figure 5 (a). The untreated group and group that received EEGL (100 and 200 mg/kg) showed normal structural organization of the hippocampal area as the hippocampus appeared normal and the neuronal cell also appeared normal. However, the group that received EEGL (400 mg/kg) showed a hippocampus with a mildly depleted neuronal cell at the CA3 region. Figure 5 (c) depicts the histology of the female hippocampal region exposed to graded doses of EEGL which showed a normal structural organization of the hippocampal region with no visible alterations.

Prefrontal cortex: The histopathological examination of the prefrontal cortex following exposures to EEGL at different dose levels and the untreated group presented in Figure 5 (b) and Figure 5 (d) for males and females showed normal structure of the cortex and stromal, with neuronal cells appearing normal without any visible alteration.

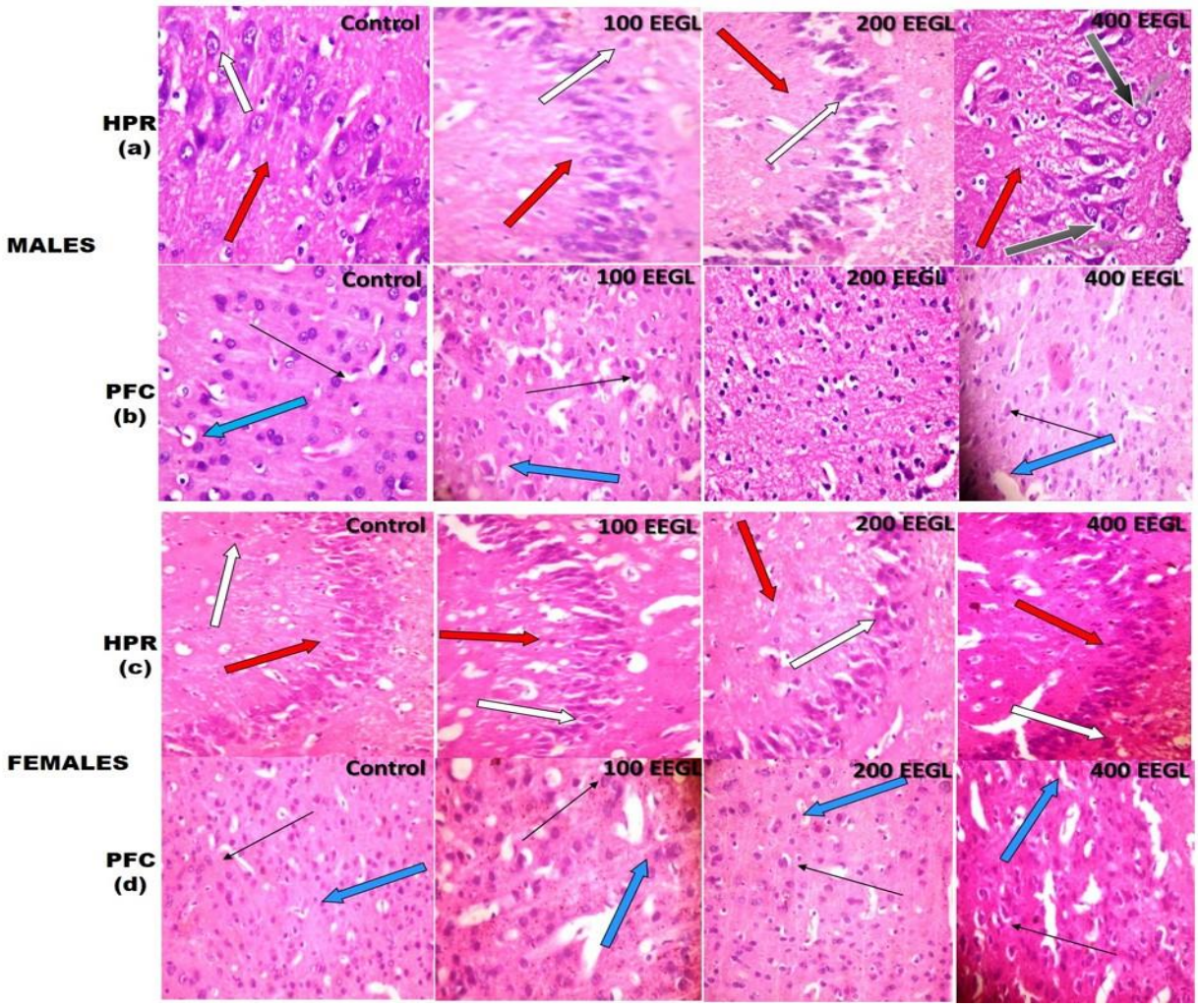

Figure 5: Representative effects of EEGL on histopathological morphology (H & E staining) of the hippocampal region and prefrontal cortex in Swiss mice. (a) HPR (male). (b) PFC (male). (c) HPR (female). (d) PFC (female). Magnification = × 400; HPR = Hippocampal region; PFC = Prefrontal cortex; EEGL = Ethanol extract of *Ganoderma lucidum*; H & E = Hematoxylin and eosin staining; Control group = distilled water; white arrow = normal hippocampus with normal neuronal cells; red arrow = structural organization of the region appeared normal; black arrow = hippocampus with mildly depleted neuronal cell at the CA3 region; blue arrow = structure of the cortex (stromal) appear normal; slender black arrow = neuronal cells appeared normal

DISCUSSION

As mushrooms are increasingly being consumed both in developed and underdeveloped countries due to their nutritional, medicinal, and health effects, it becomes pertinent that their safety in prolonged use is ascertained. Subacute exposure was used to predict the neurocognitive and anxiogenic-like effects of ethanol extract of *Ganoderma lucidum* in Swiss mice via neurobehavioral, neurochemical, and histological evaluations.

Cognitive and anxiety-like performances were used to evaluate the effect of EEGL on neurobehavior. The study showed that EEGL impairs cognition and induces anxiogenic-like symptoms in experimental mice, especially at the high doses tested. The Morris water maze is one of the "gold standards" paradigm of neurobehavioral science that is used to assess spatial and spatial learning ability as well as reference memory in experimental rodents (Nunez, 2008). Escape latency during acquisition trials (training) in male and female mice was significantly prolonged at 400 mg/kg EEGL when compared with control animals. Similarly, time spent in the platform zone was significantly decreased in treated animals compared to control, while average proximity to the platform zone increased during retention trials (memory). All these are pointers of cognitive impairment, which may eventually lead to deficiencies in working and reference memory as a result of impairment in the neuronal cell functioning of the hippocampus, which spatial/relational memory is dependent on (Soleimani *et al*., 2016). The cognitive score, which assessed the overall cognitive performance of the experimental animals, decreased significantly in males without remarkable alteration in females. It is worthy to note, that changes in memory retention and cognitive functioning tend to lower the overall performance of cognition (Dalrymple-Alford *et al*., 2010). The observed phenomenon in the different response to EEGL in either sex might be attributed to the cyclic changes in steroid hormone levels (Macoveanu *et al*., 2016; Koebele and Bimonte-Nelson, 2017). These hormones have remarkable effects on task performance, anatomy, and cell functions that are dependent on the hippocampus, and thus, EEGL may interact with these hormones, and the interplay may either be modulatory, efficacious or detrimental (Koss and Frick, 2017). On the contrary, studies have reported the neuroprotective effects of *G. lucidum* in experimental models. In 2019, Khatiani and

Aslam proved that fruiting bodies and cracked spores of *G. lucidum* extract (150 mg/kg) purchased in capsule form and administered orally have the potential of increasing the retention time of ongoing learning and memory, thereby improving memory functions, while another interesting study showed the potential of polysaccharides from *G. lucidum* (30 mg/kg) as a restorative-therapeutic agent to ameliorate neurodegeneration as a result of cognitive impairment at lower doses (Huang *et al*., 2017). The results showed it could improve cholinergic neuronal transmission and alleviate oxidative stress and mitochondrial dysfunction which could protect neurons from apoptosis, and thereby improve cognitive dysfunction (Ajith *et al*., 2009; Liu *et al*., 2020). In summary, the reported beneficial effects of *G. lucidum* were recorded at lower doses compared to the high doses in the study which probably may have accounted for the decline in cognitive manifestations remarkably significant at ≥ 200 mg/kg dose (s). Other factors such as the source and geographical location from which the *G. lucidum* mushroom was gotten from (wild) may also be considered as factors.

Light/dark box test is an animal model for the evaluation of anxiogenic and anxiolytic drugs (Aslam and Hussain, 2015). This model is dependent on the innate reluctance of experimental animals to lighted areas as well as sudden natural behaviors of animals to mild stressors, that is, light. Time spent in the dark compartment and the number of transitions, an exploratory behavior, was used to predict anxiety-like manifestations in the experimental animals (Ihne *et al*., 2012). In the study, EEGL (200 and 400 mg/kg) significantly increased the time spent in the dark compartment of the box while the number of transitions decreased in male Swiss mice. However, these indicators did not significantly affect the females, but a significant decrease in exploratory behavior was observed. Furthermore, the time spent in the corner and centre zone of the open field was used to substantiate the effect of EEGL on anxiety-like behavior in Swiss mice. The time spent in the corner zone of the open field test increased significantly in both males and females treated with higher doses of EEGL (200 and 400 mg/kg). It is noteworthy that the time spent in the corner zone of a maze measures the extent of thigmotaxis or wall-hugging behavior, which is a valid indication of anxiety-related behavior in mice (Seibenhener and Wooten, 2015; Kuniishi *et al*., 2017). Thus, the greater time spent in the corner zone instigated by higher doses of EEGL indicates an increased thigmotaxis in the open field, which increases the level of anxiety-related behavior in the experimental animals. Nasir and colleagues in 2016 reported the anxiolytic-like activity of an ethanol extract of *G. lucidum* in mice, which was specifically achieved at a lower dose of 75 mg/kg. In another study, it was demonstrated that the n-butanol fraction of the methanol extract of *G lucidum* revealed remarkable antianxiety activity at 100 mg/kg dose (Singh *et al*., 2016). The findings in this study and previous report suggest that EEGL may increase anxious behavior in Swiss mice at higher doses, with lower doses being beneficial.

Furthermore, the effects of EEGL on biochemicals in the brain were evaluated. Nitro-oxidative stress status such as MDA, SOD, CAT, GSH, and NO were assessed. This study revealed that EEGL (200 and 400 mg/kg) significantly elevated MDA and NO levels while GSH level, SOD and CAT activities were significantly reduced. Reactive species (RS) which in broad term refers to both reactive oxygen species (ROS) and reactive nitrogen species (RNS), are significant contributors and mediators of neuronal injury (Gasparovic *et al*., 2016). An imbalance between the production of reactive oxygen species (ROS) and the ability of the antioxidant systems to readily detoxify these reactive intermediates results in oxidative stress (Pizzino *et al*., 2017). MDA, a lipid peroxidation product is one of the markers of oxidative stress, while GSH is the most important antioxidant that coordinates innate defense mechanisms and capable of preventing damage to important cellular components caused by ROS such as free radicals and lipid peroxides. In other words, it maintains cell structure and function by its detoxification reactions (Sylvestre-Gonon *et al*., 2019). The significant increase in the level of MDA, and marked reduction in GSH level, SOD and CAT activities typically pointed to oxidative stress and can subsequently increase toxicity which is capable of disrupting neuronal functions, as the brain is very vulnerable to oxidative insult due to its functional, anatomic, and physiological make-up (Ren *et al*., 2019). It can be postulated that the cognitive decline and anxiety-like behaviors recorded in experimental Swiss mice might be as a result of oxidative stress induced by high doses of EEGL (200 and 400 mg/kg) which is known to implicate wide varieties of neurodegenerative disorders (Hwang, 2013). The elevated levels of NO witnessed in the study may be due to possible interaction of higher dose of EEGL (400 mg/kg) with iNOS which may lead to its induction, thereby competing with oxygen to form reactive nitrogen species capable of shutting down mitochondria respirations at multiple sites by irreversibly inhibiting the electron transport chain at the expense of ATP production, leading to more stressful influence on the overall system (Poderoso *et al*., 2019; Popov *et al*., 2021). The pro-inflammatory cytokines, TNF-alpha and IL-6 were also elevated significantly in mice that received the two high doses (200 and 400 mg/kg) of EEGL. This may be responsible for the sickness behaviors seen in animals that received EEGL at such high dose(s). Elevated levels of cytokines implicate neuroinflammation (Bhat *et al*., 2019). The observed increase may be associated with the induction of neuroinflammation in memory impairment (Kim *et al*., 2014). Oxidative stress triggers inflammatory response by activating the microglia and astrocyte as effector cells to release several pro-inflammatory cytokines in cascade of events which increase the release of TNF-alpha and IL-6, leading to inflammatory crisis and inflammation-mediated neurodegeneration (Smith *et al*., 2012). In contrast, a study reported that the administration of ethanol extract of *G. lucidum* (100 mg/kg) for 30 days (two weeks of acclimatization inclusive) significantly prevented oxido-inflammation by reducing the levels of hydrogen peroxide, MDA, and increasing the activity of antioxidant enzymes as well as the normal levels of nitrite (Adetuyi *et al*., 2020). Finally, a study that was carried out by Shevelev and Co-workers in 2015, found out that pretreatment with *G. lucidum* at a dose of 100 mg/kg enhanced the improvement in energy metabolism and balance between neurotransmitters that are inhibitory and excitatory in the CNS of rats treated with ethanol, which reflected on their behavioral profile. These reports may corroborate with the report from this study due to the fact that lower doses of EEGL (100 mg/kg) did not produce untoward toxicity seen in higher doses (200 and 400 mg/kg).

Acetylcholine levels are continuously being regulated in the brain and peripheral tissues by the hydrolytic enzyme acetylcholinesterase which rapidly degrades acetylcholine (Vaknine and Soreq, 2020). Therefore, the level of AChE, a marker of neurotransmission was assessed. In the study, administration of EEGL for 30 days significantly increased the activity of AChE in the mice brain of both sexes although

more remarkable in male mice. Consequently, in cholinergic region of the brain such as the prefrontal cortex and hippocampus, the primary activity of AChE is to terminate neurotransmission. However, increase in its activity may induce learning and memory deficits (Ikarashi *et al*., 2004; Kim *et al*., 2016). Thus, modulation of the cholinergic neurotransmission pathway via upregulation of AChE may be a potent mechanism of toxicity of high doses of EEGL.

Histopathological examinations of the regions of the male mice brain showed mild depletion of the neuronal cells at CA3 of the hippocampal region that were treated with EEGL (400 mg/kg), while others (hippocampal region of the female and prefrontal cortex of both sexes of the mice brain) appeared normal. All these are characteristic evidence of neuronal deficit which may be occasioned by high dose of EEGL. The hippocampal function is considered a major influence in spatial learning and memory performance (Jeong and Singer, 2022), structural damages to the brain cell components via elevated oxidative stress, neuroinflammation and increase in acetylcholinesterase activity may typically induce spatial learning and memory impairment and probably lead to hippocampal neurodegeneration with marked cognitive deficits. Taken together, high doses of EEGL may induce memory and anxiety-related disorders.

Conclusion

The present study evaluated the toxicity effects of *Ganoderma lucidum* ethanol extract (EEGL) on cognition and anxiogenic-like manifestation in Swiss mice. The study showed that EEGL (>100 mg/kg) may have possible CNS-related (memory and anxiety-like) toxic potential at high doses. The probable mechanism of toxicity may be via oxido-inflammatory, nitrergic, and acetylcholinesterase activity as the present study revealed. However, 100 mg/kg EEGL administered for 30 days did not produce these toxic effects in mice. These cumulatively showed that the toxicity effect of EEGL on cognition and anxiogenic-like behaviour in Swiss mice is dose-dependent.

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All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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