NEUROPHARMACOLOGICAL EFFECTS OF THE PETROLEUM ETHER/ETHYL ACETATE STEM BARK EXTRACT OF *MAERUA ANGOLENSIS D.C* (CAPPARACEAE)

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmacology, KNUST. This work has not been submitted for any other degree.

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ABSTRACT

The search for novel lead compounds from medicinal plants to ameliorate or cure various disorders is still important and valid. There is a greater and unmet medicinal need when it comes to central nervous system (CNS) disorders. *Maerua angolensis* is a useful medicinal plant found in most parts of the West-African sub-region including Ghana. It has been employed in the treatment of various CNS disorders including epilepsy and psychosis. The present study examined the anxiolytic, antidepressant and anticonvulsant potential of the petroleum ether/ethyl acetate extract of the stem bark (MAE) in murine and zebrafish models.

Preliminary neuropharmacological investigations in the Irwin test showed analgesic effects as well as a general reduction in fear response and motor activity. Further characterization indicated a general CNS depressant effect in the spontaneous locomotor activity and pentobarbitone-induced sleep tests. There was also a trend with respect to protection against death in the seizure liability test although statistical significance was absent when compared to control.

Based on the findings of the preliminary study, the anxiolytic potential was assessed in the elevated plus maze (EPM), Suok test (ST) and open field test (OFT) in mice. MAE (30-300 mg kg^{-1}), similar to diazepam, showed a significant increase in % open arm entries, a decrease in % protected head-dips and % stretch-attend postures. Oral administration of MAE also reduced anxiolytic parameters in the Suok test without affecting significantly motor coordination. However, MAE treatment revealed significant anxiolytic effects by increasing % centre time, whilst caffeine (anxiogenic agent) reduced % time spent in the central arena. The anxiolytic effects was further evaluated in the novel tank and light-dark zebrafish models.

MAE (0.1-1.0 mg mL^{-1}) treatment similar to fluoxetine and desipramine showed anxiolytic effects in both paradigms. Similar treatment with MAE also proved to be effective in reversing ethanol withdrawal-induced anxiety and anxiety induced by chronic unpredictable stress. The anxiolytic effect in the novel tank and light-dark tests was reversed by pretreatment with granisetron (a 5-HT_3 antagonist), cyproheptadine (a 5-HT_{2A} antagonist), methysergide (a 5-$HT_{2B/2C}$ antagonist) and pizotifen (a 5-$HT_{2A/2C}$ and 5-HT_1 antagonist). Also concomitant administration of MAE after flumazenil (a $GABA_A$ receptor antagonist) treatment suppressed the anxiolytic effect. Taken together, this suggest that the extract possesses true modulation of the serotonergic and GABAergic systems.

Acute administration of the extract (100-1000 mg kg^{-1}, *p.o.*) exhibited antidepressant effects by reducing the duration of immobility in both the forced swim test (FST) and tail suspension test (TST). Comparing the degree of reduction in immobility after treatment revealed that the efficacy of MAE was higher than that of fluoxetine but lower than after imipramine treatment in both tests. Significant increase in climbing and curling duration in the FST and TST respectively is suggestive of serotonergic and opioidergic interaction of MAE.

The anticonvulsant effects of MAE and possible mechanisms involved were further explored in the pentylenetetrazole-induced seizure model in rats. MAE (300 mg kg^{-1}, *p.o.*) similar to diazepam significantly delayed the onset as well as decreased the duration and frequency of PTZ-induced seizures. The $GABA_A$ receptor antagonist, flumazenil, reversed the anticonvulsant effect of MAE, further suggesting the possible involvement of the GABAergic system in its action. Furthermore, the anticonvulsant effect of MAE (300 mg kg^{-1}, *p.o.*) was reversed by pre-treatment with a sub-effective doses of L-arginine or sildenafil indicating a possible interaction with the nitricoxidergic system in the anticonvulsant effects.

In the *in vitro* antioxidant assay, the extract (0.02-0.2 mg mL^{-1}) exhibited significant DPPH scavenging activity (E_{max}=48.46 ± 3.652 %, IC_{50} =0.1817 mg mL^{-1}), superoxide scavenging activity (E_{max}=74.13 ± 8.199 %, IC_{50} =0.004357 mg mL^{-1}) and reduced the degree of lipid peroxidation (E_{max}=50 ± 0.00 %, IC_{50} =0.8245 mg mL^{-1}). However, the *in vitro* antioxidant properties of MAE was less potent than ascorbic acid, the reference antioxidant, although their efficacies were comparable. Pretreatment with MAE before pentylenetetrazole administration reduced significantly oxidative parameters suggesting the ability to prevent the damaging effects of free radicals in the brain. These findings reveal that the extract has antioxidant properties which may account for some of its neuropharmacological effects.

Results from this study suggests that MAE possesses sedative, anxiolytic, anticonvulsant, antidepressant and significant antioxidant effects.

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LIST OF ABBREVIATIONS

5-HT	5- Hydroxytryptamine
5-HTT	5-Hydroxytryptamine Transporter
AUC	Area Under Curve
ANOVA	Analysis Of Variance
BDNF	Brain-Derived Neurotrophic Factor
CAT	Catalase
cGMP	Cyclic GMP
CNS	Central Nervous System
CRF	Corticotrophin Releasing Factor
CS	CS – Chasing Stress
CUS	CUS – Chronic Unpredictable Stress
Cypro	Cyproheptadine
DA	Dopamine
DBE	DBE – Dorsal Body Exposure
Dm	Dorsal Pallium
DMSO	Dimethyl Sulfoxide
DPPH	2,2,-Diphenyl-1-Picrylhydrazyl
DSM-IV	Diagnostic And Statistical Manual IV
DTNB	5,5'-Dithiobis (2-Nitrobenzoic Acid)
Dzp	Diazepam

Emax	Maximum Effect
EDTA	Ethylenediaminetetracetic Acid
EPM	Elevated Plus Maze
FST	Forced Swim Test
Flz	Fluoxetine-
FMZ	Flumazenil
GABA	Gamma Aminobutyric Acid
$GABA_A$	GABA Receptor Subtype A
$GABA_B$	GABA Receptor Subtype A
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
Gstn	Granisetron
HD	Head Dips
HPA	Hypothalamus Pituitary Axis
HS	Heat Stress
i.p.-	Intraperitoneal
KNUST	Kwame Nkrumah University Of Science And Technology
LD	Light Dark
L-NAME	L-Arginine Methyl Ester
LPO	Lipid Peroxidation
MAE	*Maerua Angolensis* Extract
MDA	Malondialdehyde
MES	Maximum Electro Shock
Met	Methysergide

mRNA	Messenger RNA
NADH	Nicotinamide Adenine Dinucleotide
NBT	Nitro Blue Tetrazolium
NE	Norepinephrine
NO	Nitric Oxide
NT	Novel Tank
OCD	Obsessive Compulsive Disorder
OFT	Open Field Test
pHD	Protected Head Dips
Piz	Pizotifen
p.o.	Per Os
pSAP	Protected Stretch Attend Posture
PTZ	Pentylenetetrazole
q.s.	Quantity Sufficient
ROS	Reactive Oxygen Species
RS	Restrain Stress
SAP	Stretch Attend Posture
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error Of Mean
SERT	Serotonin Transporter
SI	Social Isolation
SOD	Superoxide Dismutase
SSRI	Selective Serotonin Reuptake Inhibitor
ST	Suok Test
TBA	Thiobarbituric Acid

TBARS	Thiobarbituric Reactive Substance
TC	Tank Change
TCA	Trichloroacetic Acid
TCAs	Tricyclic Antidepressants
TST	Tail Suspension Test
Tx	Treatment
uHDs	Unprotected Head Dips
uSAP	Unprotected Stretch Attend Posture
VTA	Ventral Tegmental Area
WHO	World Health Organization

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CHAPTER ONE INTRODUCTION

1.1 GENERAL INTRODUCTION

Centuries of healing with traditional medicines and a few decades of objective and directed research, interspersed with some degree of serendipity, have yielded a rich source of knowledge for the management of various ailments whether acute or chronic, infectious or non-infectious. Several of these treatment regimen have plant constituents (crude or refined) as their active principle. In these plants are a myriad of bioactive compounds that can be isolated and clinically used after showing significant efficacy with minimum toxicity to various body organs. The Ginkgolides and the artemisinins are typical examples of pure compounds derived from *Ginkgo biloba* and *Artemisia anua* respectively, two plant that have extensive usage in Chinese traditional medical practice and currently have found a place in general healthcare practice.

The WHO acknowledges the important role traditional herbal practice plays in meeting the health needs of developing nations. The WHO reports that approximately 80 % of the world's population depend on traditional remedies to meet their healthcare needs, with majority of the patronage originating from Africa (WHO, 2002; Willcox & Bodeker., 2004). Ghana cannot be left out since more than half of the population rely on this option either out of choice or due to the relative ease of acquisition. More importantly these have shown a proven consistency of effectiveness although with some reports of toxicity. With this preface, it is essential to investigate the various traditional

herbal medicines with the aim of confirming or disputing their therapeutic claims, and also exploring their inherent potential to induce toxicity.

Maerua angolensis is a tall evergreen plant distributed in the tropical and savannah regions of West Africa. Several parts of this plant have been used to manage various ailments in the traditional setting. The stem bark decoction is acclaimed to be effective in managing epilepsy and psychosis. However there is little literature to support its effectiveness for management of these CNS conditions. Hence this project seeks to investigate the pharmacological effects of the extract on selected neuropharmacological models.

1.2 *MAERUA ANGOLENSIS*

Botanical name: *Maerua angolensis* DC

Family: Capparaceae

Common names: Pugodigu, Bead-bean tree, mtunguru (Swahili)

Synonyms: *Maerua arenicola* sensu Eyles; *Maerua bukobensis* Gilg & Bened

Figure 1 *Maerua angolensis* plant (a) bark (b) fruits and leaves

1.2.1 ***Description and Distribution***

Maerua angolensis is a tall deciduous tree of the caper family, often growing in thickets fringing seasonal watercourses. It is a shrub or small tree of the coastal savanna, seldom above 5 m tall, with a greyish bark. The fruits are shaped in the form of pods usually about 25 cm in length resembling a string of beads. Leaves are soft and drooping, with petioles equal to the leaves in length, and visibly thicker at their extremities. Leaves are alternate and broadly elliptic to ovate, with rounded or notched apex and a terminal bristle. Leaf surfaces are often noticeably scratched by their rubbing against the bristles of surrounding leaves. *Maerua angolensis* is well distributed in the arid regions of tropical Africa. It is widespread in Togo, Sudan, Ghana, Mali, Nigeria, and Burkina

Faso (Ayo *et al.*, 2013).

1.2.2 ***Traditional Uses***

Most parts of *Maerua angolensis* have been traditionally formulated to treat and manage various disease conditions including:

a) **Neurological disorders**: Decoction of the leaves of *Maerua angolensis* have been used in the management of psychosis, neurosis and neurasthenia (Baerts & Lehmann , 1989). Also the root and stem bark decoction is considered sedating and have been used in the treatment of anxiety and epilepsy (Chhabra *et al.*, 1989).

b) **Aphrodisiac:** The juice obtained from the stem and root barks have been reported to reverse cases of sexual incapacity and also employed as an aphrodisiac.

c) **Gastrointestinal disorders**: In parts of West Africa, particularly Benin, the stem and leaves have been employed in the treatment of gastritis, colitis, abdominal pains and constipation (Kimpouni, 2001).

d) **Infections:** In the eastern part of Tanzania, the root and stem bark decoction have been used to effectively manage diarrhoea, dysentery and cholera (Chhabra *et al.*, 1989). The Juice obtained from the pounded leaves is also commonly employed in the treatment of candidiasis.

1.2.3 *Biological activities of Maerua angolensis*

a) ***In vitro* antioxidant activity:** *In vitro* antioxidant potential of the phenolic and flavonoid rich fractions from the leaves have been demonstrated *in vitro* (Meda *et al.*, 2013).

b) **Hypoglycaemic potential:** Mohammed and colleagues demonstrated the hypoglycaemic effects of the aqueous decoction of the stem bark in the streptozocin-induced diabetes model (Mohammed *et al.*, 2007).

c) **Antinociceptive and antinflammatory effect:** Different solvent fractions of *Maerua angolensis* leaf, root and stem bark have been demonstrated to possess antinociceptive effects mice and rats. The ethyl acetate and petroleum ether fractions of the stem bark extract were comparatively more potent at reducing the neurogenic and inflammatory phases of formalin induced paw licking (Azi *et al.*, 2014). The effect of the aqueous methanolic extract of *Maerua angolensis* has also been demonstrated to possess significant antinflammatory effects in rats (Adamu *et al.*, 2007). The analgesic effects of the petroleum ether/ethyl acetate extract and fractions prepared from the stem bark have been demonstrated using Hargreaves thermal hyperalgesia model. The effects of the extract and fractions on morphine dependence have also been assessed in mice. The authors proposed a centrally-acting mechanism for its analgesic activity (Iliya & Woode , 2014).

d) **Anxiolytic activity:** The methanolic stem bark extract have been demonstrated by Ibrahim and colleagues to possess anxiolytic effect, an effect that was partly attributed to interaction with $GABA_A$ receptors. The same authors have demonstrated the sedative potential. However they recorded no overt acute toxicity at doses of 5000 mg/kg and below (Malami *et al.*, 2014b).

e) **Anti-seizure potential:** Magaji and colleagues explored the anti-seizure potential of the methanolic fraction in maximum electric shock (MES), pentylenetetrazole and 4-aminopyridine induced seizures. However the degree of protection was unremarkable, with the highest in all the models being 50% (Magaji *et al.*, 2009).

1.3 ANXIETY AND DEPRESSION

1.3.1 *Background*

Anxiety is a common experience which usually precedes the encounter of a potential threat, with the main aim of preparing the individual to adapt to the potential threat. Surprisingly, a precise definition for anxiety is still lacking although several authors have defined and described major aspects of the disorder.

In the non-pathological form, anxiety can be divided into two categories: (1) state anxiety which is an acute adaptive response of heightened vigilance and arousal that enables an organism to navigate an unfamiliar environment of unknown danger and (2) trait anxiety which is a measure of an individual's baseline reactivity or tendency to generate anxious response. In its pathological form, anxiety is a maladaptive state that

impairs the ability of an organism to respond optimally to its environment (Leonardo &

Hen, 2008).

Six anxiety disorders are recognized by the Diagnostic and Statistical Manual of the American Psychiatric Association (American Psychiatric Association and American Psychiatric Association Task Force on DSM-IV, 2000). Four most common form of anxiety disorders; generalized anxiety disorder, panic disorders, social anxiety disorders and post-traumatic stress disorder, are associated with significant disability but less attention is given to these disorders as a whole in terms of identifying new treatment strategies (Kroenke *et al.*, 2007). In the United States alone, anxiety disorders is known to cost (direct and indirect) about 42 million dollars a year (Kroenke *et al.*, 2007) and it is further estimated that anxiety disorders may affect up to 20% of the population at some point in their lifetime (Leonardo & Hen, 2008).

Depression is also a common mental disorder, characterized by sadness, anhedonia, feelings of guilt or low self-worth, disturbed sleep or appetite, feelings of tiredness and poor concentration and often comorbid with symptoms of anxiety. It is currently estimated that 350 million people suffer from depression globally (WHO, 2012). Depression is also the leading cause of disability worldwide and a major contributor to the global burden of disease. In addition to the significant risk of suicide, depressed individuals are more likely to die from other causes such as heart disease or cancer (Rang *et al.*, 2012).

1.3.2 *Pathophysiology of anxiety*

Although anxiety is an area receiving much attention in terms of research, there is no single theory that completely explains the pathophysiology of the disorder. Review of results obtained from both human and animal experimentation strongly implicates the involvement of the GABAergic system (Kalueff *et al.*, 2007).

Anxiety and depression have been found to be naturally linked, a theory that is buttressed by the fact that they respond to similar treatments (Möhler 2012).

Predominantly, anxiety research has focused on the regulatory systems, including GABAergic and serotoninergic systems among others. Some findings on the pathophysiology of anxiety are discussed below.

1.3.3 *Involvement of the GABAergic system*

One of the most widely accepted mediators central to the development and treatment of anxiety disorders is the GABAergic system (Nemeroff, 2003).The amygdala is the site for formation and storage of fear memories and extinction memories. This area of the brain compared to other areas of the brain has a strong inhibitory tone which is attributed to the inhibitory activity of GABA. In pathological states, this inhibition can become impaired and even signals which were previously innocuous can cause nonadaptive fear and anxiety (Lydiard, 2003). It has been demonstrated by several authors that drug-induced enhancement of GABA transmission has been found to produce anxiolytic effects whilst a reduction of GABA transmission usually precipitates anxiety (Baldwin *et al.*, 2011). Also cortical deficits in $GABA_A$ receptor activity have been found to correlate with the severity of anxiety symptoms in both mice and humans

(Hasler *et al.*, 2004; Möhler 2012). Benzodiazepines act by modulation of $GABA_{A/B}$ receptors have proven efficacious the management of anxiety disorders (Cryan *et al.*, 2005). These findings support the suggestion that GABA plays a role in the pathophysiology of anxiety.

1.3.4 *Role of Oxidative Stress*

Oxidative stress is gaining grounds as a possible pathway that can affect the regulation of anxiety. Recent findings show that in comparison to healthy controls, patients with anxiety disorders have higher activity levels of the antioxidant enzymes, superoxide dismutase and glutathione peroxidase (Bouayed, 2011). Under oxidative stress, the lipid-rich constitution of the brain can undergo lipid peroxidation which can result in decreased membrane fluidity and altered neurotransmission (Bouayed *et al.*, 2009; Valko *et al.*, 2007). The anxiogenic effect of oxidative stress has been demonstrated in several models of anxiety by inducing oxidative stress (Masood *et al.*, 2008). These findings, suggest a possible link between oxidative stress and pathological anxiety.

1.3.5 *Pathophysiology of depression*

Although the exact cause of depression is still unknown, research over the past decades have led to several theories. Currently the monoamine theory, hypothalamic pituitary cortisol hypothesis, neurotrophic hypothesis as well as inflammation/oxidative stress theory have been proposed as plausible explanations.

1.3.5.1 Monoamine theory

The monoamine theory which was proposed in 1965 posits that some, if not all depressive states are caused by absolute or relative deficits in monoamines; noradrenaline and 5-hydroxytryptamine, at specific regions of the brain (Schildkraut, 1965). The main evidence in support of the monoamine theory is the ability of known antidepressant drugs to enhance monoaminergic transmission; and reserpine which inhibits vesicular transport of monoamines to cause depression (Bondy, 2002). The monoamine oxidase enzyme system which is responsible for degradation of monoamines in neurons has been used as a useful target for some antidepressant drugs. This strengthens the monoamine hypothesis since the downstream effects of these inhibitors is an elevation of serotonin and noradrenaline levels. Also data gathered from positron emission studies have shown a 30% increase in brain monoamine oxidase activity in depressed patients, in further support of the theory (Belmaker & Agam, 2008). However the monoamine theory is unable to resolve the discrepancy between the relatively rapid increase in brain monoamine levels and the delay in the antidepressant effects. Additionally, the increases in monoamines induced by cocaine and amphetamine administration are ineffective in relieving symptoms of depression (Belmaker & Agam, 2008).

1.3.5.2 Hypothalamic-Pituitary-Cortisol Hypothesis

Chronic stress has been identified as risk factor for major depression in individuals with genetic vulnerability. In rodent models repeated stress have been used to model depression-like behaviours such as anhedonia, reduced sexual activity and increased

corticosterone levels. The hippocampus is especially vulnerable to stress due to the enrichment of glucocorticoid receptors and this could underlie the decreased neurogenesis and impaired short term memory that results from chronic stress.

Hypersecretion of corticotropin-releasing factor (CRF), inadequate glucocorticoid feedback and increased cortisol levels have been observed in depressed patients suggesting a possible link between the hypothalamic-pituitary-adrenal (HPA) axis and depression (Bondy, 2002). Also stressful life events appear to have a strong causal association with depression. The link between genetic predisposition and life stressors in the etiology of depression has been most clearly demonstrated (Caspi *et al.*, 2003).

1.3.5.3 Neurotrophic Hypothesis

The neurotrophic hypothesis suggests that a loss of brain derived neurotrophic factor (BDNF) is directly involved in pathophysiology of depression and that its restoration may underlie the therapeutic efficacy of antidepressant treatment (Groves, 2007). BDNF promotes neuronal survival by acting on tyrosine kinase receptors (TrkB). Some evidence has been offered in support of this theory. Firstly, post-mortem analysis has detected decreased BDNF and TrkB expression in the hippocampi of depressed suicide patients and increased levels in patients medicated with antidepressants before death (Lauterborn *et al.*, 2003). Antidepressant treatment increases neurotrophic factor expression and increases adult neurogenesis. Non-pharmacologic strategies such as electroconvulsive shock therapy, transcranial magnetic stimulation and exercise also show increased mRNA or protein BDNF levels in the rat brain. When considered in isolation, this evidence appears to provide substantive support for the BDNF theory;

however other studies have generated data that are inconsistent with such a theory. Elevated hippocampal BDNF protein increases depressive-like behaviour in the mouse forced swim test (Branchi *et al.*, 2006) (Branchi *et al.* 2006). Interestingly, BDNF infusions into the ventral tegmental area (VTA) increases depressive-like behaviour in the mouse forced swim test whilst suppression of BDNF receptor expression within the VTA causes anti-depressive-like behaviour in the rat forced swim test (Eisch *et al.*, 2003). Finally, the SSRI fluoxetine has no effect or decreases BDNF mRNA in the rat hippocampus (Miró *et al.*, 2002; Russo-Neustadt *et al.*, 1999).

1.3.5.4 Inflammation and Oxidative Stress

Mounting evidence shows that clinical depression is accompanied by increased oxidative and nitrosative stress (Berk *et al.*, 2013). There have been many consistent findings over the years of increased levels of pro-inflammatory cytokines in patients with depression, e.g. interferon-γand tumour necrosis factor-α (Schiepers *et al.*, 2005). Likewise, depression was found to be characterized by increased levels of malondialdehyde, a by-product of polyunsaturated fatty acid peroxidation and arachidonic acid (Sarandol *et al.*, 2007). Although the brain metabolizes 20% of the total oxygen in the body, it has a limited amount of antioxidant capacity, so it is particularly vulnerable to the production of reactive oxygen species (Xu *et al.*, 2014). Most, if not all antidepressants have specific anti-inflammatory effects (Maes *et al.*, 2009) and the clinical efficacy of antidepressants may be enhanced by concurrent administration of anti-inflammatory drugs such as celecoxib. Emerging data from

animal models reveals that some antioxidants possess antidepressant activity at relatively lower doses than commonly used antidepressants such as imipramine or fluoxetine, although the mechanism of action has not been well elucidated (Xu *et al.*, 2014).

1.4 EXPERIMENTAL MODELS OF ANXIETY AND DEPRESSION

1.4.1 *Light dark test*

The light dark test explores the behaviour of rodents to at least two mild stressors; novel and well illuminated environment. Rodents have a natural aversion to brightly lit areas and also tend to explore less when placed in a novel environment. The time spent in each arena as well as the latency to emerge from the dark arena have been shown to be sensitive to classical anxiolytic and anxiogenic agents in mice. Benzodiazepines as well as the newer anxiolytic-like compounds acting through the serotonergic system can be detected using this paradigm (Hascoët *et al.*, 2001). The light dark test has the advantages of being quick and easy to use, without requiring the prior training of animals. Compared to the light dark test however, the elevated plus-maze may be more sensitive to the anxiolytic effects of benzodiazepines (Chaouloff *et al.*, 1997).

1.4.2 *Open field test*

The initial behaviour of rodent when placed in a novel environment, in this case the open field, has been taken as an indicator of the emotional state of the animal (Katz *et al.*, 1981). The novel environment is usually demarcated into three sections at the base,

and the time spent in each section as well as the entry frequency into these sections can be used to assess the anxiety state of the rodent. A detailed ethological assessment by Choleris *et al.,* 2001 identified that anxiolytics such as diazepam and chlordiazepoxide; were able to significantly reduce the typical anxiety-like behaviour such as stretch attend posture and thigmotaxis, whilst increasing time spent in central arena (Choleris *et al.,* 2001). Although the test has proved to be sensitive to most anxiogenic and anxiolytic agents, a few compounds such as adinazolam, alprazolam and selective serotonin reuptake inhibitors are poorly effective as anxiolytics in the open field test (Prut & Belzung, 2003).

1.4.3 *Elevated plus maze*

The elevated plus maze is a simple method for assessing anxiety responses of rodents. Unlike other anxiety models the responses obtained from this test does not rely upon the presentation of noxious stimuli (i.e. electric shock, exposure to predator odour, etc.) that typically produce a conditioned response. The elevated plus maze relies upon rodents' proclivity toward dark, enclosed spaces and an unconditioned fear of heights/open spaces (Barnett, 2007). A five minute testing period can reveal behaviours that can be used not only in the assessment of the anxiety state but also locomotor activity and risk assessment behaviours. The duration and frequency of closed/open arm entry , protected head dips and stretch attend postures have been used as measures of anxiety whilst the total head dips and stretch attend postures have been used to study exploratory and risk assessment behaviours respectively (Rodgers *et al.*, 1997).

1.4.4 *Suok test (ST)*

The Suok test is a fast and simple model which has been used to assess sensorimotor coordination and anxiety related behaviours in rats and mice (Dow *et al.*, 2011; Kalueff *et al.*, 2008; Kalueff *et al.*, 2005). The test explores the natural spectrum of behaviours of mice when placed on an elevated rod. It combines different principles from the elevated plus maze, open field and beam walk tests. The novelty, elevation, and instability of the rod allow assessment of both anxiety-like behaviour and motor coordination simultaneously. Behaviours such as rearing, head-dips, side-looks, and frequency and duration of freezing bouts are used as endpoints for assessing of anxiogenic/anxiolytic potential of a chemical entity whilst the number of falls and missteps are known to predict closely the degree of sensorimotor coordination (Kalueff *et al.*, 2008).

There is currently a modification to the regular Suok test which employs a well-lit environment as an additional anxiogenic parameter (Kalueff *et al.*, 2008). The latter modification might offer an advantage in exploring anxiolytic potential of treatments or drugs in mice strains with a higher anxiety threshold by assessing the distance and time spent in the light and dark regions. In the regular Suok test, a one trial test without any prior training can be used to evaluate motor performance between different strains as well as drug treated groups (Kalueff *et al.*, 2005).

1.4.5 *Zebrafish novel tank test*

The novel tank diving test, conceptually similar to the open field test in rodents, is the most extensively studied model of anxiety in the zebrafish. This test explores the fact that adult zebrafish spend about 50 % of a 5 min session in the bottom of a novel tank (Maximino *et al.*, 2012). Exposure to a novel environment evokes a robust anxiety response in zebrafish as they dive to the bottom (geotaxis) until they feel safe to swim in the upper regions of the tank. This behaviour, called ―bottom-dwelling‖ is reduced by diazepam, buspirone, chronic fluoxetine or acute ethanol treatment (Maximino *et al.*, 2010b).

1.4.6 *Zebrafish light dark test*

The light dark test for zebrafish, in its present format, was initially proposed by Serra *et al.* and further validated by Maximino and colleagues (Maximino *et al.*, 2012; Maximino *et al.*, 2010a). The light/dark or scototaxis test can be used to assess anxiety in zebrafish and is similar to the light dark test used to assess anxiety in rodents (Chakravarty *et al.*, 2013).

Behaviour in this task reflects a conflict between the preference of the animal for dark protected areas and an innate motivation to explore new environments. It has been demonstrated to be sensitive to both anxiogenic and anxiolytic drugs. Spending longer periods of time in the dark chamber reflects increased anxiety. An increased anxiety level is also indicated by a decrease in the latency to enter the dark chamber, similar to the light/dark box test for rodents (Maximino *et al.*, 2010b).

1.4.7 *Chronic unpredictable stress*

Chronic unpredictable stress (CUS) involves the application of a series of stressors in an unpredictable fashion over an extended period of time. Chronic stress has been demonstrated to induce affective disorders such as anxiety, depression and other related mood disorders (Egan *et al.*, 2009). This paradigm has been used to model affective like disorders such as depression in rats, mice and zebrafish. Chronic models are considered more naturalistic in the induction of a depressive-like state, such models are suggested to have better potential homology to the human affective disorders (Duman, 2010). The CUS model has been employed in studying stress and other related disorders such as anxiety and by extension screen possible drug candidates that might be useful in affective and stress disorders (Chakravarty *et al.*, 2013; Piato *et al.*, 2011).

1.4.8 *Tail suspension test*

The tail-suspension test (TST) is a mouse behavioural test useful in the screening of potential antidepressant drugs and assessing other manipulations that are expected to affect depression related behaviours (Steru *et al.*, 1985). The tail suspension test was developed as a rodent screening test for potential antidepressant drugs (Can *et al.*, 2011). It is based on the assumption that an animal will actively try to escape an aversive (stressful) stimulus by assuming escape oriented behaviours such as curling of the body, pedaling of the limbs or a general swinging of the body interspersed with periods of immobility. Longer periods of immobility are characteristic of a depressivelike state. The validity of this test stems from the finding that treatment with an antidepressant drug will decrease the time the animal spends immobile.

1.4.9 *Forced swim test*

The forced swim test (FST) is a rodent behavioural test used for evaluation of antidepressant drugs, and experimental manipulations that are aimed at rendering or preventing depressive-like states (Steru *et al.*, 1985). It remains one of the most used test for antidepressant screening due to its ease of use and high predictive value (Cryan *et al.*, 2005). In the FST, the animal is placed in a cylindrical container of water from which it cannot escape. The rodents' response to the threat of drowning is to attempt escape oriented behaviours interspersed with immobility where the animal makes no attempt to escape apart from making necessary movement to keep its head above (Can *et al.*, 2012). An animal that gives up relatively quickly is thought to be displaying characteristics similar to human depression. Furthermore, it is suggested that immobility reflects a state of lowered mood (Slattery & Cyran, 2012; Steru *et al.*, 1985).

The validity of this test stems from the finding that physical or psychological stress (which can induce depression in humans) administered prior to the test causes animals to give up sooner and treatment with an antidepressant drug will increase the time an animal spends in escape attempts (Can *et al.*, 2012).

1.5 MANAGEMENT OF ANXIETY AND DEPRESSION

Treatment options include psychological therapy and medications. All patients with anxiety disorder require supportive or psychological therapy and pharmacological

treatment should be viewed as complimentary and not alternative therapies (Bandelow *et al.*, 2012).

Cognitive behavioural therapy has been demonstrated to be highly effective in anxiety disorders (Westra, 2004). Exposure therapy and response prevention are also effective especially in specific phobia, agoraphobia, social phobia and obsessive compulsive disorders (OCD). Patient can also be assisted with supportive talks and psychoeducational advice (Bandelow *et al.*, 2012).

Selective serotonin re-uptake inhibitors (SSRIs), tricyclic antidepressants (TCAs) and benzodiazepines have proved to be effective in the treatment of anxiety. Evidence from multiple clinical trials supports the use of SSRIs as first-line pharmacotherapy in anxiety disorders (Hoffman & Mathew, 2008). However a delay of at 2-4 weeks may precede their anxiolytic effect (Bandelow *et al.*, 2012). Benzodiazepines have a much quicker onset of action, although their potential for abuse and the lack of antidepressant properties limit their use (Hoffman *et al.*, 2008).

Treatment for anxiety is challenging because current medications are neither consistently beneficial nor particularly precise. Although current pharmacological and psychological therapies are efficacious for some patients; others experience significant residual symptoms or intolerable side effects. Side effects such as nausea, tremors and sexual dysfunction have been reported with the use of SSRIs (Hoffman *et al.*, 2008). Benzodiazepines are widely used drugs for the management of anxiety disorders; however their use is associated with development of tolerance and dependence and a

lack of efficacy in many patients (Pinna & Rasmusson, 2012). Even first line treatments such as SSRIs require a period of 2-4 weeks before their anxiolytic effect may be observed. This is a challenge in management of anxiety in acute cases.

1.6 ZEBRAFISH AS AN EXPERIMENTAL ORGANISM

Danio rerio has risen from being a mere aquarium fish to being one with diverse experimental uses including the preliminary screening of newly identified compounds. The genetic makeup of this fish is not entirely homologous to that of humans though gene comparisons reveal similarities in genes that code proteins necessary for many pharmacological processes. With high reproducibility and relatively low cost of experiments, it is advantageous to employ the zebrafish as an alternative to the rodents used in most laboratories (Goldsmith & Jobin , 2012; Patton & Zon, 2001).

In nature, the zebrafish is found in the Indian subcontinent mainly in the Ganges and Brahmaputra river basins. It is a small cyprinid that habituates slow-moving or stagnant water bodies. In the laboratory, the domestication/adaptation process seeks to conserve natural traits in the fish such as response to predator cues while enhancing survival and improving the reproductive life (Harper *et al.*, 2012).

The anatomy of zebrafish has been extensively studied and characterized with vast differences existing between the larvae, juvenile and adult stage in terms of functioning phenotype (Kimmel *et al.*, 1995). Even amongst the adult *Danio rerio* species obvious genetic differences are present depending on the gender.

It is fascinating to know that the developmental cycle lasts for typically 3-4 months (Kimmel *et al.*, 1995) making the fish suitable for use in generational toxicity and selection experiments. The zebrafish, immediately after hatching, exhibit an innate defensive ability to shoal. Alarming substances increase shoaling cohesion (Engeszer *et al.*, 2007). They are group spawners with an average number of embryos per female ranging from 100-200 per spawning. They spawn every 2-3 days and the eggs are fertilized externally. When relocated to a laboratory they respond to the same spawning cues such as green vegetation; artificial or natural, drop in temperature, shallow water and the onset of light. The optical transparency of the larval stage (up to 1 month) makes them an ideal choice for use in studying the general effects of xenobiotics on internal organs such as the heart because pericardial effusion can easily be seen through their skin. Recently, several domestic and systematically bred strains have been identified. Currently recognized Wild type lines include AB, Tubingen, AB/Tubingen, Tubingen Long fin, Singapore etc.

1.7 JUSTIFICATION OF RESEARCH

Despite availability of several treatment regimens for the management of most CNS disorders, pharmacotherapy still remains the common and easily accessible treatment option for many patients. However, most drug treatments cause undesirable side effects. For example, some current anticonvulsant therapies are associated with dose-related cognitive impairment (Trimble & Thompson, 1983) whilst others induce structural lesions and negatively affect prognosis. Also some antidepressants not only cause sexual dysfunction and weight gain but there is a significant delay in the onset of pharmacologically relevant actions (Segraves, 1998). This has made the search for newer drugs with a superior pharmacological profile, possibly effective at multiple relevant targets, important.

There is mounting evidence that oxidative stress is a common feature of most CNS pathologies (Bouayed, 2011) and therapies with additional antioxidant effects may prove beneficial over existing therapies. Increased inflammatory responses have been implicated in the pathology or prognosis of depression, anxiety and epilepsy (Raison *et al.*, 2006; Salim *et al.*, 2012; Vezzani *et al.*, 2012). Recent pharmacological

investigations demonstrate that the plant possesses significant *in vivo* antioxidant (Meda *et al.*, 2013), anti-inflammatory and central analgesic (Iliya & Woode, 2014) properties which may prove beneficial in selected CNS disorders such as anxiety, depression and epilepsy.

Plants have also served as a rich source of new molecules with important pharmacological properties hence it is very important to source for new compounds with potentially effective properties. *Maerua angolensis* stem bark is a traditional plant used in the treatment of psychosis and epilepsy, two conditions that are intimately linked with anxiety and depression. Hence it would be important to investigate the potential of *Maerua angolensis* extract in anxiety, epilepsy management as well as other related disorders to provide some amount of scientific evidence for the plant's folkloric use.

1.8 AIMS OF STUDY

The aim of this study is to investigate the anxiolytic, antidepressant and anti-seizure potential of the petroleum ether/ethyl acetate extract of the stem bark of *Maerua angolensis* in murine and zebrafish. This study may provide pharmacological evidence for the traditional uses of the plant in epilepsy treatment and also explore the potential benefits of the extract in other conditions that share similar neurobiology.

1.8.1 *Specific Objectives of Research*

The specific objectives of this study is to investigate the:

i. Anxiolytic potential of the petroleum ether/ethyl acetate extract of the stem bark of *Maerua angolensis* in both mice and zebrafish

ii. Anxiolytic potential of the extract in experimentally-induced anxiety in the zebrafish and the involvement of the serotonergic and GABAergic systems in the anxiolytic effects.

iii. Acute antidepressant activity of the extract in mice.

iv. Anti-seizure potential of the extract in the pentylenetetrazole-induced seizures in mice and rats.

v. Involvement of the GABAergic system in the anti-seizure potential of the extract.

vi. *In vitro* antioxidant effects of the extract vii. Protective effects of the extract in PTZ-induced oxidative stress in the rat brain.

viii. Involvement of L-arginine-NO-cGMP pathway in the anti-seizure activity of the extract

CHAPTER TWO MATERIALS AND METHODS

2.0 PLANT COLLECTION AND EXTRACTION

2.0.1 Plant Collection

The stem bark of *Maerua angolensis* were collected from Kwahu Tafo in the Eastern region of Ghana (6.415360° N, 0.363160°W) and authenticated by Dr. Kofi Annan of the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical sciences. A voucher specimen (KNUST/FP/12/051) was kept at the herbarium of the Faculty.

2.0.2 Plant extraction

The fresh stem bark was chopped and room-dried for 14 days. The dried bark was then pulverized into a fine light-brown powder. The powder (1.8 kg) was then extracted by cold percolation with 5 L of petroleum ether and ethyl acetate mixture (50:50). The solvent mixture was drained 72 h later to obtain a dark green extract which was further concentrated in a rotary evaporator at 60 °C under reduced pressure. The concentrate obtained was further dried in a hot air oven at 55°C for 72 h to obtain a green semisolid mass (8.5 g) which was then stored in the freezer at -20 °C until use. Throughout this study the crude extract will be referred to as MAE.

2.1.0 *Animals*

ICR mice (20-25 g) and Sprague-Dawley rats (150-200 g) were obtained from the animal house of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. The animals were housed in groups of 5 in stainless steel cages ($34 \times 47 \times 18$ cm^3) with soft wood shavings as bedding and housing conditions controlled– temperature maintained at 24-25 °C, relative humidity

60-70 %, and 12 h light-dark cycle. They had free access to tap water and food (commercial pellet diet, GAFCO, Tema, Ghana).

Zebrafish were obtained from a commercial source in Malaysia through a local aquarist (Aquarium Marshall and Business Ventures Ltd., Accra, Ghana). Twenty (20) fish were housed in glass tanks (10 l) containing filtered and dechlorinated water maintained at a 22-25 °C. To mimic their natural habitat each housing tank was filled with gravels to a height of about 2 cm and a fresh water plant (*Cabomba aquatica*) was submerged in each tank. Adult fish were fed twice daily with commercial fish flakes alternated with high protein pellets. However larval and juvenile fish were fed three times daily with *Paramecium* culture alternated with freshly hatched brine shrimp larvae.

All studies were conducted in accordance with accepted principles for laboratory animal use and care (Barthold *et al.*, 2011). Approval for this study was obtained from the Faculty Ethics Committee.

2.1.1 Chemicals and drugs

Granisetron hydrochloride (Corepharma LLC, Middlesex, England); pizotifen maleate, methysergide (Novartis Pharmaceutical cooperation, Basel, Switzerland); sildenafil (Pfizer Inc., Brooklyn, NY, USA); fluoxetine (Eli Lilly and Co., Indianapolis, IN, USA); diazepam (Intas Gujarat, India); imipramine, cyproheptadine (Actavis UK Ltd, Devon, UK); flumazenil (Roche Pharmaceutical Ltd, Garden City, UK.); adrenaline (Indus Pharma. PVT, India); protease inhibitor cocktail-EDTA free (Santa Cruz Biotechnology Inc., Dallas, Texas, USA).

Pentylenetetrazole, caffeine, trichloroacetic acid, thiobarbituric acid, *o*-dianisidine, sodium dodecyl sulphate, sodium chloride, Tween 80, *n*-butanol, Triton-X, L-NAME (L-arginine methyl ester) , L-arginine, DTNB (5,5'-Dithiobis (2-nitrobenzoic acid)),

nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), ethylenediamineteraacetic acid (EDTA), dimethyl sulfoxide (DMSO), DPPH (2,2,diphenyl-1-picrylhydrazyl), NADH (nicotinamide adenine dinucleotide), and Tris-HCl were obtained from Sigma-Aldrich Inc., St. Louis, MO, USA.

2.2 PRELIMINARY NEUROPHARMACOLOGICAL SCREENING

2.2.1 Irwin test

ICR mice (20 -25 g) were randomly distributed into five groups (n=5) and left to acclimate for 24 h. Mice were fasted overnight but had free access to glucose solution. The animals were treated with 5 oral doses of MAE (30-5000 mg kg^{-1}, *p.o.*) while control group received distilled water 10 ml kg^{-1}, *p. o*. The neurologic, autonomic and behavioural state of the animals were observed at 0 to 15, 30, 60, 120, 180 min, 24 h and 48 h as described by Irwin (Irwin, 1968).

2.2.2 Activity meter test

The effect of MAE on spontaneous locomotor activity was evaluated using an activity cage (Ugo Basile, model 7401, Comerio, VA, Italy). Male ICR mice (20-25 g) randomly divided into eleven groups (n=6-8) were treated with MAE (30, 100, 300, 1000 mg kg^{-1}, *p.o.*), diazepam (0.1, 0.3, 1.0 mg kg^{-1}, i.p.), caffeine (10, 30, 100 mg kg^{-1} , *p.o.*) or distilled water (10 ml kg^{-1} , *p.o.*). Animals were then placed individually in the activity cage and their activity scored in 5 min blocks for 30 min.

2.2.3 Convulsive threshold test

Male ICR mice randomly assigned to twelve (12) groups received either MAE *p.o* (301000 mg kg^{-1}), diazepam i.p. (8 mg kg^{-1}) or distilled water (10 ml kg^{-1}) *p. o*. One

hour after *p.o.* or 30 minutes after i.p. treatment, seizure was induced by subcutaneous administration of pentylenetetrazole (85 mg kg^{-1}) at the nape of the neck and placed in plastic observational cages. The seizure characteristics after injection were recorded for thirty minutes with the aid of a camcorder. The video output was then analyzed for the latency to clonic seizure and death with the behavioural analysis software, JWatcher® version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia. available at http://www.jwatcher.ucla.edu/).

2.2.4 Pentobarbitone induced sleeping time test

To investigate the effect of MAE in the pentobarbitone-induced sleeping time, male ICR mice were assigned randomly to seven groups (n=7) and received either MAE (30-1000 mg kg^{-1}), diazepam (8 mg kg^{-1}), caffeine (16 mg kg^{-1}) or distilled water (10 ml kg^{1}) *p. o.* Sodium pentobarbitone (50 mg kg^{-1}) was administered i.p. 1 h after respective drug treatments. Latency to sleep (time between pentobarbitone injection and loss of righting reflex) and duration of sleep (time between loss of and regaining of righting reflex) were recorded with a stopwatch.

2.3 ANXIETY SCREENING IN MICE

2.3.1 Open field test

Testing was conducted in a transparent Plexiglas box (40 × 40 × 30 cm^3) whose floor is divided into 16 equal squares. The floor of the arena was designated as either corner (any of the four corner squares), periphery (squares along the walls) or center (inner four squares). ICR mice were randomly selected and distributed into ten groups of 7 animals each. Animals received either MAE (30,100,300 mg kg^{-1}, *p.o.*), diazepam (0.1,

0.3, 1.0 mg kg-1, i.p.), caffeine (10, 30,100 mg kg^{-1}, i.p.) or distilled water (10 ml kg^{-1} , *p.o.*). Thirty minutes after intraperitoneal administration and sixty minutes after oral dosing mice were introduced gently in the center of the open field and their behaviours exhibited were recorded with a camcorder after for a period of 5 minutes; representing a single session. To remove olfactory cues produced by previous mice, sessions were conducted only after the field has been wiped with 10 % v/v ethanol. The frequency of entry and time spent in each region (corner, center and periphery) was assessed.

2.3.2 Elevated plus maze

The elevated plus maze consist of two closed (30 × 5 × 1 cm^3) and two open arms (30 × 5 × 1 cm^3) with a central arena (5 × 5 cm^2). The maze is elevated 60 cm above the floor with the aid of a platform. Behavioural testing was performed under dim light in a noise- attenuated room. ICR mice were randomly selected and distributed into eleven groups of 7 animals each. Animals received either MAE (30,100,300 mg kg^{-1}, *p.o.*), diazepam (0.1, 0.3, 1.0 mg kg^{-1}, i.p.), caffeine (10, 30,100 mg kg^{-1} i.p.) or distilled water (10 ml kg^{-1} *p.o.*). The maze was cleaned with 10 % v/v ethanol after each session to remove olfactory cues. With the aid of camcorder and JWatcher software, the following behaviours were recorded and scored; (i) number of closed and open arm entries (ii) time spent in exploring open and closed arms (iii) frequency and duration of protected and unprotected head dips (iv) number and duration of protected and unprotected stretch attend postures (v) frequency of protected and unprotected grooming (vi) frequency of rearing. An arm entry was defined as a mouse having entered an arm of the maze with all four limbs. A behaviour was defined as protected if it occurs in the closed arms or center and unprotected when exhibited in the open arm region of the maze.

2.3.3 Suok test

The regular Suok test was carried out as described by Kalueff *et al.*,(2008) with slight modifications (Kalueff *et al.*, 2008). The setup consisted of a 2 meter aluminum rod (diameter 2 cm) divided into 10 equal segments and elevated 25 cm high. To avoid or reduce harm to mice falling from the rod, the base of the setup was covered with paper towels.

Forty two (42) mice were allowed to acclimate for 24 h in a dimly lit experimental room for an hour before drug treatment and testing. Mice were the randomly selected and distributed into seven groups of 6 animals each. Animals received either MAE (30,100,300 mg kg^{-1}, *p.o.*), diazepam (0.1, 0.3, 1.0 mg kg^{-1}, i.p.), or distilled water (10 ml kg^{-1}, *p.o.*). One hour after oral and thirty minutes after intraperitoneal administration, mice were placed in the central region of the rod. The behaviour on the rod were captured for five (5) minutes with the aid of a camcorder approximately 2 meters away from the rod. The exploratory activity as well as specific behaviours were then scored and analyzed with the aid of JWatcher software. Behaviours assessed included the frequency and duration of (a) stretch attend postures (b) side looks (c) head dips and (d) immobility and (e) number of leg slips.

2.4 ANXIETY SCREENING IN ZEBRAFISH

2.4.1 Sedative and Locomotor activity

Adult zebrafish (4 months old) were gently netted and introduced into non-reflective transparent plastic containers (21 × 16.5 × 12 cm^3) containing either MAE (0.03, 0.3, 3 mg mL^{-1}) or diazepam (30, 100, 300 µM). To assess the effect of MAE on locomotor activity, the activity of fish (n=5) were recorded for 30 minutes with the aid of a vertical

(75 cm high) camcorder mounted. Videos were converted into .avi (mjpeg) format and spliced to generate fifteen, 120 s videos. The spliced videos were then analyzed in ImageJ® (MJTrack Plugin) for the total distance travelled (cm) and mean velocity (cm/s). Mean Velocity (cm/s) and mean total distance (cm) were then compared to the solvent control. The time course of the sedation as well as the pattern of movement was also be generated from the data obtained.

2.4.2 Novel tank test

The novel tank (30 × 10 × 25 cm^3) was divided into three equal 6 cm height sections. Baseline anxiety behaviour of the zebrafish (n=15) was assessed in the novel tank by introducing fish gently into the novel environment after been netted from their home tank.

On a later day, zebrafish were treated with either MAE (0.1, 0.3, 1.0 mg mL^{-1}), fluoxetine (30, 100, 300 µg L^{-1}), desipramine (10, 30, 100 µg mL^{-1}) or diazepam (0.15, 15, 30 µg L^{-1}) for 20 minutes before being gently netted into the novel tank. Their behaviour was recorded for 5 minutes and stored for further analysis. The videos were then analyzed and scored with the aid of JWatcher version 1.0 for the following parameters; total time spent and latency to enter the upper 2/3.

2.4.3 Light-Dark test

The baseline preference to dark or well-lit environment was assessed in a light-dark paradigm (50 × 10 × 10 cm^3) with the length divided into two equal parts. Each portion was either covered with a dark or white non-reflective material except the top region. Animals were introduced gently into the novel environment after being netted from their home tank. Zebrafish were then treated, on a later day, with either MAE (0.1, 0.3, 1.0 mg mL^{-1}), fluoxetine (30, 100, 300 µg L^{-1}), desipramine (10, 30, 100 µg mL^{-1}) or

diazepam (0.15, 15, 30 µg L^{-1}) for 20 minutes before being introduced gently into the mid region of the setup. Their behaviour were then recorded for 6 minutes with the aid of a camcorder mounted vertically above the setup. Videos obtained were then stored and further analyzed for total time spent in the light region.

2.5 ETHANOL-WITHDRAWAL INDUCED ANXIETY

Ethanol-withdrawal induced anxiety in zebrafish was based on methods described by Holcombe and colleagues with slight modifications (Holcombe *et al.*, 2013). The baseline preference to novel environment and brightly lit arenas were assessed in the novel tank (n=30) and light dark paradigm (n=20) respectively. In the novel tank the preference for the upper 2/3 was assessed whilst the scototaxis behaviour was assessed in the light dark test as described above. Zebrafish were then divided into five groups and exposed to system water or 0.5% / ethanol by immersion (20 minutes) for eight (8) continuous days according to the scheme below: Group I – Ethanol naïve control group, Group II-V – Ethanol treated Control (0.5% /).

Zebrafish were randomly selected and subjected to the novel tank and light dark tests on day five of ethanol exposure. To induce withdrawal, the administration of ethanol was replaced with system water from day 8 onwards. To assess the effect of ethanol withdrawal on anxiety levels, zebrafish were tested in the light-dark and novel tank tests on day four (4) and day eight (8) post withdrawal.

On the ninth day of ethanol withdrawal, the fish were acutely treated MAE (0.1, 0.3. 1.0 mg mL^{-1}), diazepam (0.15 µg L^{-1}), ethanol (0.5 % /) or system water according to the scheme below.

Group I – system water, Group II – ethanol (0.5% /), Group II – diazepam (0.15 µg L^{-1}), Group III – MAE (0.1 mg mL^{-1}), Group IV – MAE (0.3 mg mL^{-1}), Group V – MAE (1.0 mg mL^{-1})

2.6 CHRONIC UNPREDICTABLE STRESS IN ZEBRAFISH

Chronic stress was induced according to the pattern described by Piato and colleagues with slight modifications (Piato *et al.*, 2011). Forty eight (48) zebrafish were subjected to a series of stressors for a period of 14 days. To avoid habituation to stressors, unpredictability was maintained by changing the time and sequence of stressors daily during the 14 days of the stress paradigm. The description of the seven stressors are elaborated in table 1.0. Fish were subjected to a twice daily regimen of stressing according to the table below (Table 2). Aeration and temperature were controlled during the presentation of each stressor, except during heating and cooling stresses. The non-stressed control group was maintained in the same room during the 14-day stress period.

Table 1: Description of stressors employed in the chronic unpredictable stress par

Adigm **Ressor**	**Description**
Cold stress (CS)	exposed to system water at 23°C for 30 minutes
Heat stress (HS)	exposed to system water at 33°C for 30 minutes
Social isolation (SI)	placed individually in separate 100 mL beaker for 60 minutes,
Tank Change (TC)	were transferred into six other tanks in a period of 10 minutes
Overcrowding (OC)	10 animals were placed in 250 mL beaker half filled with system water.

Restrain stress (RS)	gently introduced into a 10 mL test tube half filled with system water for 10 minutes
Dorsal body exposure (DBE)	Water level in home tank lowered to expose the dorsal body surface for 10 minutes

Table 2: Schematic representation of chronic unpredictable stress paradigm

		Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Week 1	Morning	TC	OC	RS	SI	DBE	RS	CS
	Evening	DBE	CS	HS	CS	CS	HS	O
Week 2	Morning	CS	O	SI	HS	DBE	CS	HS
	Evening	TC	DBE	RS	TC	CS	SI	OC
Week 3	Morning	**Tx & testing**	**Tx**	**Tx & testing**				
	Evening							

RS-Restrain stress, CS-Cold stress, HS-Heat stress, SI-Social isolation, OC-Overcrowding, DBE-Dorsal body exposure, TC-Tank change, CS-Chasing stress Tx-Treatment.

2.6.1 Novel tank test

The novel tank test was carried out according to an adaptation of the method used by Chakravarty *et al.,* 2013 (Chakravarty *et al.,* 2013). After treatment, fish were placed individually in a narrow (15×10 × 25 cm^3) transparent glass tank with a water depth of 18 cm partitioned into two equal, virtually horizontal sections marked by lines on the outside of the tank. The fish were then recorded for 5 minutes using a video camera mounted about 1 m away facing the tank horizontally. The time spent by fish in upper section of the tank was measured using the public domain software JWatcher Version 1.0®

2.6.2 Light dark test

The light dark test was carried out as according to an adaptation of the method used by Chakravarty *et al.*, 2013 (Chakravarty *et al.*, 2013). The setup for the test consisted of a (50×10×10 cm^3) glass tank divided equally into light and dark regions with a water depth of 6 cm. The light region was illuminated by a lamp mounted at the edge of the tank. After initial treatment where required, the fish were placed in turns in the centre of the light/dark tank facing either direction. The fish were then recorded for 5 minutes using a video camera suspended 80 cm above the tank. The time spent by the fish in the light and dark chambers of the tank was measured using the public domain software JWatcher® Version 1.0®.

2.6.3 Shoaling response

Three zebrafish from each group was introduced into the novel tank and imaged from above for a ten minute period. The shoaling response was assessed by measuring the average area between the fish using MTrackJ plugin for ImageJ®.

2.7 INVOLVEMENT OF 5-HT SYSTEM

The experiment was intended to characterize the behavioural responses of four serotonin receptor antagonists: granisetron, cyproheptadine, pizotifen and methysergide, in a novel tank test and light dark paradigm. The anxiolytic potential of *Maerua angolensis* extract was then assessed after treatment with the antagonists mentioned above.

Zebrafish (n=8-10) were exposed by immersion in a 1 L solution or suspension of fluoxetine (10 µg L^{-1}), MAE (1 mg mL^{-1}), granisetron (1 mg L^{-1}), cyproheptadine (1 mg L^{-1}), pizotifen (1 mg L^{-1}) , methysergide (1 mg L^{-1}) or system water for 20 minutes

before behavioural testing in the novel tank and subsequently the light dark paradigm. Doses of the antagonists and MAE were chosen based on pilot experiments and previous reports.

In a separate experiment zebrafish (n=5-8) were pretreated for 20 minutes with either granisetron (1 mg L^{-1}), cyproheptadine (1 mg L^{-1}), pizotifen (1 mg L^{-1}) or methysergide (1 mg L^{-1}) followed by a 20 minute MAE (1 mg mL^{-1}) or fluoxetine (10 µg L^{-1}) treatment. Zebrafish were placed in novel tank and subsequently in the light dark tank to quantify anxiety behaviours.

Video recordings were made from the front and upper view of the novel tank and light dark tanks respectively. The video output were then analyzed for the time spent in the light and upper half of the light dark and novel tanks test respectively.

2.8 INVOLVEMENT OF GABAERGIC SYSTEM

The intent of the experiment was to characterize the behavioural responses of adult zebrafish to a $GABA_A$ antagonist, flumazenil, and also to assess the possible interaction of *Maerua angolensis* extract with the $GABA_A$ receptor. The anxiolytic potential of the extract was assessed in the novel tank and light dark test after flumazenil administration. Zebrafish were treated with system water, flumazenil (1 µg mL^{-1}), diazepam (0.15 µg L^{-1}), or MAE (1 mg L^{-1}). In a follow up experiment performed on the same day, fish were treated with MAE (1 mg mL^{-1}) or diazepam (0.15 µg L^{-1}) following twenty minutes exposure to flumazenil. After the various treatment, fish were gently placed in the novel tank and imaged for five minutes each. Doses of flumazenil

and diazepam were chosen based on pilot experiments and previous reports. The video output were then analyzed for the time spent in the upper half of the novel tank.

2.9 ACUTE ANTIDEPRESSANT ACTIVITY IN MICE

2.9.1 Tail suspension test

ICR mice were randomly assigned to ten groups of seven animals each. After acclimatization, mice were dosed with either MAE (100, 300, 1000 mg kg^{-1}, *p.o.*), fluoxetine (3, 10, 30 mg kg-^{1}, *p.o.*), desipramine (10, 30,100 mg kg^{-1}, *p.o.*) or distilled water (10 ml kg^{-1}, *p.o.*). One hour after oral dosing animals were suspended at their tail (1 cm from the tip) with an adhesive tape on a horizontal bar raised 50 cm from a table top. Behaviours exhibited by the mice were recorded for a period of 6 min and subsequently analyzed for escape oriented-behaviours such as pedaling, curling and swinging and immobility time were quantified for the last 4 min of each session.

Behaviours assessed included: (1) *immobility* – a mouse was judged to be immobile when it hung by its tail without engaging in any active behaviour; (2) *swinging* – a mouse was judged to be swinging when it continuously moved its paws in the vertical position whilst keeping its body straight and/or it moved its body from side to side; (3) *curling* – a mouse was judged to be curling when it engaged in active twisting movements of the entire body and (4) *pedaling* – defined as when the animal moved its paws continuously without moving its body. Mice that climbed on their tail were gently pulled down and the test continued.

2.9.2 Forced swim test

Seventy (70) ICR mice were randomly assigned to ten groups of seven animals each. After acclimatization, mice were dosed with either MAE (100, 300, 1000 mg kg^{-1}, *p.o.*), fluoxetine (3, 10, 30 mg kg^{-1}, *p.o.*), desipramine (10, 30,100 mg kg^{-1} *p.o.*) or distilled water (10 ml kg^{-1} *p.o.*) 60 minutes before behavioural testing. Mice were gently dropped individually into identical transparent cylindrical tanks (25 cm high and 10 cm deep) containing water (26 ± 1 °C) up to 20 cm for a total of 6 minutes. Each session was videotaped with a camcorder suspended above the cylinder. The duration of immobility and other escape oriented behaviours (climbing and swimming) during the last 4 minutes were quantified using JWatcher Version 1.0®. After the end of each session animals were removed from the cylinders dried with a towel and placed near a heater until they were completely dry.

2.10 PTZ-INDUCED CONVULSIONS IN RATS

Pentylenetetrazole (65 mg kg^{-1}, s.c.) was used to induce clonic convulsions. Rats were divided into 7 groups and received MAE (300 mg kg^{-1}, *p.o.*), diazepam (1.0 mg kg^{-1}, i.p.) or normal saline (10 ml kg^{-1} i.p.) 30 min (i.p.) or 1 h (*p.o.*) before the injection of PTZ, respectively. After PTZ injection, animals were placed in testing chambers (15 ×15 ×15 cm^3). A mirror angled at 45 ° below the floor of the chamber allowed a complete view of the convulsive event. Behaviour of the animals was captured with a camcorder for a 30 min period after PTZ challenge and the latency to myoclonic jerks and the incidence of generalized tonic–clonic seizure were recorded

2.10.1 Involvement of GABAergic system

To investigate the involvement of $GABA_A$ receptor in the anticonvulsant effects of MAE, rats were treated with diazepam (1.0 mg kg^{-1} i.p.) or MAE (300 mg kg^{-1} *p.o.*) 15 minutes after flumazenil (2 mg kg^{-1} i.p.) administration. The rats were then challenged; 30 min after diazepam and 60 min after MAE treatment, with a single subcutaneous dose of PTZ (65 mg kg^{-1}) to induce clonic convulsions. The latency, frequency and duration of clonic convulsions were assessed for a period of 30 min.

2.10.2 Involvement of Nitric oxide pathways

To investigate the involvement of L-arginine-NO-cGMP pathway, rats were pretreated with L-arginine (150 mg kg^{-1}, *i.p.*), sildenafil (5 mg kg^{-1}, *i.p.*) or vehicle 15 minutes before MAE (300 mg kg^{-1} , *p.o.*). Treated animals were then challenged with PTZ (65 mg kg^{-1}, s.c.), 45 min after MAE administration. The latency, frequency and duration of clonic convulsions were assessed for a period of 30 min.

2.11 *IN VITRO* ANTIOXIDANT ASSAY

2.11.1 DPPH radical scavenging activity

The method of Blois (1958) with modification was used for the determination of scavenging activity of MAE against DPPH free radicals. DPPH (1.0 mL, 0.135 mM) prepared in absolute methanol was mixed with 1.0 mL of dissolved extracts (200-2000 µg mL^{-1}) or ascorbic acid (20-200 µg mL^{-1}). The reaction mixture was shaken vigorously and left in the dark at room temperature for 30 minutes. The absorbance was measured spectrophotometrically at 517 nm after transferring 100 µL of each treatment into designated wells of a 96-well plate. Control experiment contained only methanol

and DPPH free radical. All experiments were conducted in triplicates. The percentage scavenging activity of DPPH-radical was calculated using the formula:

$$\%\text{scavenging activity of DPPH} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\ \%$$

Where $A_{control}$ is the absorbance of the control reaction and A_{sample} is the absorbance in the presence of the sample extracts or standard.

2.11.2 Superoxide anion scavenging activity

The superoxide anion scavenging activity was measured as described by Robak & Gryglewski, (1988) with slight modifications. Three mL of Tris-HCl buffer (16 mM, pH 8.0), containing 0.5 mL of NBT (0.3 mM), 0.5 mL NADH (0.936 mM) solution was added to 1.0 mL MAE (200-2000 µg mL^{-1}) or ascorbic acid (20 – 200 µg mL^{-1}) in 0.5 mL Tris-HCl buffer (16 mM, pH 8.0). The reaction was started by adding 0.5 mL PMS solution (0.12 mM) to the mixture, incubated at 25 °C for 5 minutes. The absorbance change was measured at 560 nm against a blank sample containing Tris-HCl buffer only. Ascorbic acid was employed as the standard antioxidant in this assay. The percentage inhibition of superoxide anion was calculated using the following equation:

$$\%\ \text{inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

Where $A_{control}$ is the absorbance of the control reaction and A_{sample} is the absorbance in the presence of the sample extracts or standard.

2.11.3 Estimation of anti-lipid peroxidation

A modified thiobarbituric acid-reactive species (TBARS) assay described by Dasgupta & De (2007) was used to measure the lipid peroxide formed using egg-yolk

homogenates as lipid-rich media.

An aliquot of egg yolk homogenate (10% $^{v}/_{v}$) in distilled water and 0.1 mL of the extract (200-2000 µg mL^{-1}) or standard (20-200 µg mL^{-1}) were mixed separately in a test tube and the volume was made up to 1 mL with distilled water. Finally, 0.05 mL Fe_2SO_4 (0.07 M) was added to the mixture and incubated for 30 minutes to induce lipid peroxidation. Thereafter, 1.5 mL of 20 % acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 mL of 0.8 % w/v TBA (prepared in 1.1 % sodium dodecyl sulfate) and 0.05 mL 20% TCA were added, vortexed and heated in a boiling water bath at 100 °C for 60 minutes. To eliminate non-MDA interference, another set of samples was treated in the same way, but incubating without TBA, so as to subtract the absorbance of the nonMDA interference from the test and standards absorbance. After cooling, the coloured TBA–MDA complex was extracted with 5 mL *n*-butanol by vigorously shaking and centrifuging at 3000 × g for 10min. The absorbance of the organic upper layer was measured at 532 nm. For control, 0.1 mL of distilled water was used in place of the extract or standard.

$$\%\ \text{anti} - \text{lipid peroxidation} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

Where $A_{control}$ is the absorbance of the control reaction and A_{sample} is the absorbance in the presence of the sample extracts or standard

2.12 PTZ INDUCED OXIDATIVE STRESS

The animals from the PTZ-induced seizure experiments above were killed by cardiac puncture and the brains were quickly excised, washed with saline and stored at - 80 ° C.

On the day of the experiment the brain tissue was thawed, weighed and homogenized in TNG-T buffer to obtain 10 % $^{w}/_{v}$ of homogenate. The tissue homogenate obtained was used to assay superoxide dismutase and reduced glutathione levels. The supernatant obtained after centrifuging at 20,000 ×g for one hour (4 °C) was assayed for catalase levels and the degree of lipid peroxidation.

Each assay below was carried out in a single unused 96 round well plates (Nunc™, Thermo Fischer Scientific, Waltham, MA, USA). All absorbances were measured with Synergy H1® hybrid plate reader at wavelengths specified below. Three readings were taken for each treatment sample and the direct absorbances or derived data represented as mean ± SEM. The protein content of the brain homogenates were assayed using Flexor EL150™ (Vital Scientific) biochemistry analyzer. All readings were normalized with the protein content of the respective tissue sample, hence all readings; except reduced glutathione assay, were expressed as Unit of absorbance per mg of protein (U/mg).

2.12.1 Lipid peroxidation in brain tissue

The degree of lipid peroxidation (LPO) was estimated in brain tissue supernatant in terms of malondialdehyde (MDA) levels which was determined as per the methods described by Heath & Parker (1968) with slight modifications.

MDA content was estimated by addition of 3 mL trichloroacetic acid (20 $\%^{w}/_{v}$) containing thiobarbituric acid (0.5%$^{w}/_{v}$) to 1 mL of tissue supernatant. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice bath. The mixture was then centrifuged at 10 000 g for 10 minutes (23 °C), and then absorbance of the supernatant was read at 532 nm. The value for the nonspecific absorption at 600 nm was subtracted from the 532 nm reading. The concentration of MDA was calculated using MDA's extinction coefficient of 155 $mM^{-1}cm^{-1}$.

2.12.2 Brain Superoxide dismutase activity

Brain superoxide dismutase activity was measured based on the ability of SOD to inhibit autoxidation of adrenaline to adrenochrome (Misra & Fridovich, 1972).

To 0.5 mL of tissue homogenate, 0.75 mL of ethanol (96%$^{v}/_{v}$) and 0.15 mL of chilled chloroform were added. The resulting solution was centrifuged at 2000 g at 30°C for 20 minutes to obtain a clear supernatant. 0.5 mL of EDTA (0.6 mM) was added to the supernatant solution containing 1 mL of bicarbonate buffer (0.1M, pH 10.2). 50 µL of adrenaline (1.3 mM) was added to the mixture to initiate the reaction and the absorbance of the adrenochrome formed was measured at 480 nm at 25 °C. The absorbance of a sample blank containing all reagents apart from tissue homogenate was also measured at 480 nm.

2.12.3 Brain Catalase activity

The catalase activity was assayed colorimetrically as described by the method of Aebi (1984). It is based on the ability of the enzyme source to break down H_2O_2. A 100 µL of the supernatant was added to 130 µL of potassium buffer (50 mM, pH=7.0). The reaction was initiated in well by the addition of 65 µL of H_2O_2 (10 mM). The reaction

mixture was then incubated at 25°C for 5 minutes. The absorbance of the resulting product was read at 620 nm with an appropriate blank.

2.12.4 Brain reduced glutathione levels

Reduced glutathione (GSH) was determined according to the method described by Cribb *et al.* (1989). An aliquot of 10 % tissue homogenate was mixed with 2.4 mL of EDTA (0.02 M) solution and kept on ice bath for 10 minutes. To the resulting homogenate, 2 mL of distilled water and 0.5 mL of trichloroacetic acid (TCA) 50 % ($^{w}/_{v}$) were added and centrifuged at 3000 × g for 20 minutes at 4 °C to remove precipitate. 1 mL of supernatant was then mixed with 2.0 mL of Tris buffer (0.4 M, pH=8.9) and 0.05 mL of 5' - dithiobisnitro benzoic acid solution (10 mM) was added and vortexed thoroughly. The absorbance was measured at 412 nm against a reagent blank with no homogenate after addition of DTNB and incubation at 23 °C for 5 minutes. The amount of reduced glutathione was extrapolated from a standard curve and expressed as µM/mg of protein.

2.13 STATISTICS

Data are presented as mean ± SEM. However some data are presented as box and whisker plots with the lower and upper margins of the boxes representing 25th and 75th percentiles with the extended arms representing the 10th and 90th percentiles respectively.

Data were analyzed using one-way analysis of variance (ANOVA). When ANOVA was significant, multiple comparisons between treatments was done using Sidak *post hoc* test. Significance between grouped data was assessed using a two-way ANOVA followed by a Sidak *post hoc* test.

Dose-responses curves are constructed using iterative curve fitting with the following nonlinear regression (three parameter logistic) equation:

$$Y = \frac{1}{1 + 10^{(LogED_{50} - X) \times b}}$$

Where, X is the logarithm of dose and Y is the response. Y starts at a (the bottom) and goes to b (the top) with a sigmoid shape. The fitted midpoints (ED_{50}s) of the curves were compared statistically using *F* test (Miller, 2003; Motulsky, 2003) with GraphPad Prism for Windows version 6.01 (GraphPad® Software, San Diego, CA, USA)

CHAPTER THREE RESULTS

3.1 PRELIMINARY NEUROPHARMACOLOGICAL SCREENING

3.1.1 *Irwin Test*

Oral administration of MAE caused analgesia and reduced fear response at all tested doses. The onset of action of these responses was observed to be shorter at higher doses. A reduction in touch response and increased in jumps (lasting 15 min) were also recorded after administration of 3000 and 5000 mg kg^{-1} respectively. MAE (100-5000 mg kg^{-1}, *p.o.*) administration did not have any lethal effects over the 48 h observation period.

Table 3 Effects of *Maerua angolensis* stem bark extract in the Irwin test

Dose (mg/kg)	Effects (onset- end)	Deaths/Treated
0	No effect (0-180 min)	0/5
100	Analgesia, reduced fear response (60-120 min)	0/5
300	Analgesia, reduced fear response (30-120 min)	0/5
1000	Analgesia, reduced fear and touch response (30-120 min)	0/5
3000	Analgesia, reduced fear, touch response and activity (30-120 min)	0/5
5000	Analgesia, reduced fear, touch response and activity (30-120 min). Increased jumps (15-30 min).	0/5

3.1.2 *Activity meter test*

Maerua angolensis stem bark extract and diazepam at doses of 1000 mg kg^{-1} and 1.0 mg kg^{-1} respectively reduced (P<0.0001) spontaneous locomotor activity. Caffeine on the other hand significantly increased the locomotor at 30 and 100 mg kg^{-1}(P<0.0001).

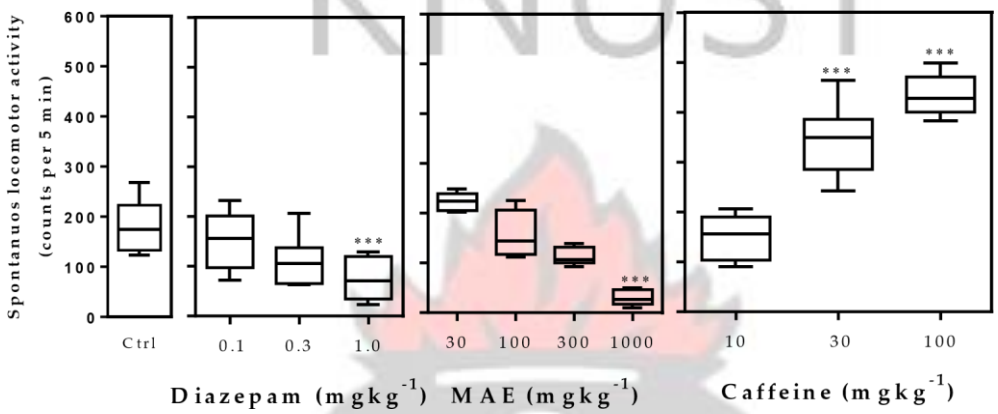


Figure 1: Effects of MAE (30-1000 mg kg^{-1}, *p.o.*), diazepam (0.1-1.0 mg kg^{-1}, *i.p.*) and caffeine (10-100 mg kg^{-1}, *p.o.*) in the activity meter test. Significantly different from control: ***P < 0.001 (One-way ANOVA followed by Sidak *post hoc* test)

3.1.3 *Convulsive threshold test*

MAE (1000 mg kg^{-1} *p.o.*) delayed onset of PTZ-induced convulsions, significantly (P<0.05, Fig. 2.0). However due to some mortalities recorded before the 30th minute in the solvent control and MAE-treated groups, the frequency and duration of convulsions were not assessed. Consequently a survival analysis (Fig. 3.0) was rather employed to reveal the degree of protection. Although the survival analysis revealed a significant increase in the degree of protection offered by MAE, comparison with the solvent control did not reveal any significant statistical difference. Diazepam (8 mg kg^{-1}) the reference anticonvulsant increased the latency to convulsions (P<0.0001) whilst decreasing the lethality significantly.

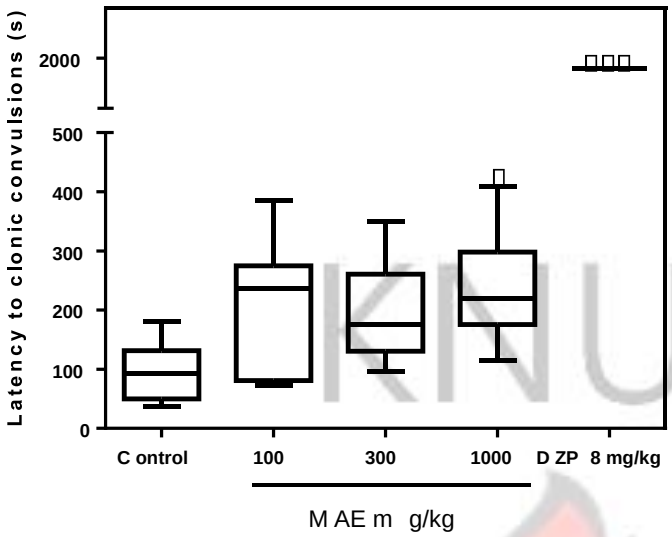


Figure 2: Effect of MAE (100- 1000 mg kg^{-1} *p.o.*) and diazepam (8 mg kg^{-1} *p.o.*) on the latency to clonic seizures in mice. Significantly different from control: **P*<0.05, ****P*<0.001 (One-way ANOVA followed by Sidak post hoc test)

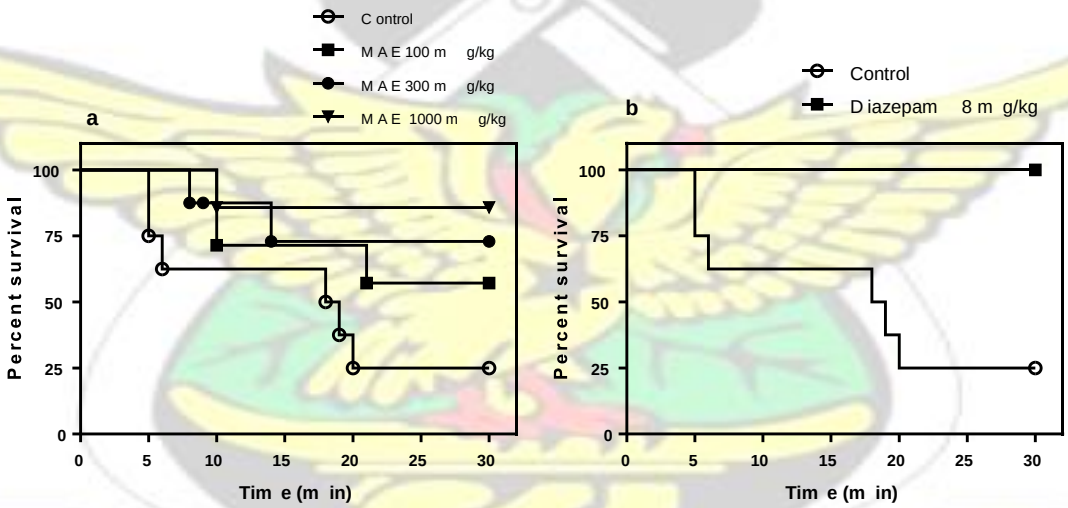


Figure 3: Kaplan–Meier estimates of overall survival of animals treated with (a) MAE (100 1000 mg kg^{-1} *p.o.*) and (b) diazepam (8 mg kg^{-1} *p.o.*) in the pentylenetetrazole-induced seizure test over a 30 min observation period.

3.1.4 Pentobarbitone-induced sleeping time

Acute administration of MAE (*p.o.*) at all doses exhibited a sedative property after pretreatment with pentobarbitone. There was a dose-dependent and significant decrease

in latency to loss of righting reflex ($F_{6, 42}$= 71.06, P < 0.0001) (Fig 4.0). Diazepam (8 mg kg^{-1}) and caffeine (16 mg kg^{-1}) significantly decreased and increased the latency to sleep respectively. Figure 5.0 also shows a significant increase in duration of sleep after MAE administration ($F_{6, 42}$ = 71.06 P < 0.0001) and diazepam. In contrast, a CNS stimulant (caffeine) did not significantly reduce the duration of sleep as compared to the vehicle control group.

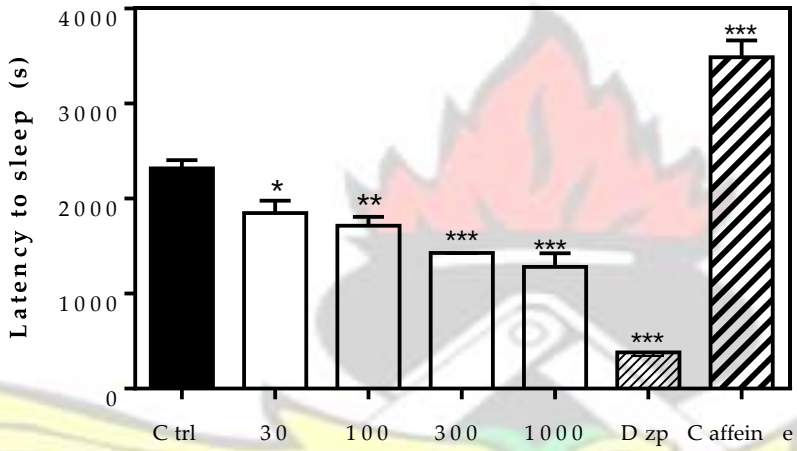


Figure 4: Effects of acute MAE (30-1000 mg kg^{-1} *p.o.*), diazepam (8 mg kg^{-1} *p.o.*) and caffeine (16 mg kg^{-1} *p.o.*) on latency to sleep in the pentobarbitone-induced sleeping time. Data are presented as group mean ± SEM. Significantly different from control: *P<0.05, **P<0.01. ***P<0.001

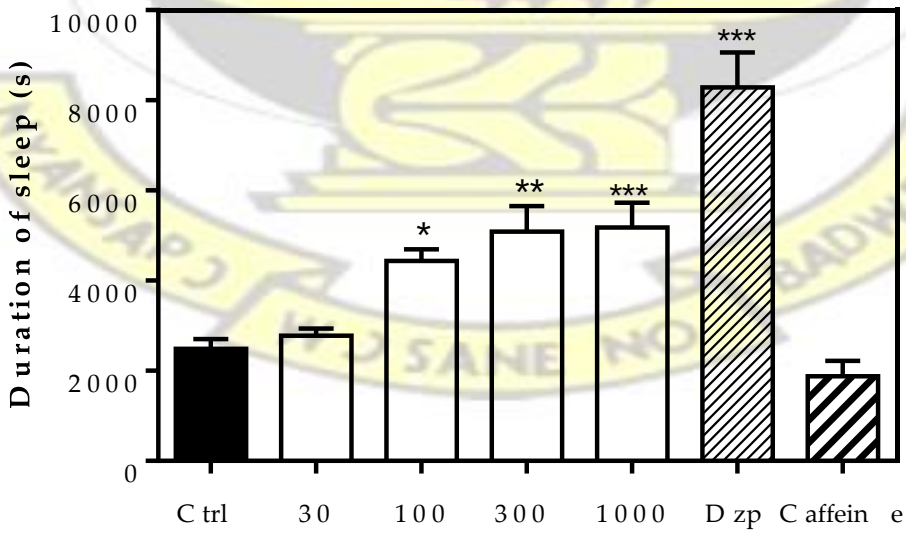


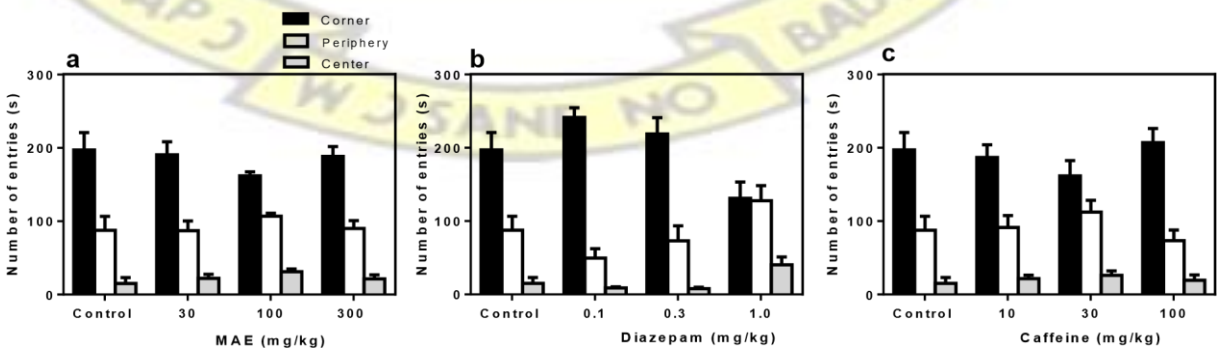
Figure 5: Effects of acute MAE (30-1000 mg kg-$^{-1}$, *p.o.*), diazepam (8 mg kg^{-1} *p.o.*) and caffeine (16 mg kg^{-1} *p.o.*) on sleep duration in the pentobarbitone-induced sleeping time. Data are presented as group mean ± SEM. Significantly different from control: **P*<0.05, ***P*<0.01. ****P*<0.001

3.2 ANXIETY SCREENING IN MICE

3.2.1 Open field test

Figures 6-7 represent the effect of acute administration of MAE (30-300 mg kg^{-1} *p.o.*), the anxiolytic drug diazepam (0.1-1.0 mg kg^{-1}, *p.o.*) and the anxiogenic agent caffeine (10-100 mg kg^{-1}, *p.o.*) on mice behaviours in the open field test.

A two-way ANOVA on all treated groups revealed no significant difference in both the number and duration entries into the various zones compared to saline control. However MAE treated mice exhibited anxiolytic-like activity by significantly increasing the percentage number of entries ($F_{3, 21}$=4.158, *P*=0.0185) and percentage time spent ($F_{3, 21}$=3.802, *P*=0.0254) in the central zone. Treatment of mice with diazepam, produced effects that were similar to those produced by MAE. Diazepam significantly increased the number of entries ($F_{3, 24}$=6.862, *P*=0.0017) but not the time spent ($F_{3, 24}$=0.9577, *P*=0.4287) in the central arena. On the other hand the anxiogenic agent, caffeine, was able to significantly reduce the total time spent ($F_{3, 23}$=2.754, *P*=0.0656) in the central arena but not the frequency of entry.



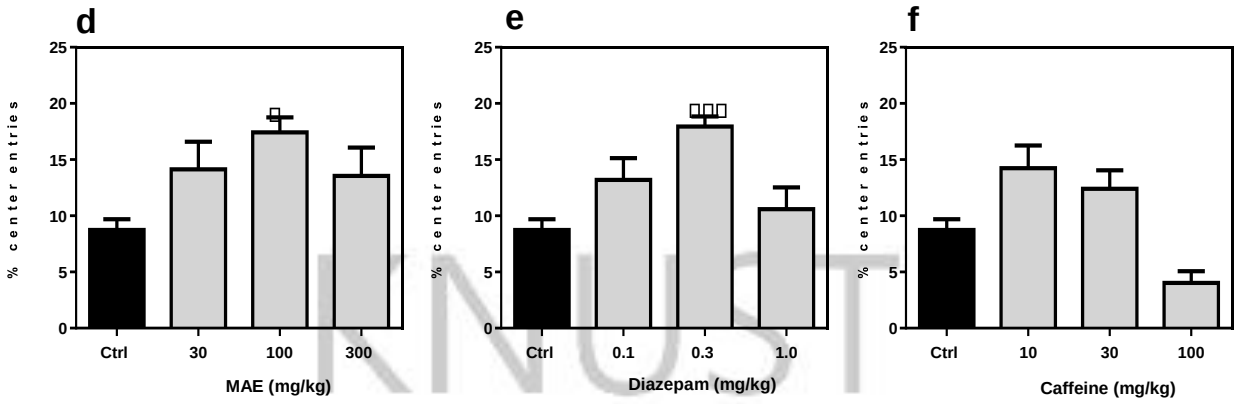


Figure 6: Effects of acute MAE (30-300 mg kg^{-1}), diazepam (0.1-1.0 mg kg^{-1}) and caffeine (10-100 mg kg^{-1}) treatment on the number of zonal entries (a, b, c) and percentage entries into central zone (d, e, f) in the open field test. .Data are presented as group mean ± SEM. Significantly different from control: ***P*<0.01, ****P*<0.001. (One-way ANOVA followed by Sidak *post hoc* test).

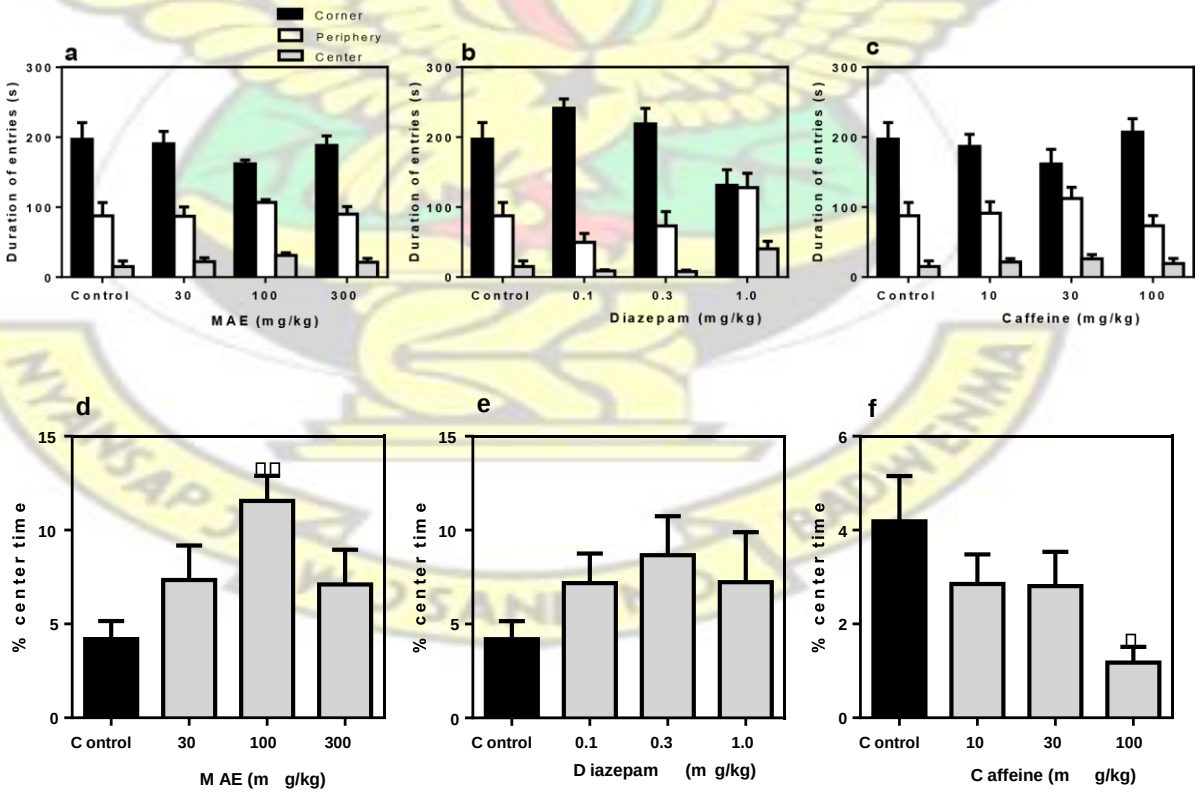
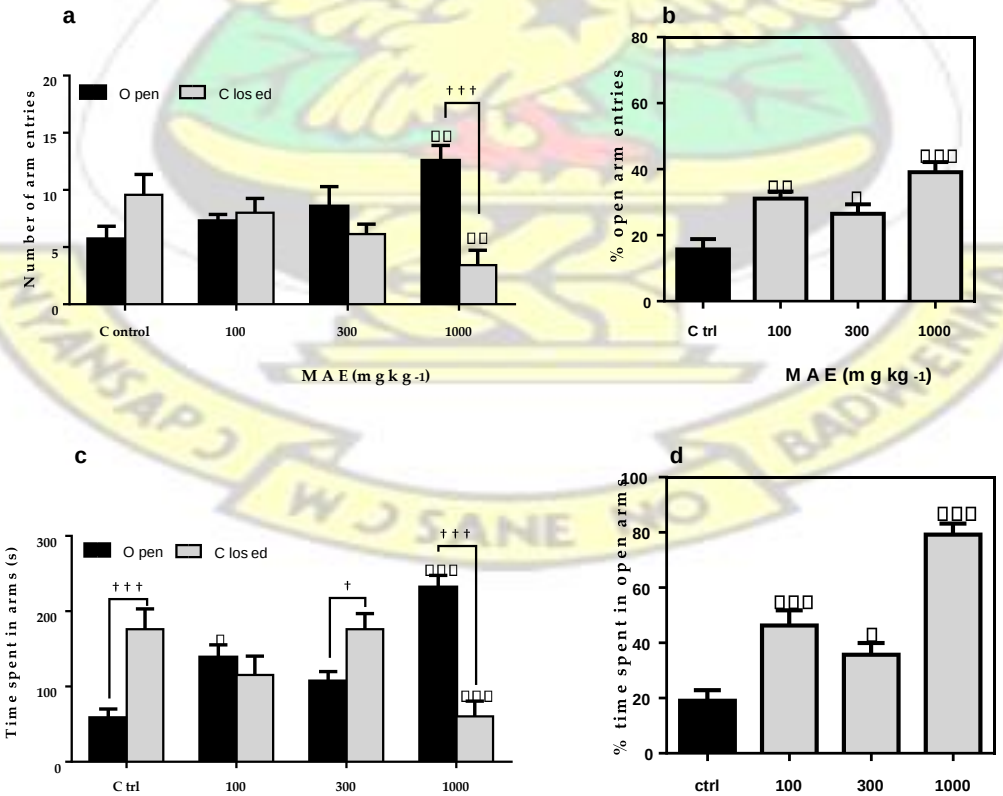


Figure 7: Effects of acute MAE (30-300 mg kg^{-1}), diazepam (0.1-1.0 mg kg^{-1}) and caffeine (10-100 mg kg^{-1}) treatment on the total time spent in the zones (a, b, c) and percentage time in central zone (d, e, f) in the open field test. Data are presented as group mean ± SEM. Significantly different from control: **P*<0.05, ***P*<0.01. (One-way ANOVA followed by Sidak *post hoc* test).

3.2.2 Elevated plus maze

Testing in the elevated plus maze after acute MAE administration significantly favoured anxiolytic parameters as shown in Fig 8.0. A two-way ANOVA (*closed* ×*open arms*) revealed a significant increase in number of open arm entries and a decrease in closed arm entries ($F_{3, 48}$ =9.22 *P*<0.0001) as well as significant increase in time spent in the open arms ($F_{3, 48}$ =21.34 *P*<0.0001). There was also a significant increase in % open arm entries ($F_{3, 24}$ =12.10 *P*<0.0001) and % time spent in the open arms ($F_{3, 24}$ =32.58 *P*<0.0001) at all tested doses.



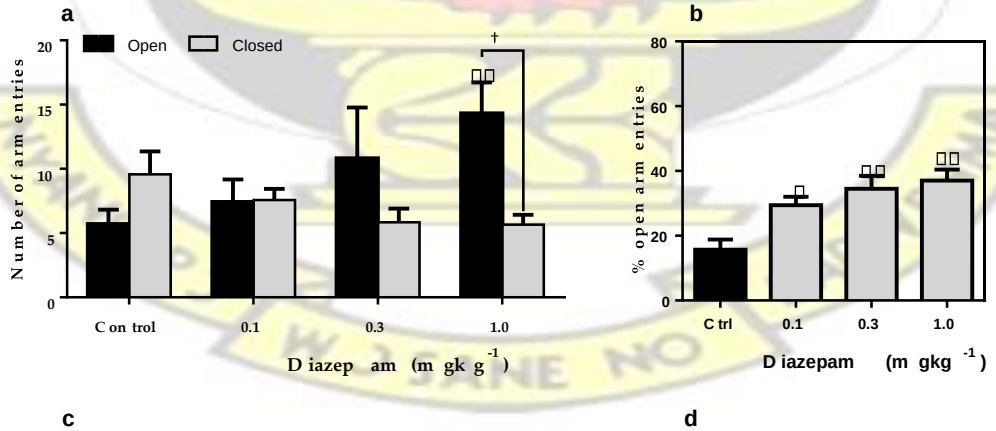
A E (m g kg -1)

M A E (m g kg -1) M

Figure 8: Effects of MAE (100-1000 mg kg^{-1}) on mice behaviour in the EPM test, over a 5 min test period. (a, c) Number of entries and time spent in open and closed arms. (b, c) Percentage open arm entries and percentage time spent in open arms. Data are presented as group mean ± SEM. Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.01 (one-way ANOVA followed Sidak *post hoc* test) and significant difference when open arm and closed arm were compared: †*P*<0.05, †††*P*<0.0001 (twoway repeated measures ANOVA followed by Bonferroni's *post hoc* test).

A two-way ANOVA (*closed* × *open arms*) revealed a significant increase in number of open arm entries and a decrease in closed arm entries ($F_{3, 44}$ =4.21 *P*<0.0001) as well as significant increase in time spent in the open arms ($F_{3, 44}$ = 11.57 *P*<0.0001) after acute diazepam (0.1-1.0 mg kg^{-1}) treatment. There was also a significant increase in % open arm entries ($F_{3, 22}$ =8.68 *P*=0.0005) and % time spent in the open arms ($F_{3, 22}$ =9.09

P<0.0004).



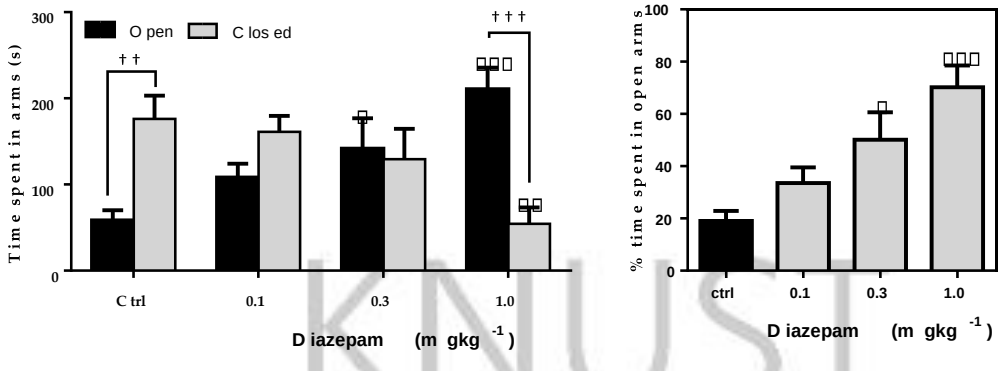
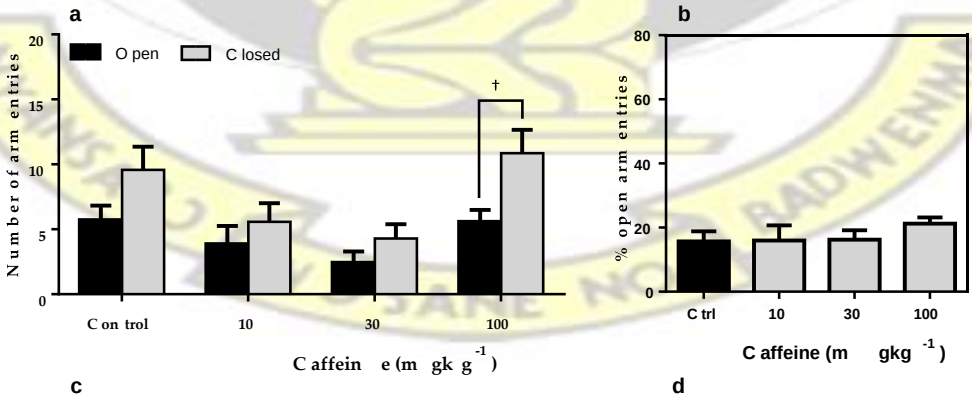


Figure 9: Effects of diazepam (0.1-1.0 mg kg^{-1}) on mice behaviour in the EPM test, over a 5 min test period. (a, c) Number of entries and time spent in open and closed arms. (b, d) Percentage open arm entries and percentage time spent in open arms. Data are presented as group mean ± SEM. Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.01 (one-way ANOVA followed Sidak *post hoc* test) and significant difference when open arm and closed arm were compared: †*P*<0.05, ††*P*<0.001, †††*P*<0.0001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

Caffeine administration did not significantly alter % time spent in open arm ($F_{3, 24}$ =1.50 p=0.2392) and % open arm entries ($F_{3, 24}$ =0.63 P=0.6021). A two-way ANOVA also did not reveal significant differences between number of arm entries ($F_{3, 48}$ =0.81 P=0.4958) and time spent in arms ($F_{3, 48}$ =1.84 P=0.152).when compared to the solvent control.



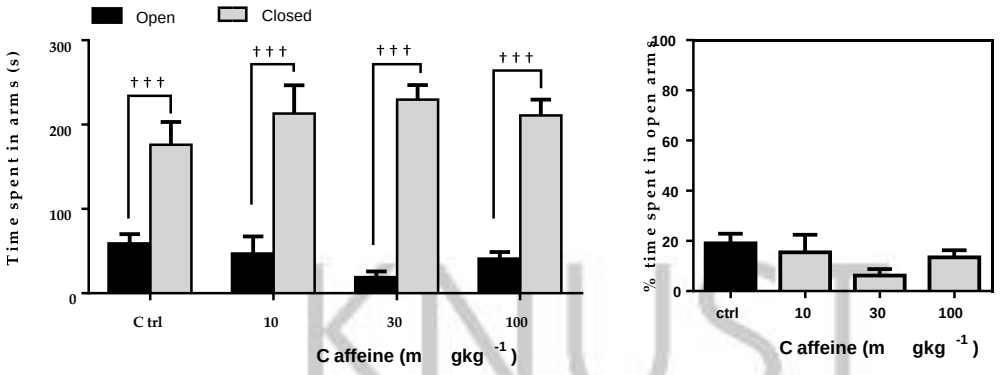


Figure 10: Effects of Caffeine (10-100 mg kg^{-1}) on mice behaviour in the EPM test, over a 5 min test period. (a, c) Number of entries and time spent in open and closed arms. (b, d) Percentage open arm entries and percentage time spent in open arms. Data are presented as group mean ± SEM. Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.01 (one-way ANOVA followed Sidak *post hoc* test) and significant difference when open arm and closed arm were compared: †*P*<0.05, †††*P*<0.0001 (twoway repeated measures ANOVA followed by Bonferroni's *post hoc* test).

There was a significant decrease ($F_{3, 24}$ =6.07 p=0.0032) in % protected head-dips (PHDs) duration only at 1000 mg kg^{-1} of MAE and a significant decrease ($F_{3, 24}$ =13.30 *P*<0.0001) of % PHDs frequency at all tested doses. A two-way ANOVA (*protected* × *unprotected*) revealed a significant increase in the number ($F_{3, 48}$ =14.81 *P*<0.0001) and duration ($F_{3, 48}$ =8.50 *P*=0.0001) of UHDs. Also, there were significant decreases in the number but not the duration of protected head dips.

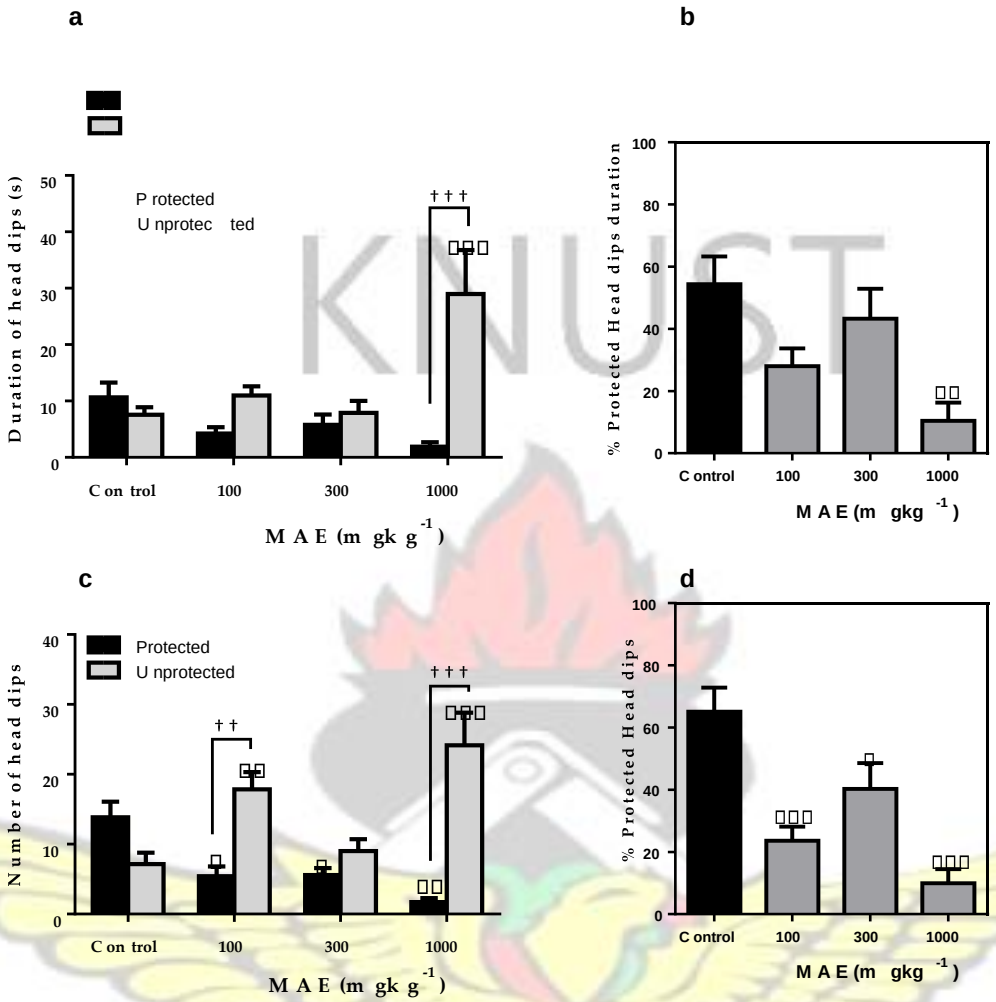


Figure 11: Effects of MAE (100-1000 mg kg^{-1}) on risk assessment behaviour (frequency and duration of head dips) in the EPM. (a, c) Duration and number of unprotected and protected head dips. (b, d) Percentage protected head dips duration and percentage protected head dips frequency. Data are presented as group mean ± SEM. Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.01 (one-way ANOVA followed Sidak *post hoc* test) and significant difference when open arm and closed arm were compared: ††*P*<0.01, †††*P*<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

There was a significant decrease ($F_{3, 22}$ =6.64 *P*=0.0023) in % protected head-dips (PHDs) duration and % PHDs frequency ($F_{3, 22}$ =10.43 *P*=0.0002) only at 1.0 mg kg^{-1} of diazepam. A two-way ANOVA (*protected* × *unprotected*) revealed a significant increase in the number ($F_{3, 44}$ =9.26 *P*<0.0001) and duration ($F_{3, 44}$ =6.86 *P*=0.0007)

of UHDs. Also, there were significant decreases in the number and duration of protected head dips at 1.0 mg kg^{-1} of diazepam.

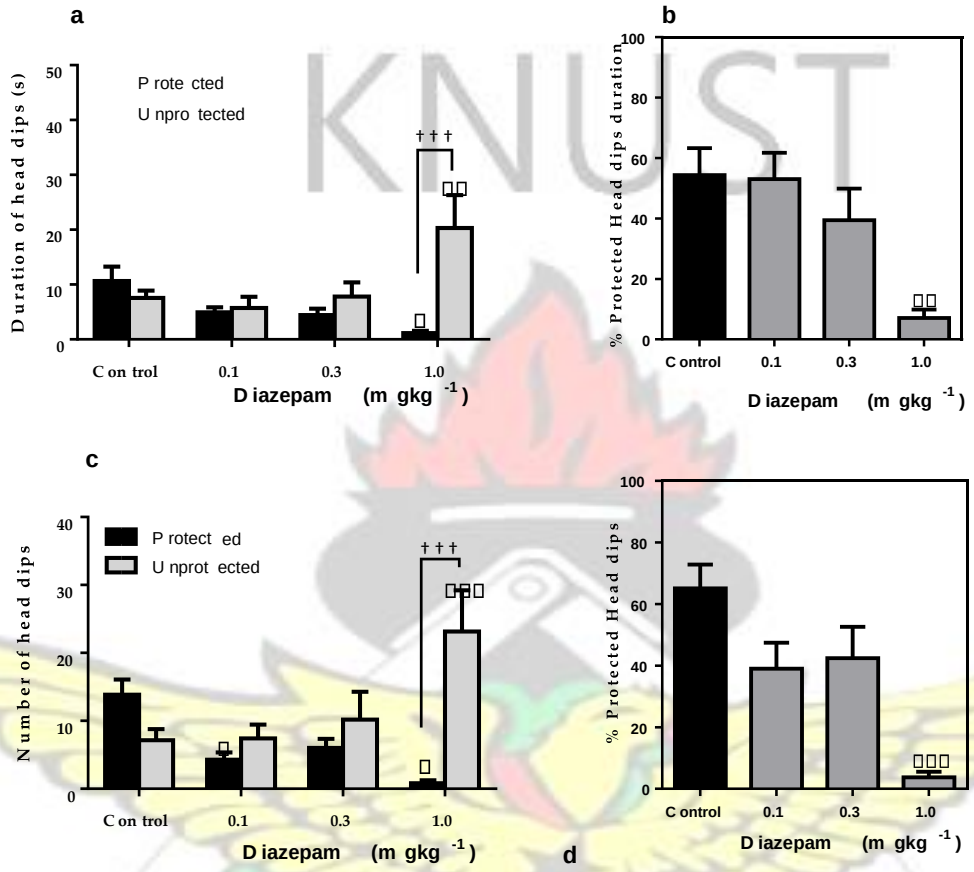


Figure 12: Effects of diazepam (0.1-1.0 mg kg^{-1}) on risk assessment behaviour (frequency and duration of head dips) in the EPM. (a, c) Duration and number of unprotected and protected head dips. (b, d) Percentage protected head dips duration and percentage protected head dips frequency. Data are presented as group mean ± SEM. Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.01 (one-way ANOVA followed Sidak *post hoc* test) and significant difference when open arm and closed arm were compared: †††*P*<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

Caffeine administration did not significantly increase ($F_{3, 23}$ =0.47 *P*=0.7066) % protected head-dips (PHDs) duration and % PHDs frequency ($F_{3, 22}$ =1.02 *P*=0.4030)

at all tested does. A two-way ANOVA (*protected* × *unprotected*) revealed a no significant decrease in the number ($F_{3, 48}$ =1.98 P=0.1296) of PHDs at all tested doses.

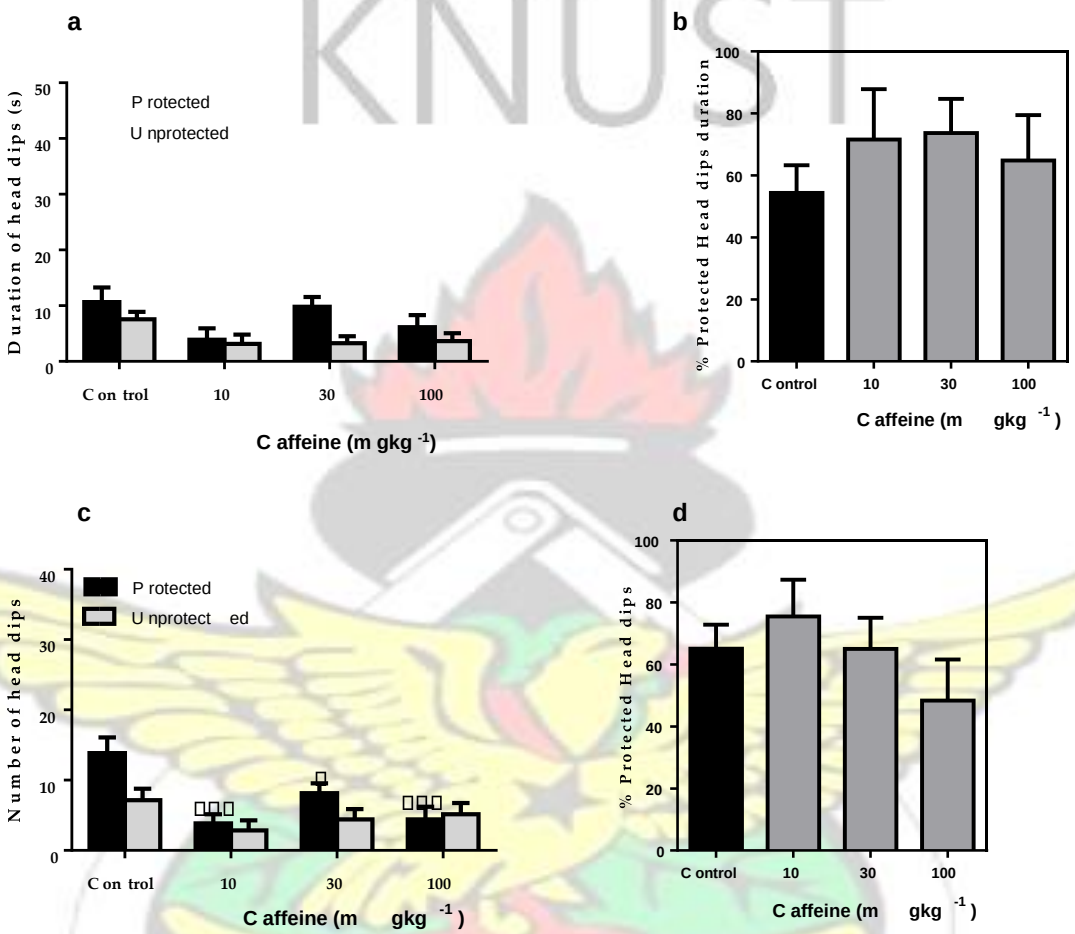


Figure 13: Effects of Caffeine (10-100 mg kg^{-1}) on risk assessment behaviour (frequency and duration of head dips) in the EPM. (a, c) Duration and number of unprotected and protected head dips. (b, d) Percentage protected head dips duration and percentage protected head dips frequency. Data are presented as group mean ± SEM. Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.01 (one-way ANOVA followed Sidak *post hoc* test) and significant difference when open arm and closed arm were compared (two-way repeated measures ANOVA followed by Bonferroni's *post ho* test *c*).

The duration of protected and unprotected SAPs were significantly different

(P<0.0001) in the control group. The duration ($F_{3, 24}$ =15.87 P<0.0001) and frequency ($F_{3, 24}$ =8.06 P=0.0007) of SAP for the 5 minutes test period were significantly decreased at 100-1000 mg kg^{-1} and 1000 mg kg^{-1} of MAE respectively. A two-way

ANOVA (*protected* × *unprotected*) revealed a significant increase in the number ($F_{3, 48}$ =9.27 P<0.0001) and duration ($F_{3, 48}$ =12.25 P=0.0001) of USAPs.

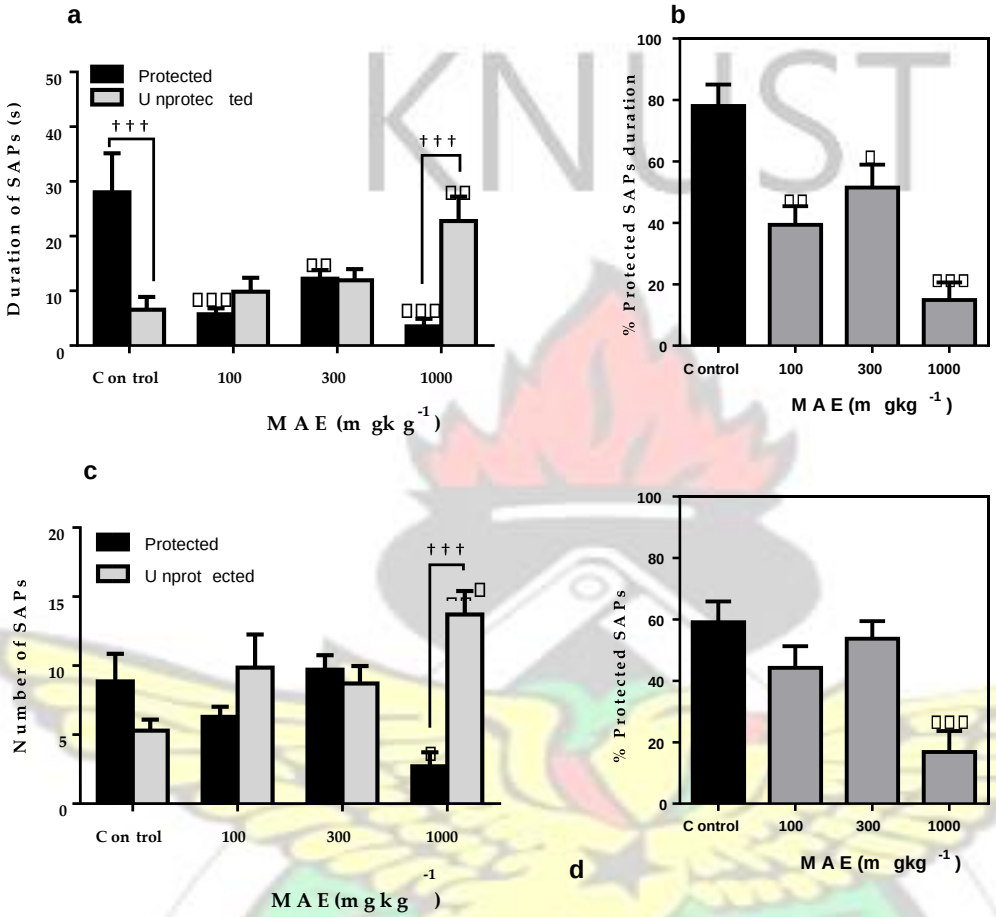


Figure 14: Effects of MAE (100-1000 mg kg^{-1}) on risk assessment behaviour (frequency and duration of SAPs) in the EPM. (a, c) Duration and number of unprotected and protected SAP (b, d) Percentage protected head dips duration and percentage protected SAP frequency. Data are presented as group mean ± SEM. Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.01 (one-way ANOVA followed Sidak *post hoc* test) and significant difference when open arm and closed arm were compared: †††*P*<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

After diazepam treatment the duration ($F_{3, 22}$ =7.94 P=0.0009) and frequency ($F_{3, 24}$ =8.06 P=0.0192) of SAP, for the 5 minutes test period, were significantly decreased. A two-way ANOVA (*protected* × *unprotected*) revealed a significant decrease in duration

of USAPs ($F_{3, 44}$ =6.76 P=0.0008). However there was no significant change in number of protected and unprotected SAPs ($F_{3, 44}$ =2.59 P=0.0641).

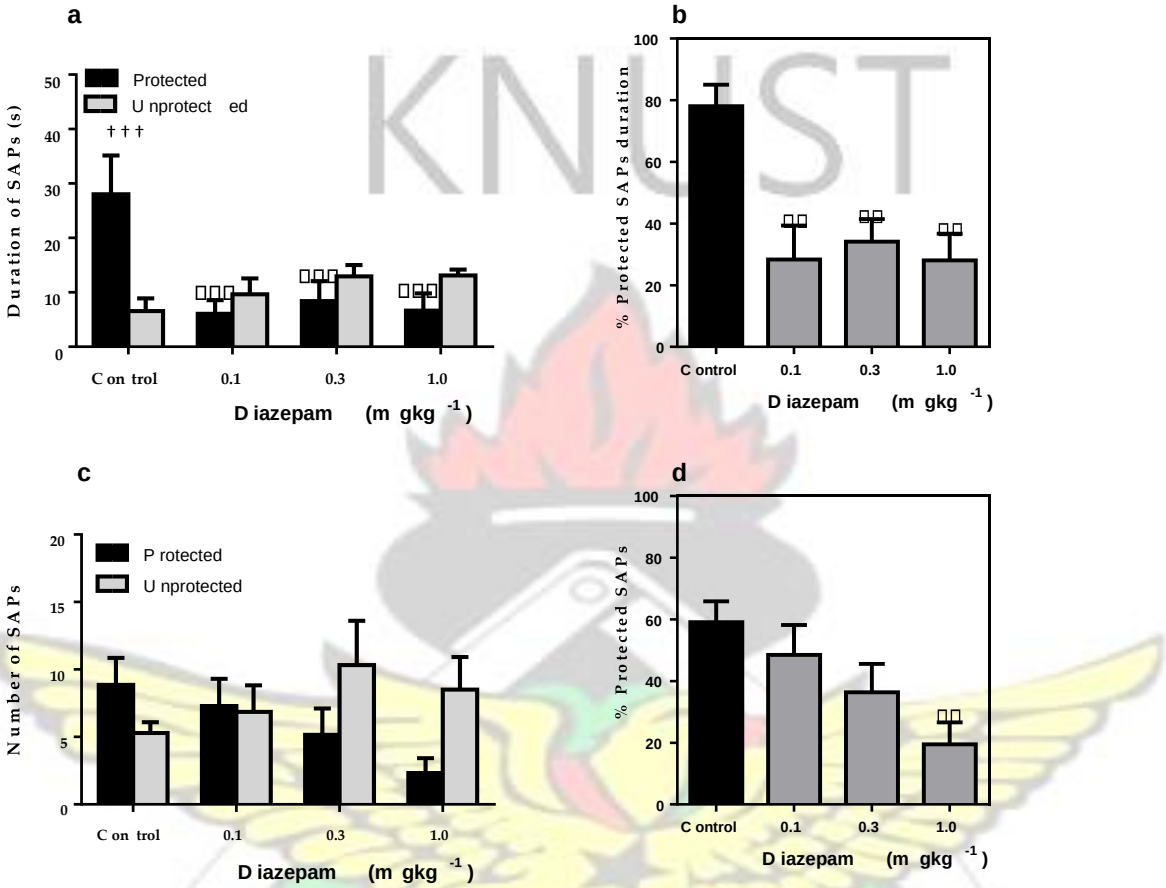


Figure 15: Effects of diazepam (0.1-1.0 mg kg^{-1}) on risk assessment behaviour (frequency and duration of SAPs) in the EPM. (a, c) Duration and number of unprotected and protected SAP. (b, d) Percentage protected head dips duration and percentage protected SAP frequency. Data are presented as group mean ± SEM. Significantly different from control: *P<0.05, **P<0.01, ***P<0.01 (one-way ANOVA followed Sidak *post hoc* test) and significant difference when open arm and closed arm were compared: †††P<0.001 (two-way repeated measures ANOVA followed by

Bonferroni's *post hoc* test).

After caffeine treatment the % duration of PSAP ($F_{3, 22}$ =1.37 P=0.2773) was not significantly altered at all doses. However there was a significant increase in % frequency ($F_{3, 23}$ =3.76 P=0.0247) only at 10 mg kg^{-1}. A two-way ANOVA (*protected* × *unprotected*) revealed a significant decrease in duration of USAPs ($F_{3, 48}$ =1.35 P=0.0138) at 10 and 100 mg kg^{-1}. However there was no significant change in number of protected and unprotected SAPs ($F_{3, 44}$ =0.57 P=0.6374).

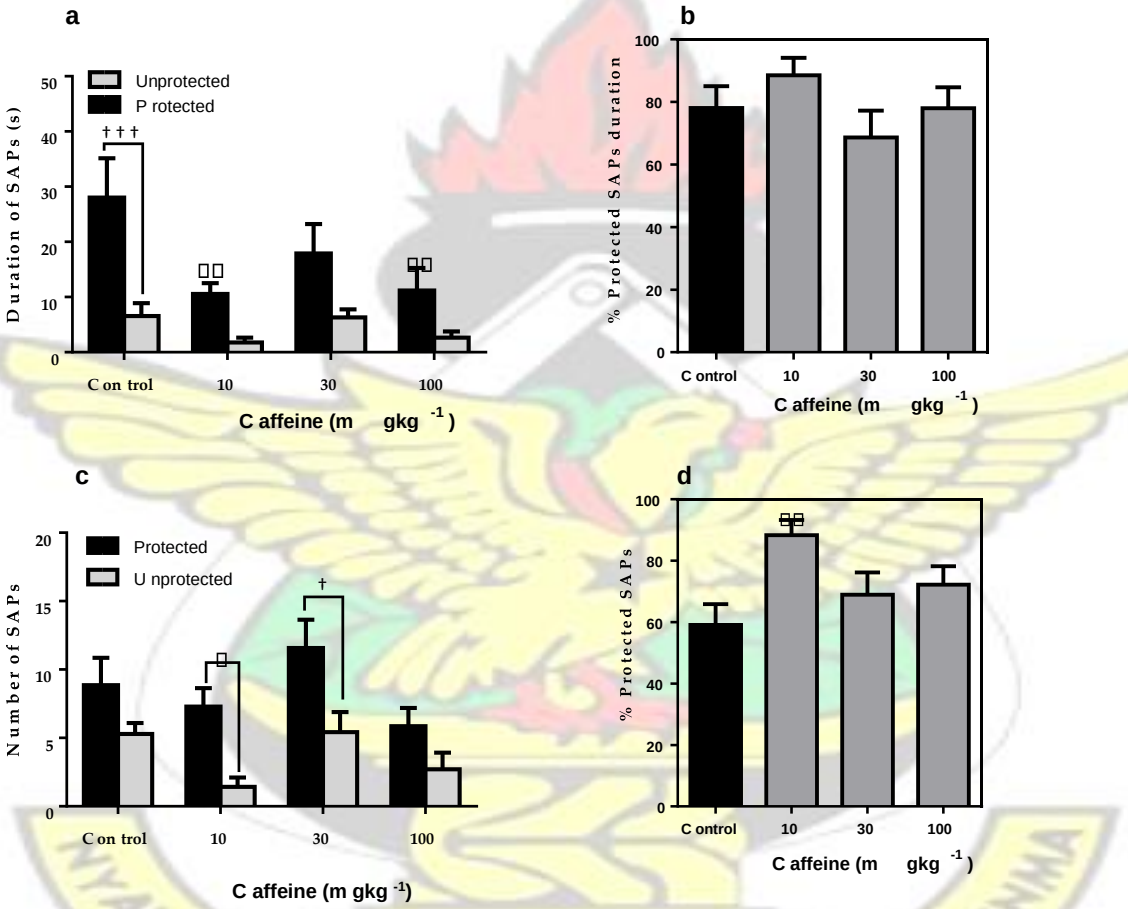


Figure 16: Effects of Caffeine (10-100 mg kg^{-1}) on risk assessment behaviour (frequency and duration of SAPs) in the EPM. (a, c) Duration and number of unprotected and protected SAP. (b, d) Percentage protected head dips duration and (d) percentage protected SAP frequency. Data are presented as group mean ± SEM. Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.01 (one-way ANOVA followed Sidak *post hoc* test) and significant difference when open arm and closed arm were compared: †*P*<0.05, †††*P*<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

3.2.3 Suok test

Typical behaviours assessed include: horizontal locomotion, side-directed exploration, stretch-attend posture, head-dip, freezing (immobility) and misstep. Acute diazepam or MAE treatment did not significantly alter the duration (MAE= $F_{3, 17}$ = 2.452 P=0.0987, Dzp= $F_{3, 18}$ = 0.7338 P=0.5453), and frequency (MAE= $F_{3, 17}$ = 1.441 P=0.2702, Dzp =

$F_{3, 18}$ = 1.389 P=0.2822) of head dips as compared to the vehicle-treated group. However the duration of side-looks were significantly increased for both diazepam ($F_{3, 17}$ = 2.820 P=0.072) and MAE ($F_{3, 16}$ = 22.4 P<0.0001) treated groups. Interestingly the frequency of side looks was significantly reduced at 0.1-1.0 mg kg^{-1} ($F_{3, 17}$ = 6.228 P=0.0053) for diazepam and 100-300 mg kg^{-1} for MAE ($F_{3, 16}$ = 2.452 P=0.001) treated groups. Although the duration of freezing (MAE= $F_{3, 17}$ = 1.827 P=0.1829, Dzp = $F_{3, 18}$ = 1.220 P=0.3311) was not significantly altered the frequency of freezing bouts was significantly reduced at all dose levels of diazepam ($F_{3, 17}$ = 7.59 P=0.0026) and 300 mg kg^{-1} of MAE ($F_{3, 18}$ = 4.653 P=0.0172). The number of leg-slips which is an indication of the degree of locomotor impairment was not significantly different from the vehiclecontrol when animals were treated with MAE ($F_{3, 18}$ = 1.315 P=0.3003). However there was a significant increase ($F_{3, 17}$ = 7.876 P=0.0019) in number of leg-slips when mice were treated with 1.0 mg kg^{-1} of diazepam.

a **b**

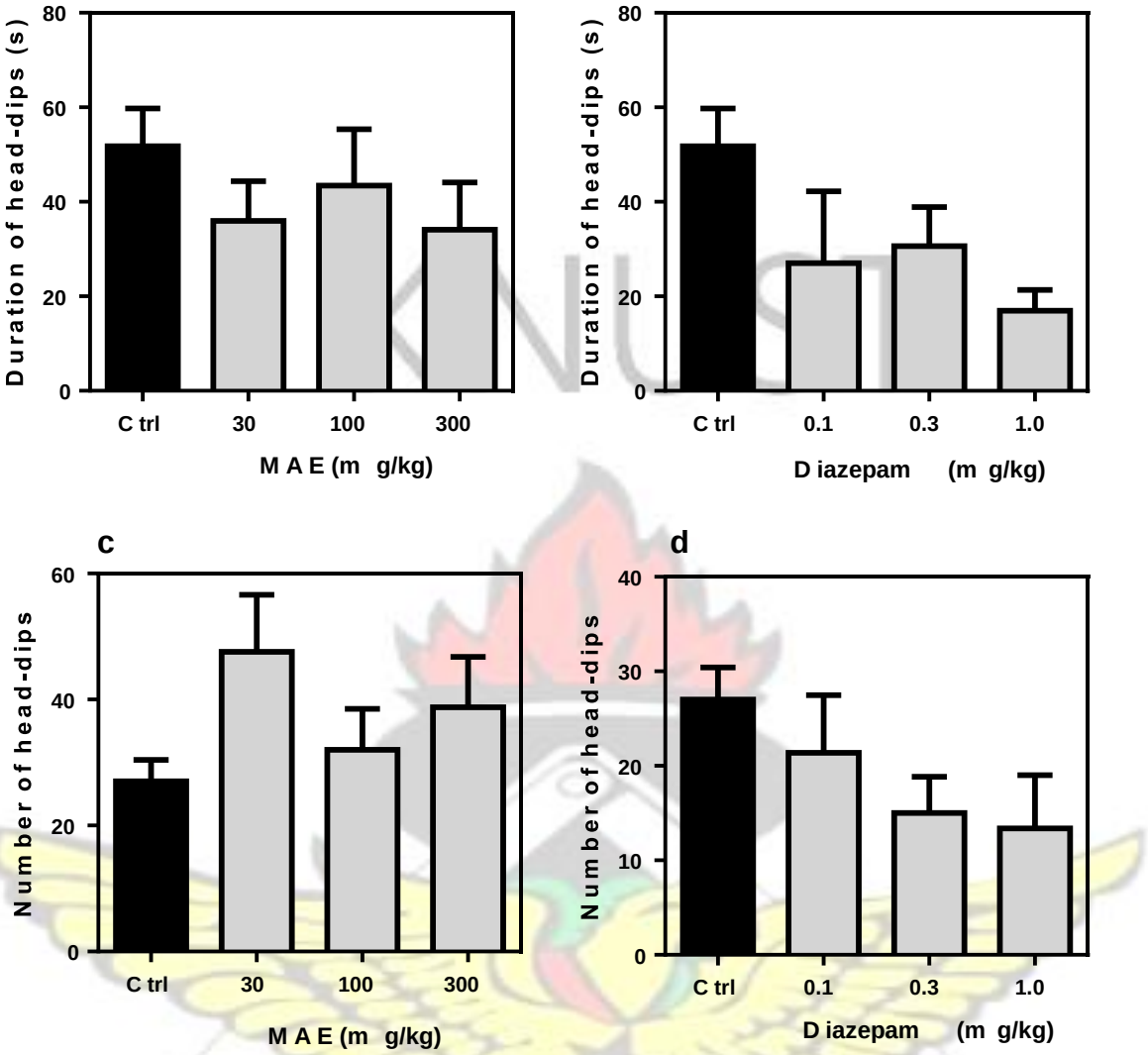


Figure 17: Effects of MAE (30-300 mg kg^{-1}) and diazepam (0.1-1.0 mg kg^{-1}) on duration (a & b) and number of head dips (c & d) over a 5 min test period in the regular Suok test. Data are expressed as group mean ± SEM. Significant difference: compared to control group (one-way ANOVA followed by Sidak *post hoc* test).

a b

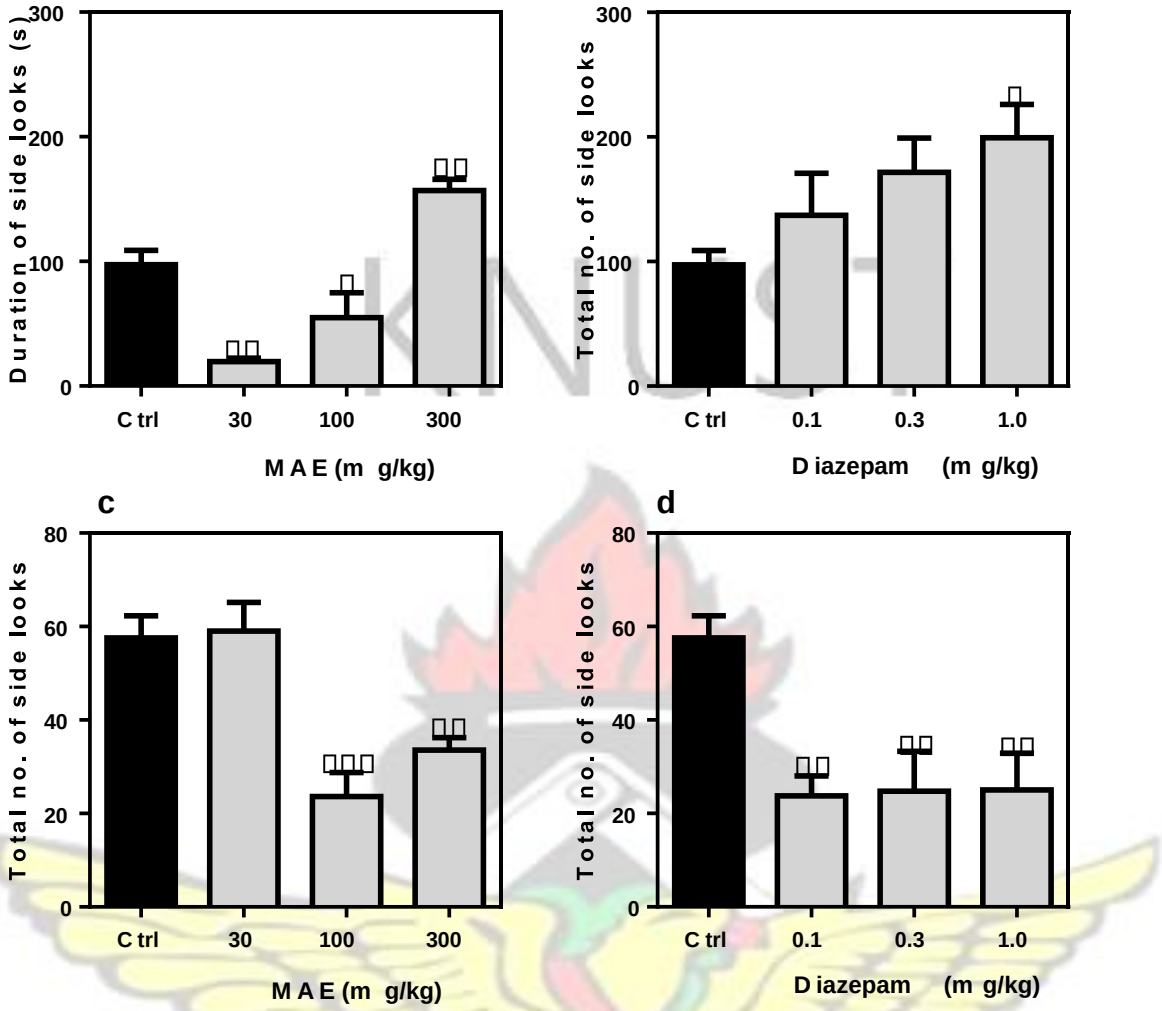


Figure 18: Effects of MAE (30-300 mg kg^{-1}) and diazepam (0.1-1.0 mg kg^{-1}) on duration (a & b) and total number of side looks (c & d) over a 5 min test period in the regular Suok test. Data are expressed as group mean ± SEM. Significant difference: **P*<0.05, ***P*<0.01, ****P*<0.001 compared to control group (one-way ANOVA followed by Sidak *post hoc* test).

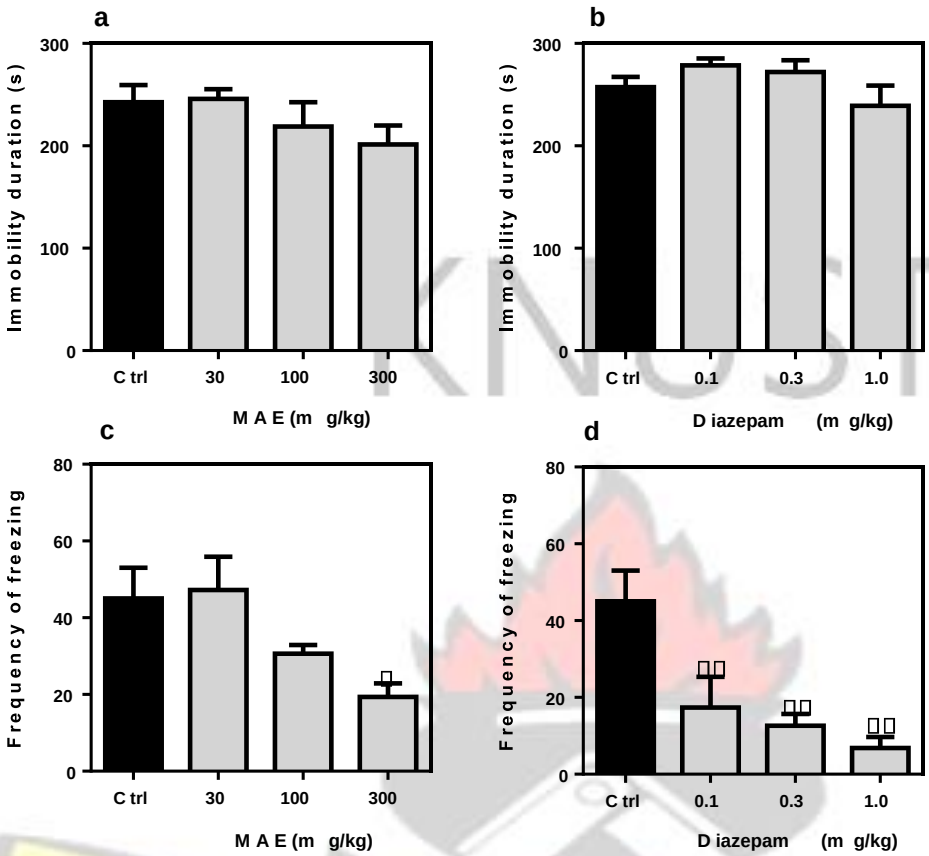


Figure 19: Effects of MAE (30-300 mg kg^{-1}) and diazepam (0.1-1.0 mg kg^{-1}) on duration of immobility (a & b) and number of freezing bouts (c & d) over a 5 min test period in the regular Suok test. Data are expressed as group mean ± SEM. Significant difference: **P*<0.05, ***P*<0.01, ****P*<0.001 compared to control group (one-way ANOVA followed by Sidak *post hoc* test).

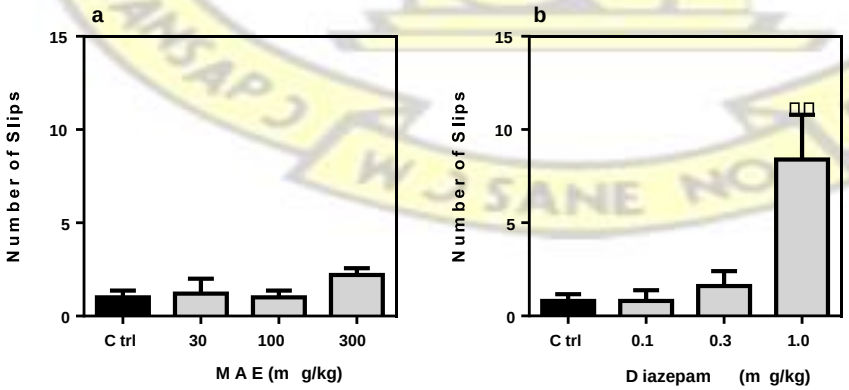


Figure 20: Effects of MAE (30-300 mg kg^{-1}) and diazepam (0.1-1.0 mg kg^{-1}) on the number of leg slips over a 5 min test period in the regular Suok test. Data are expressed as group mean ± SEM. Significant difference: *P<0.05, **P<0.01, ***P<0.001 compared to control group (one-way ANOVA followed by Sidak *post hoc* test).

3.3 ANXIETY SCREENING IN ZEBRAFISH

3.3.1 Sedative and locomotor activity

The effect on locomotor activity after continuous exposure of adult zebrafish to MAE, diazepam and system water was evaluated using the mean velocity and total distance per 120 s bocks for a total period of 30 minutes. Velocity below an arbitrary velocity limit of 1 cm/s ; based on observational studies carried out previously, was taken as an indication of sedative or and locomotor impairment. Based on the preamble above the sedative/locomotor impairment potential of MAE (3 mg mL^{-1}) was significantly reduced after 10 minutes of exposure. The onset for diazepam (100 & 300 µM) induced sedation/locomotor impairment was relatively faster, occurring at ~2-4 minutes after immersion. Similar responses were also obtained with regards to the path length (cm).

A one-way ANOVA of the AUCs derived from the velocity and path length curves revealed a significant reduction; compared to vehicle-control, in average velocity and path length after diazepam (100 and 300 µM) treatment. However, a similar analysis did not reveal any significant difference in velocity/distance after MAE (0.03-0.3 mg mL^{-1}) treatment.

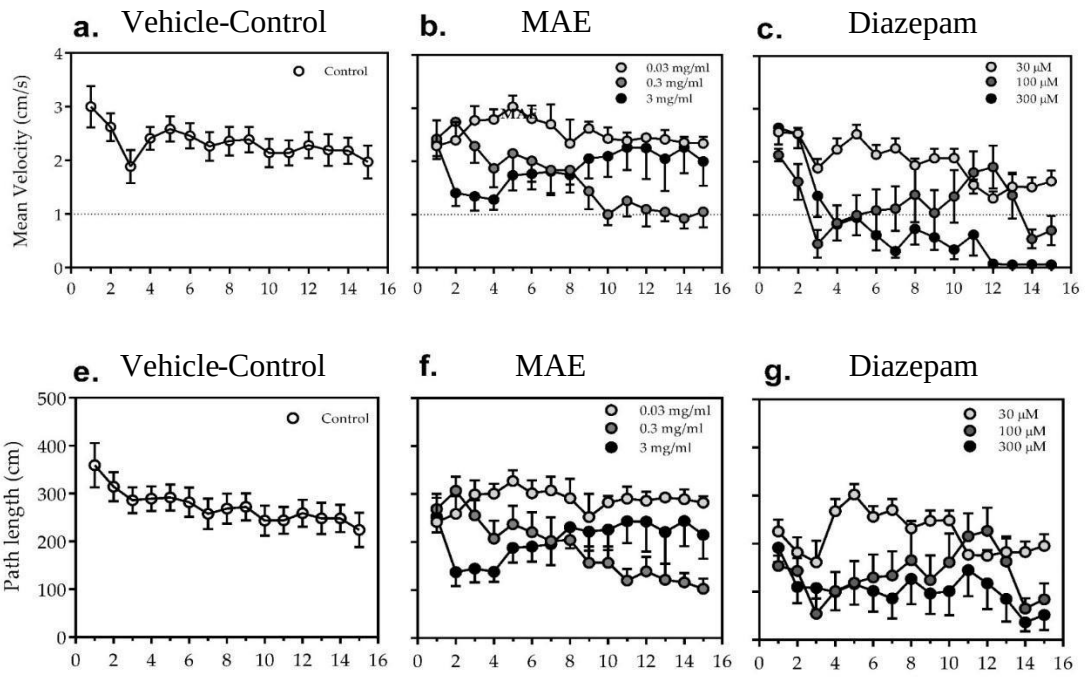


Figure 21: Effects of MAE (0.03 -3 mg mL^{-1}) and diazepam (30-300 µM) on locomotor activity (a-c)- Velocity and (e-g) distance travelled in adult zebrafish. X- axis represents activity per 2 min for 30 min (15 data points).

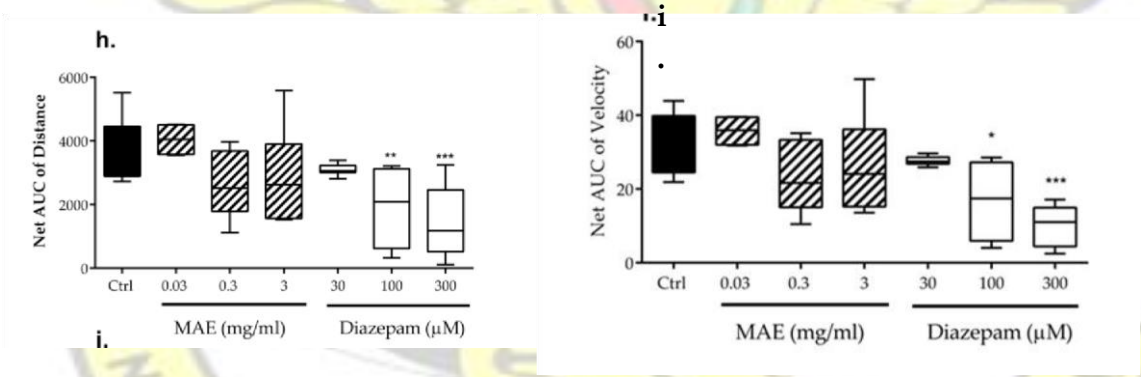


Figure 22: Box and whisker plot of net AUCs derived from the time course curves of zebrafish activity, (h) Total distance (i) mean velocity.

3.3.2 Novel tank test

The innate bottom dwelling behaviour were significantly reversed by acute administration of MAE ($F_{3, 24}$ = 11.43 P<0.0001), fluoxetine ($F_{3, 18}$ = 3.649 P=0.0324), and desipramine ($F_{3, 18}$ = 15.84 P<0.0001). However selected doses of diazepam

exhibited a sedative rather than anxiolytic effect. A significant ($F_{3, 18}$ =4.297 P=0.0188) reversal of anxiolysis due to locomotor /sedative effect was observed at 30 µg/l (Fig 23). The latency to the upper section of the novel tank was significantly reduced after acute administration of MAE ($F_{3, 23}$ = 24.45 P<0.0001), fluoxetine ($F_{3, 18}$ = 3.925 P=0.0256), and desipramine ($F_{3, 18}$ = 16.09 P<0.0001) only. Diazepam treatment, however, did not significantly ($F_{3, 18}$ = 2.448 P<0.0970) alter the latency to upper section at all dose levels.

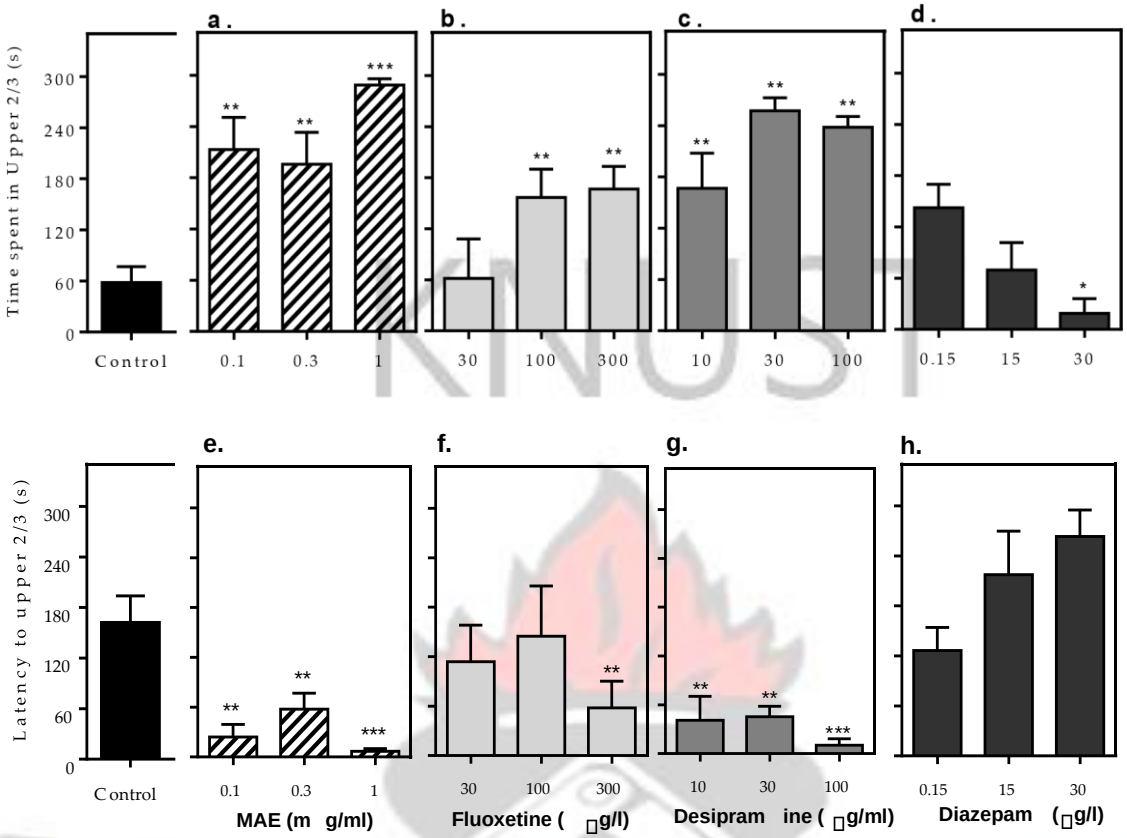


Figure 23: Effects of acute administration of MAE (0.1, 0.3, 1.0 mg mL^{-1}), fluoxetine (30, 100, 300 µg L^{-1}), desipramine (10, 30,100 µg mL^{-1}), diazepam (0.15, 15, 30 µg L^{-1}) on (a-d) time spent in upper 2/3 and (e-h) latency to upper 2/3 entry in the novel tank test. Data are expressed as group mean ± SEM. Significant difference: **P*<0.05, ***P*<0.01, ****P*<0.001 compared to control group (one-way ANOVA followed by Sidak *post hoc* test).

3.3.3 Light/Dark paradigm

The preference to light was assessed based on the time spent in the lit section of the light dark paradigm. Time spent in the light compartment was significantly increased after acute MAE ($F_{3, 21}$ = 4.485 *P*<0.0139) and desipramine ($F_{3, 16}$ = 3.748 *P*=0.0326) treatment. The time spent in the light section after acute treatment of fluoxetine ($F_{3, 16}$ = 0.9536 *P*=0.4384) and diazepam ($F_{3, 15}$ = 2.021 *P*=0.1543) was not significantly different from the vehicle control.

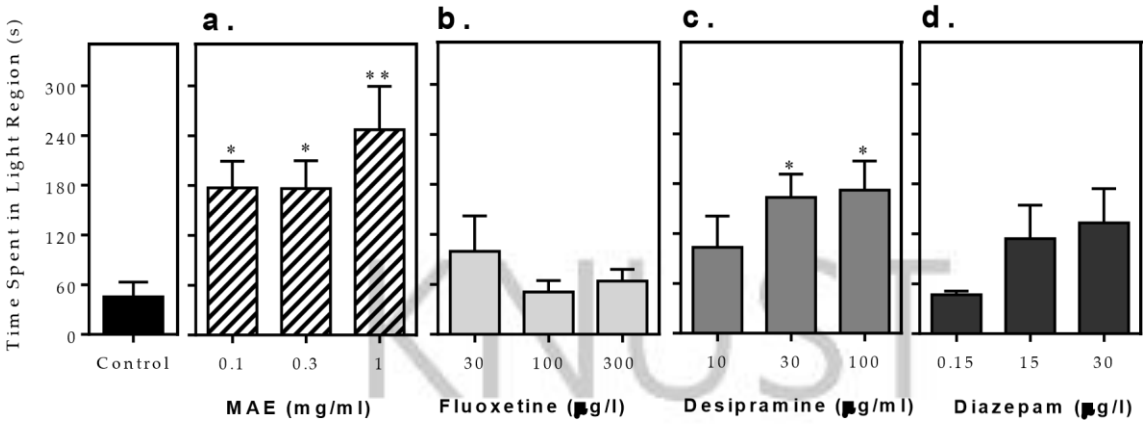


Figure 24: Effects of acute administration of MAE (0.1, 0.3, 1.0 mg mL^{-1}), fluoxetine (30, 100, 300 µg L^{-1}), desipramine (10, 30,100 µg mL^{-1}), diazepam (0.15, 15, 30 µg L^{-1}) on time spent in the light section of light-dark test. Data are expressed as group mean ± SEM. Significant difference: **P*<0.05, ***P*<0.01, ****P*<0.001 compared to control group (one-way ANOVA followed by Sidak *post hoc* test).

3.4 ETHANOL WITHDRAWAL-INDUCED ANXIETY

Continuous exposure of adult zebrafish to ethanol (0.5 % /) produced anxiolytic-like effect in the light dark and novel tank test. The time spent in the upper section of the novel tank at treatment day 5 and withdrawal day 8 was significantly ($F_{3, 24}$ = 10.23 *P*=0.0002) increased and reduced respectively compared to the control group. However the light-dark test did not reveal any significant difference between control group and withdrawal day 4 or 8 as seen in Fig 25b. Similar to the novel tank test the light-dark test revealed that ethanol exposure produced significant ($F_{3, 24}$ = 18.98 *P*<0.0001) anxiolytic effect after 5 days of treatment.

The novel tank proved to be relatively more sensitive at detecting anxiety induced by ethanol withdrawal as seen in fig 25a. Hence this test was adapted to assess the potential of MAE to reverse the recorded anxiogenic behaviour induced by ethanol withdrawal. Testing revealed a significant ($F_{6, 24}$ = 25.78 *P*<0.0001) increase in the total time spent

in the upper section after treatment with diazepam and all dose levels of MAE as seen in fig 26. Reinstating ethanol after 8 days of withdrawal did not produce a significant change in the time spent in the upper section of the novel tank.

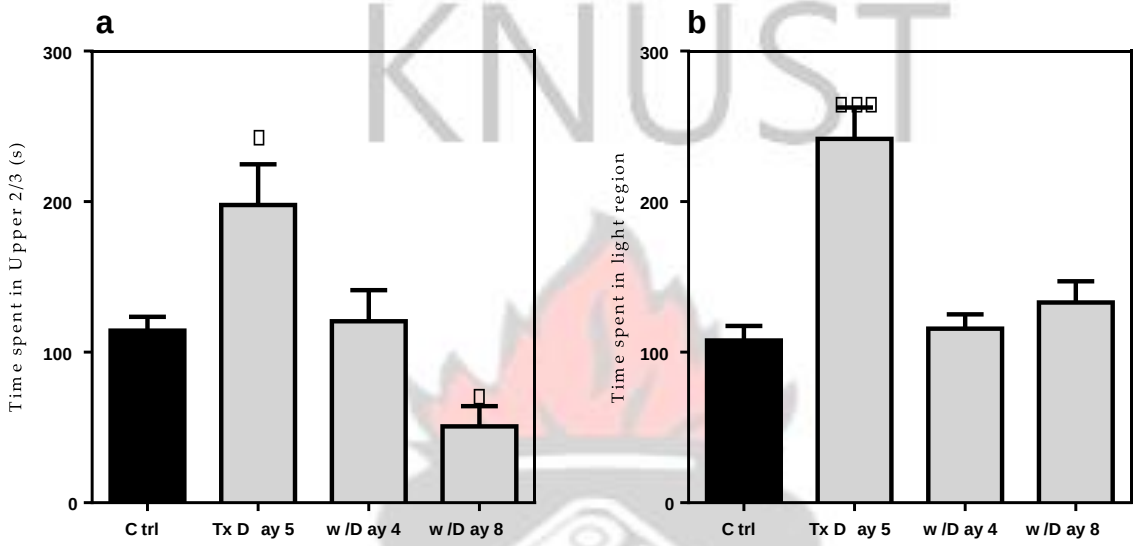


Figure 25: Behaviour of zebrafish in the (a) novel tank and (b) light dark tests during ethanol treatment and withdrawal phase. Data are expressed as group mean ± SEM. Significant difference: **P*<0.05, ****P*<0.001 compared to solvent control group (one-way ANOVA followed by Sidak *post hoc* test).

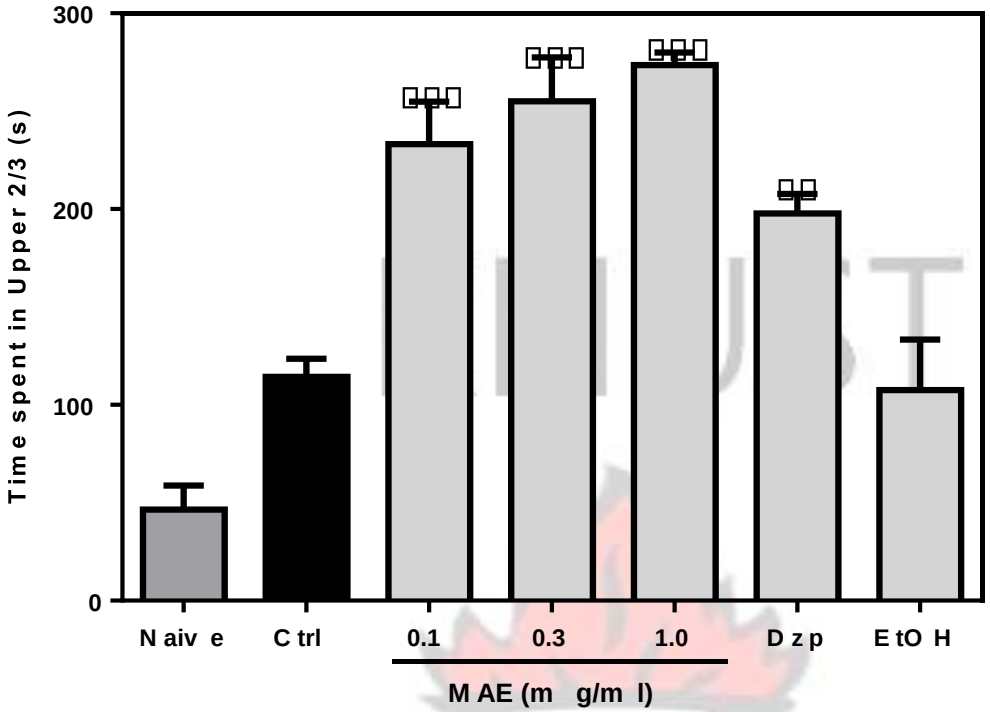
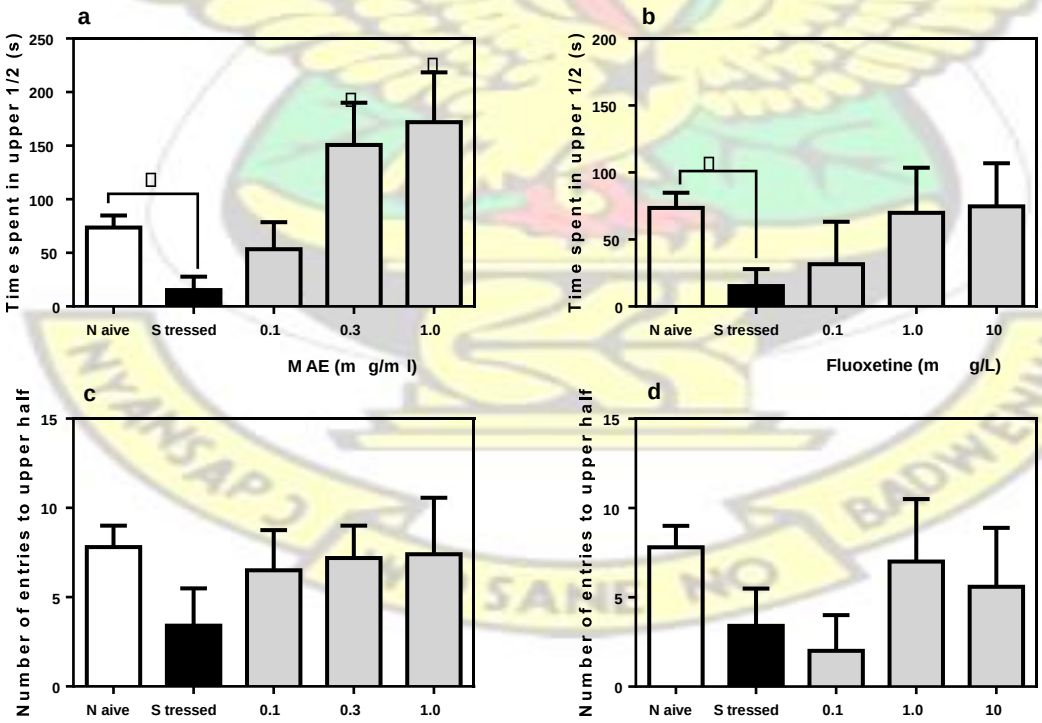


Figure 26: Behaviour of zebrafish in the novel tank test after MAE (0.1-1.0 mg mL^{-1}), diazepam (0.15 µg L^{-1}) or ethanol (0.5 % /) treatment on withdrawal day 8. Data are expressed as group mean ± SEM. Significant difference: ***P*<0.01, ****P*<0.001 compared to treatment naive group (one-way ANOVA followed by Sidak *post hoc* test).

3.5 CHRONIC UNPREDICTABLE STRESS IN ZEBRAFISH

3.5.1 Novel tank

Acute treatment with MAE ($F_{4, 18}$ = 4.136 P<0.0150) unlike fluoxetine ($F_{4, 13}$ = 1.592 P<0.2350) significantly increased the time spent in the upper half of the novel tank, an indicator of anxiolytic effect. However there was no significant ($F_{4, 19}$ = 0.6751 =0.6150, $F_{4, 16}$ = 0.845 P=0.5170) change in the number of upper-half entries for both treatments. A three-day (10 min) daily exposure to MAE or fluoxetine significantly increased the time spent ($F_{4, 16}$ = 14.93 P<0.0001, $F_{4, 19}$ = 6.505 =0.0018) in the upper half entry frequency ($F_{4, 17}$ = 9.778 P=0.0003, $F_{4, 20}$ = 4.136 P<0.0001) as seen in fig 28. Chronic treatment ($F_{4, 18}$ = 14.46 P<0.0001, $F_{4, 21}$ = 9.228 P=0.0002) as opposed to acute treatment ($F_{4, 19}$ = 1.4820 P=0.2470, $F_{4, 16}$= 1.398 P=0.2794) significantly reduced the transition latencies at all doses



M A E (m g/m l) F luoxetine (m g/L)

Figure 27: Effects of acute administration of MAE (0.1, 0.3, 1.0 mg mL^{-1}), fluoxetine (30, 100, 300 µg L^{-1}) on (a & b) time spent and(c & d) number of entries in the upper 1/ 2 of the novel tank. Data are expressed as group mean ± SEM. Significant difference:*P<0.05 compared to stressed group (one-way ANOVA followed by Sidak *post hoc* test).

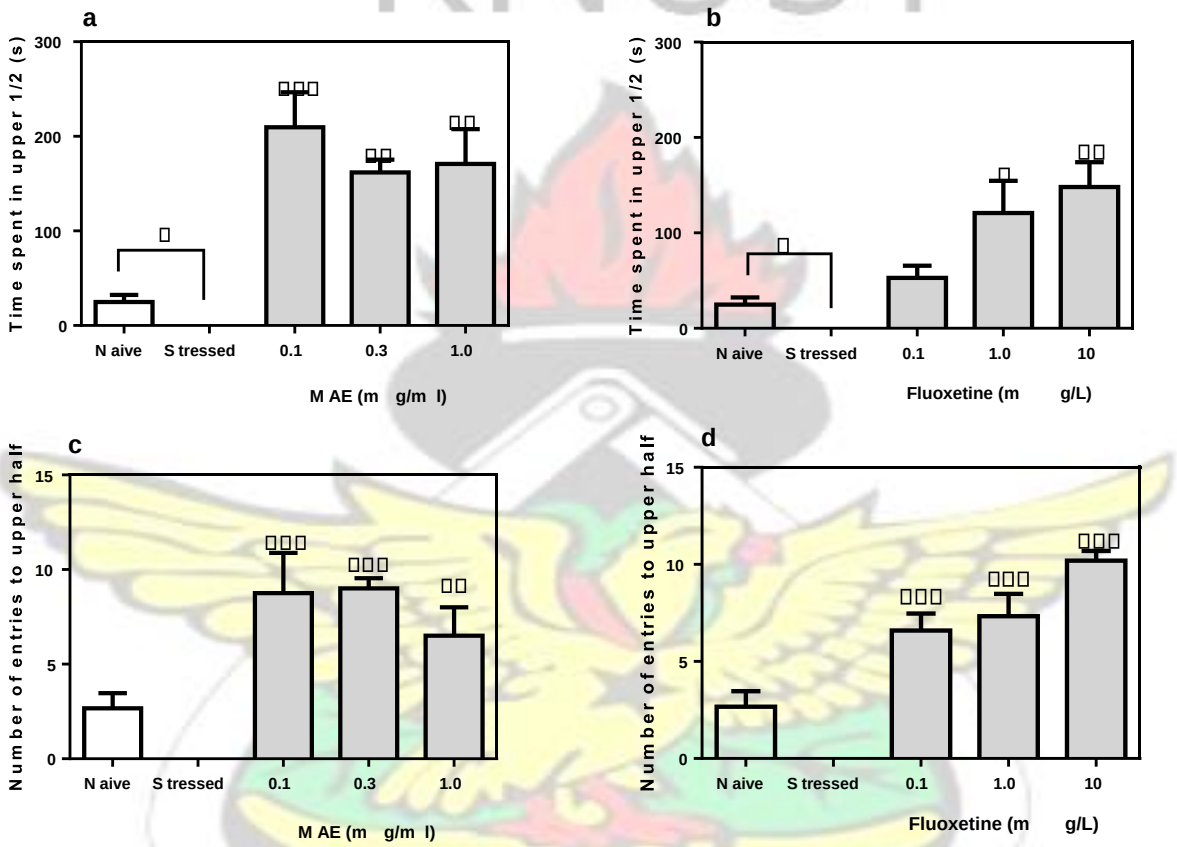


Figure 28 Effects of chronic administration of MAE (0.1, 0.3, 1.0 mg mL^{-1}), Fluoxetine (30, 100, 300 µg L^{-1}) on (a & b) time spent and(c & d) number of entries to the upper 1/ 2 of the novel tank. Data are expressed as group mean ± SEM. Significant difference:

*P<0.05, **P<0.01, ***P<0.001 compared to stressed group (one-way ANOVA followed by Sidak *post hoc* test).

a b

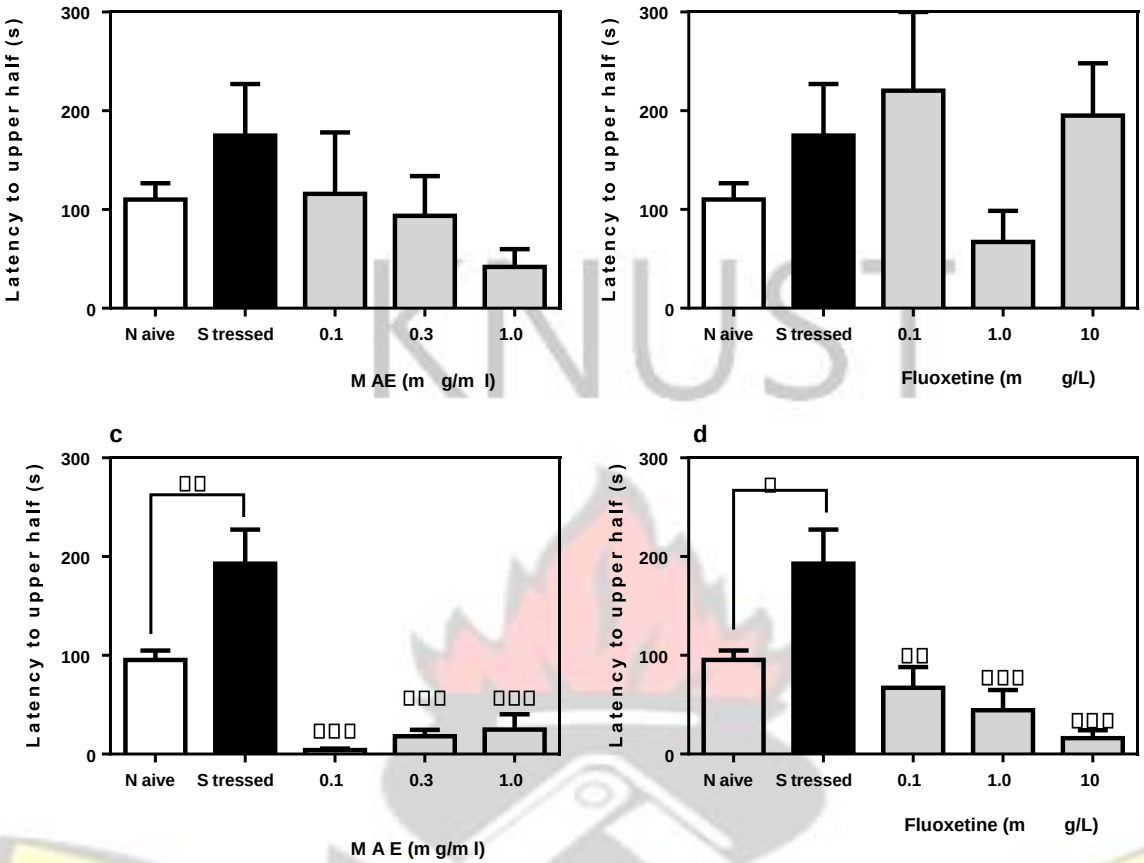


Figure 29: Effects of (a & b) acute and (c& d) chronic administration of MAE (0.1, 0.3, 1.0 mg mL^{-1}) or fluoxetine (30, 100, 300 µg L^{-1}) on latency to upper 1/ 2 of the novel tank. Data are expressed as group mean ± SEM. Significant difference: **P*<0.05, ***P*<0.01, ****P*<0.001 compared to stressed group (one-way ANOVA followed by Sidak *post hoc* test).

3.5.2 Light- dark

Acute treatment with 0.3 mg mL^{-1} MAE ($F_{4, 18}$ = 2.949 *P*=0.0489) unlike fluoxetine significantly increased the time spent in the light region of the light-dark apparatus.

There was also a significant increase ($F_{4, 16}$ = 5.335 P=0.0063) in number of entries into the light region after MAE (0.1 mg mL^{-1}) treatment. However, there was no significant ($F_{4, 19}$ = 0.6751 P=0.6150) change in the number of entries after fluoxetine treatment. A three day (10 min) daily exposure to MAE significantly increased the time spent in the light region ($F_{4, 17}$ = 5.307 P=0.0058) but not fluoxetine ($F_{4, 20}$ = 1.683 P =0.1933).

Chronic MAE administration also increased the frequency of entry into the light region ($F_{4, 17}$ = 3.594 P =0.0268).

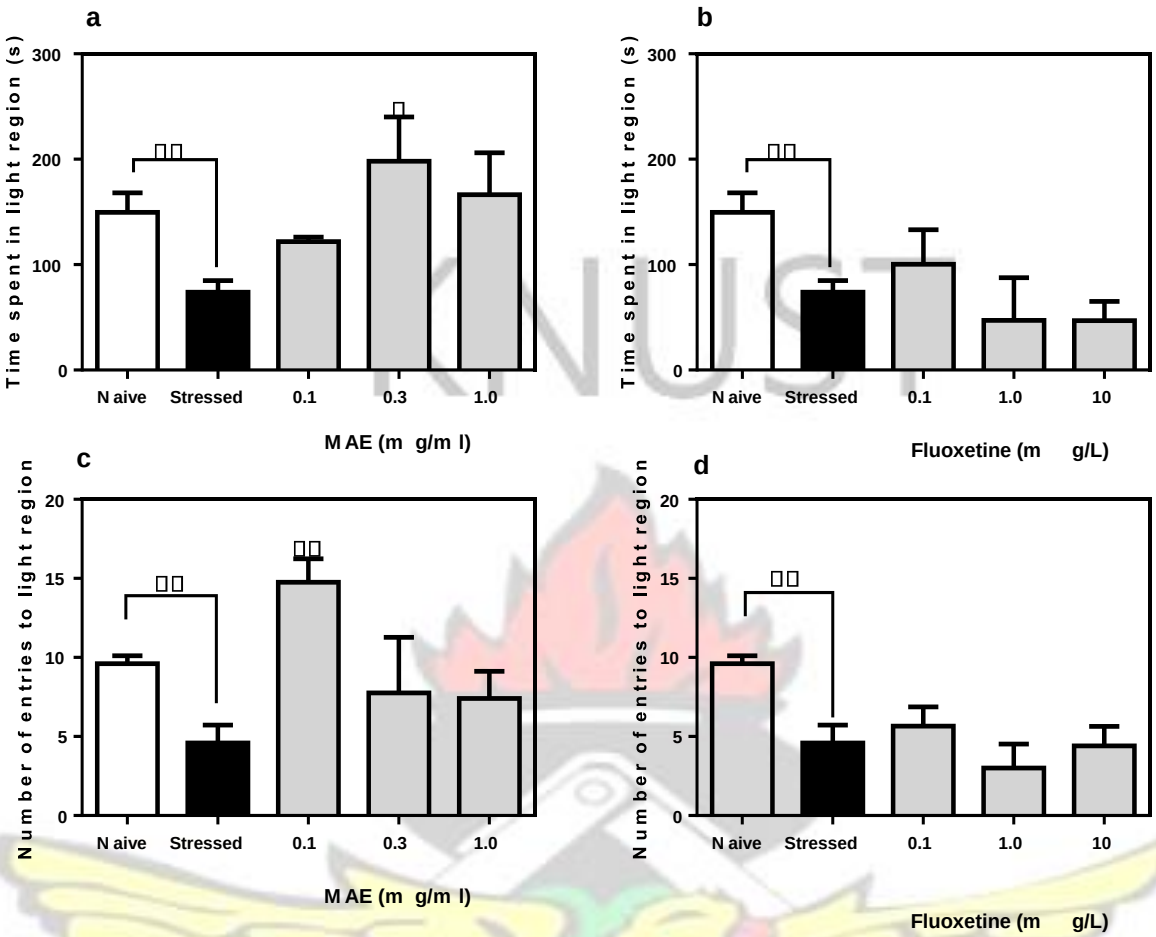


Figure 30: Effects of acute administration of MAE (0.1, 0.3, 1.0 mg mL^{-1}), fluoxetine (30, 100, 300 µg L^{-1}) on (a & b) time spent and(c & d) number of entries to the dark region of the light dark test. D ata are expressed as group mean ± SEM. Significant

difference: **P*<0.05, ***P*<0.01, ****P*<0.001 compared to stressed group (one-way ANOVA followed by Sidak *post hoc* test).

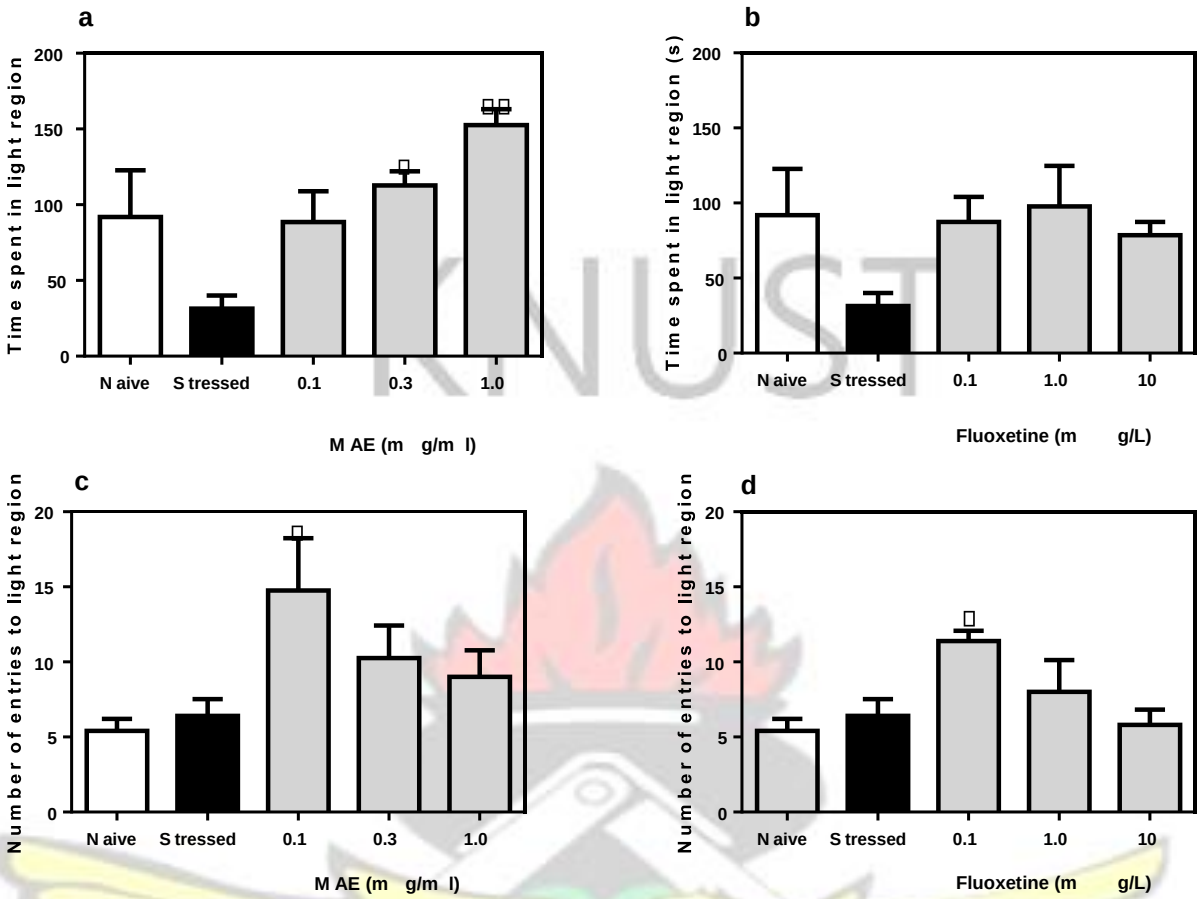


Figure 31: Effects of Chronic administration of MAE (0.1, 0.3, 1.0 mg mL^{-1}), fluoxetine (30, 100, 300 µg L^{-1}) on (a & b) time spent and (c & d) number of entries into to the dark region of the light dark test. Data are expressed as group mean ± SEM. Significant difference: **P*<0.05, ***P*<0.01, ****P*<0.001 compared to stressed group (one-way ANOVA followed by Sidak *post hoc* test).

3.5.3 Shoaling response

Shoaling response was assessed by using the average area between three adult fish over a ten-minute period. An increase in area between zebrafish is considered as decreased shoal cohesion and vice versa. Shoal cohesion is an innate behaviour that is exhibited on exposure to an anxiogenic agent or environment. This behaviour is known to be reversed by standard anxiolytics.

A one-way ANOVA revealed a significant increase in shoaling response in the treatment naïve group when compared with the treatment naïve group (P<0.001). Chronic MAE (1 mg mL^{-1}) and fluoxetine (0.1-1.0 mg L^{-1}) treatment also significantly increased the average area and hence decreased shoal cohesion. (Fig. 32)

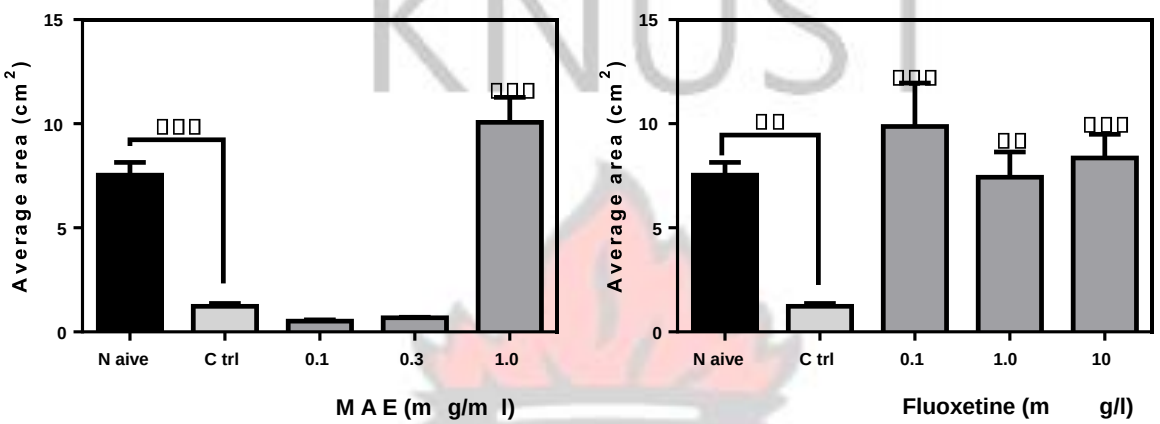


Figure 32: Effects of Chronic administration of MAE (0.1, 0.3, 1.0 mg mL^{-1}), fluoxetine (30, 100, 300 µg L^{-1}) on shoaling behaviour in a novel tank.Data are expressed as group mean ± SEM. Significant difference: *P<0.05, **P<0.01, ***P<0.001 compared to unstressed control group (one-way ANOVA followed by Sidak *post hoc* test).

3.6 INVOLVEMENT OF 5-HT SYSTEM

3.6.1 Novel tank test

Exposure to MAE (1 mg mL^{-1}) or fluoxetine produced a significant increase in time spent in the upper half of the novel tank, an indicator of anxiolytic effect. The anxiolytic potential of MAE and fluoxetine was assessed after pre-treatment with either methysergide (Met), granisetron (Gstn), pizotifen (Piz) or cyproheptadine (Cypro). Treatment with 1 mg L^{-1} methysergide, granisetron or pizotifen did not significantly alter the total time spent in the upper half. Cyproheptadine (1 mg L^{-1}) treatment, however, increased the duration. The anxiolytic effects of MAE was significantly

reversed by pre-treatment with methysergide ($P<0.05$), cyproheptadine ($P<0.01$), granisetron ($P<0.05$) and pizotifen ($P<0.01$). The anxiolytic effects of fluoxetine was similarly reversed only after granisetron ($P<0.05$) or methysergide ($P<0.01$) pretreatment.

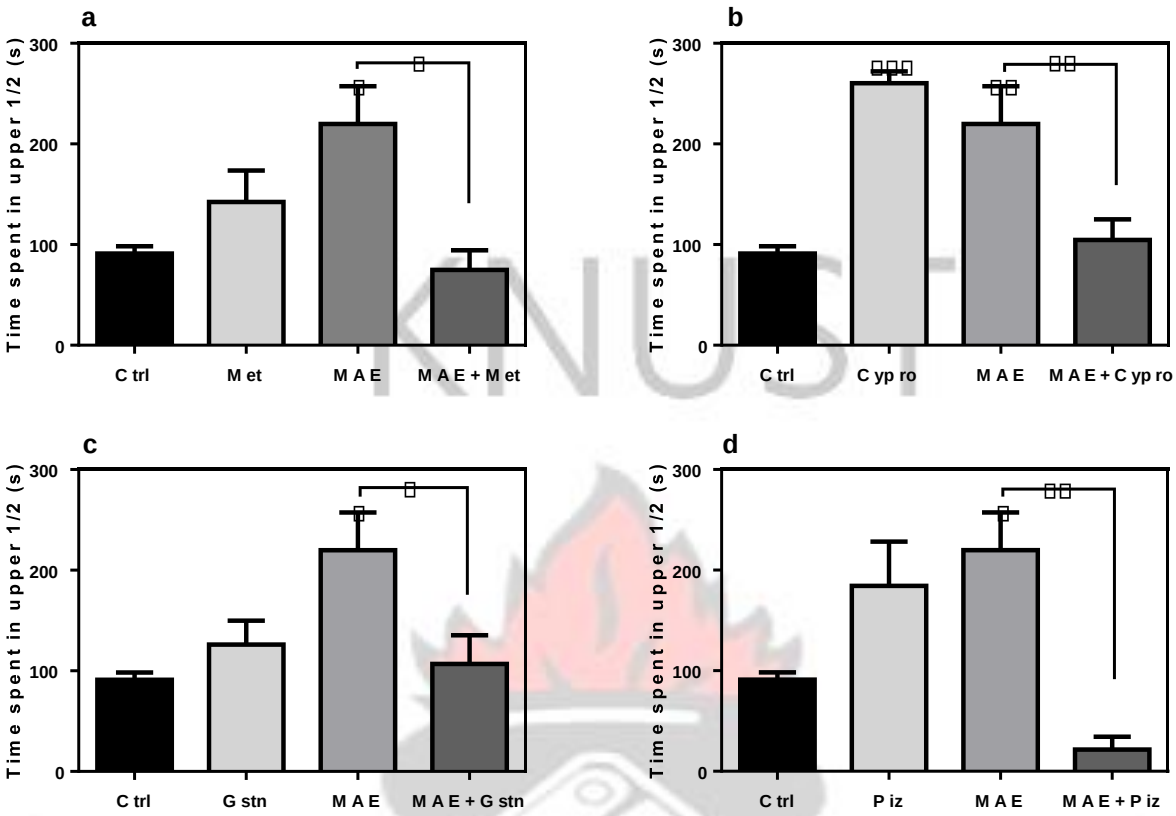
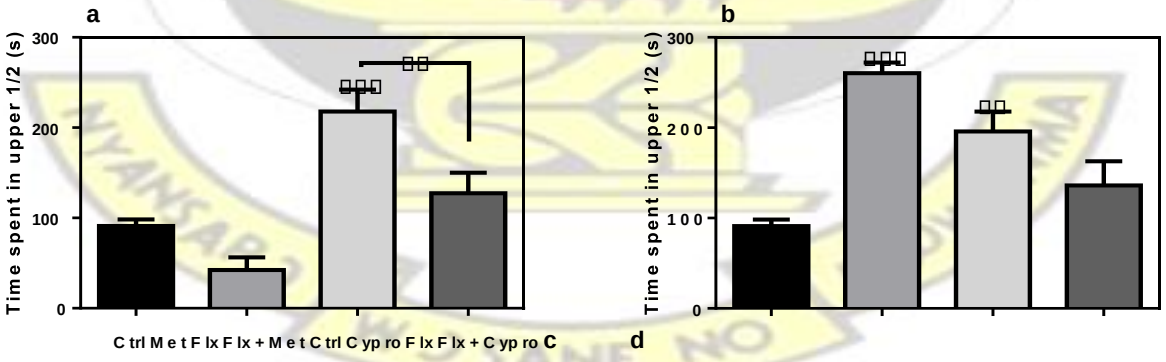


Figure 33: Effects of acute administration of (a) methysergide (b) cyproheptadine (c) granisetron or (d) pizotifen alone or in combination with MAE on the time spent in the upper half of the novel tank. Data are expressed as group mean ± SEM.

Significant difference: **P*<0.05, ***P*<0.01, ****P*<0.001 compared control group. (one-way ANOVA followed by Sidak *post hoc* test).



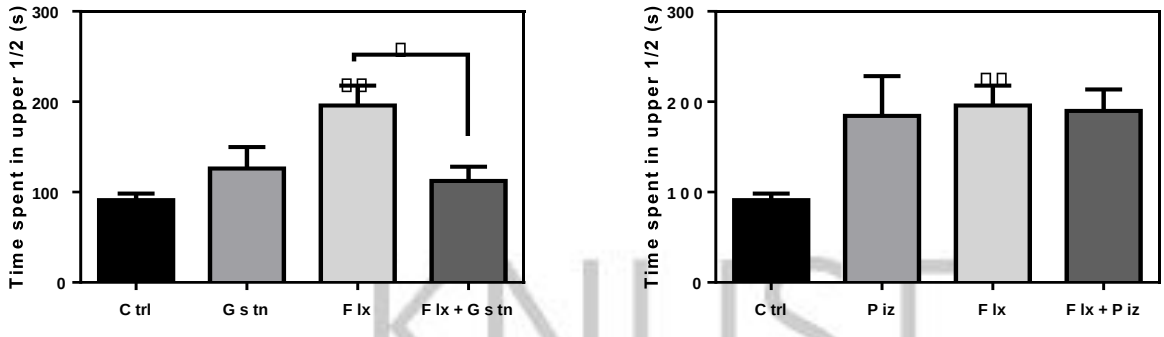


Figure 34: Effects of acute adminstration of (a) methysergide (b)cyproheptadine (c) granisetron or (d) pizotifen alone or in combination with fluoxetine on the time spent in the upper half of the novel tank. Data are expressed as group mean ± SEM. Significant difference: **P*<0.05, ***P*<0.01, ****P*<0.001 compared to control group. (one-way ANOVA followed by Sidak *post hoc* test).

3.6.2 Light-dark test

Exposure to MAE (1 mg mL^{-1}) or fluoxetine produced a significant increase in time spent in the light region of the light dark test, an indicator of anxiolytic effect. The anxiolytic potential of MAE and fluoxetine was assessed after pre-treatment with either methysergide, granisetron, pizotifen or cyproheptadine.

Treatment with 1 mg L^{-1} methysergide, granisetron or pizotifen did not significantly alter the time spent in the light region compared to the solvent control. Cyproheptadine (1 mg L^{-1}) treatment however increased the duration significantly (P<0.05). The anxiolytic effects of MAE was significantly reversed by pre-treatment with methysergide (P<0.001), cyproheptadine (P<0.05), granisetron (P<0.05) and pizotifen (P<0.001). The anxiolytic effects of fluoxetine was similarly reversed only after cyproheptadine (P<0.05), granisetron (P<0.05) or pizotifen (P<0.001) pre-treatment.

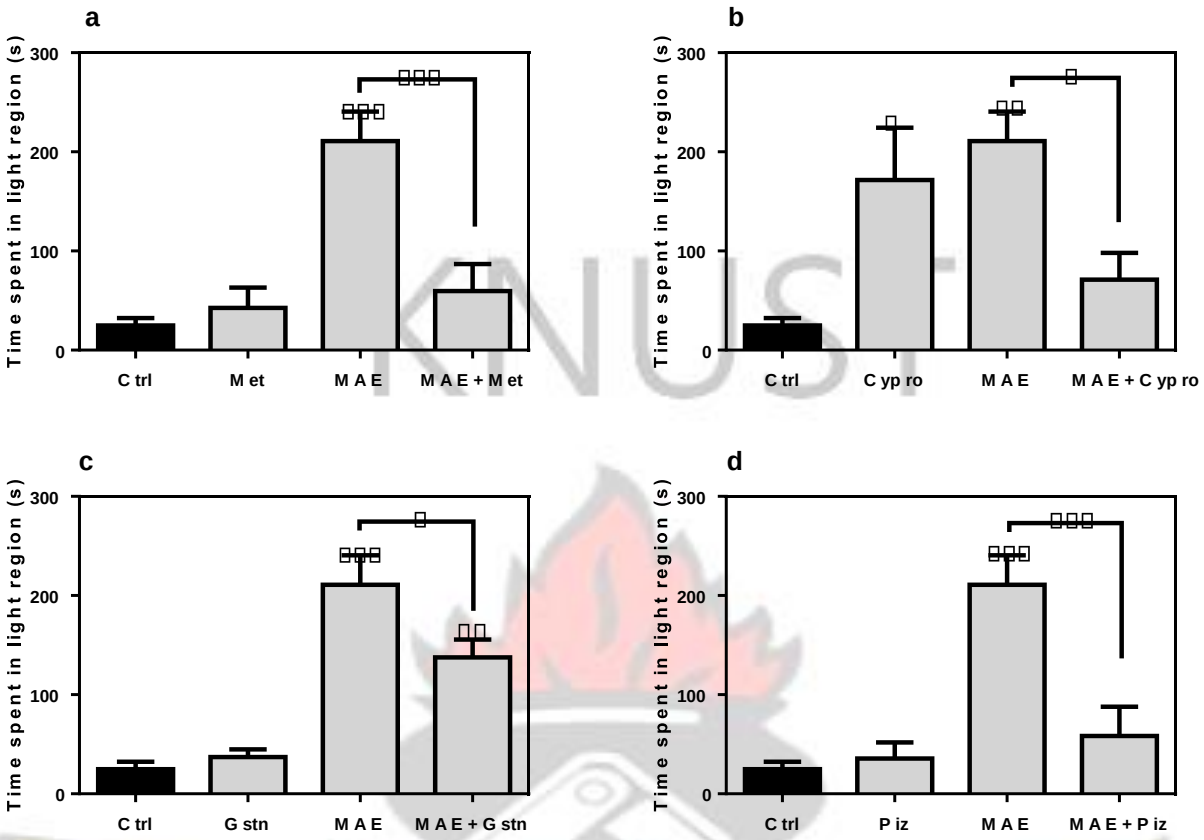
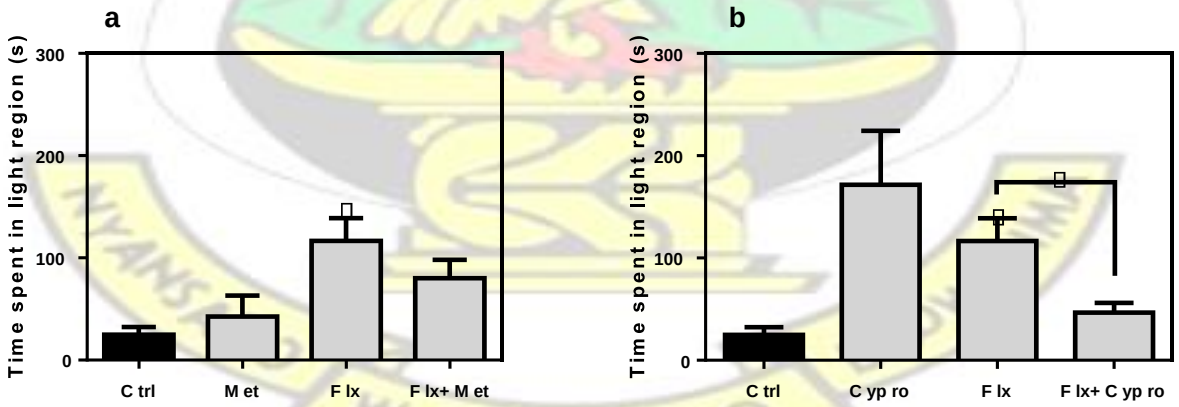


Figure 35: Effects of acute administration of (a) methysergide (b)cyproheptadine (c) granisetron or (d) pizotifen alone or in combination with MAE on the time spent in dark half in the light dark test. Data are expressed as group mean ± SEM. Significant difference: **P*<0.05, ***P*<0.01, ****P*<0.001 compared to control group (one-way ANOVA followed by Sidak *post hoc* test).



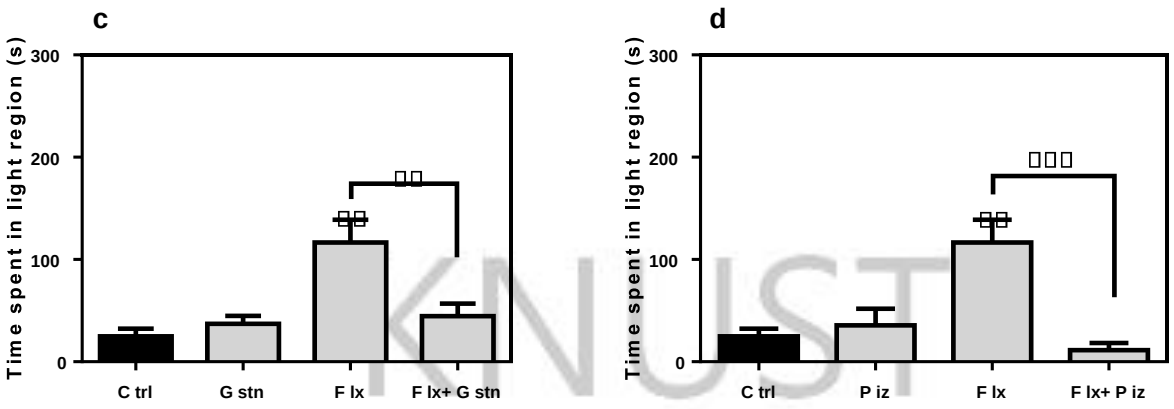


Figure 36: Effects of acute administration of (a) methysergide (b)cyproheptadine (c) granisetron or (d) pizotifen alone or in combination with fluoxetine on the time spent in the dark half of the light dark test. Data are expressed as group mean ± SEM. Significant difference: **P*<0.05, ***P*<0.01, ****P*<0.001 compared to control group (oneway ANOVA followed by Sidak *post hoc* test).

3.7 INVOLVEMENT OF GABAERGIC SYSTEM

3.7.1 Novel tank test

Twenty minutes of flumazenil (1 µg mL^{-1}) administration did not significantly alter the time spent in the upper-half of the novel tank although a general erratic movement was observed.

Treatment with 1 mg mL^{-1} of MAE and 0.15 µg L^{-1} of diazepam increased the time spent in the upper half (*P*<0.0001 for both), an indicator of anxiolytic effect. Pretreatment with flumazenil (1 µg mL^{-1}) significantly reversed the anxiolytic effects of MAE (*P*<0.001) and diazepam (*P*<0.0001).

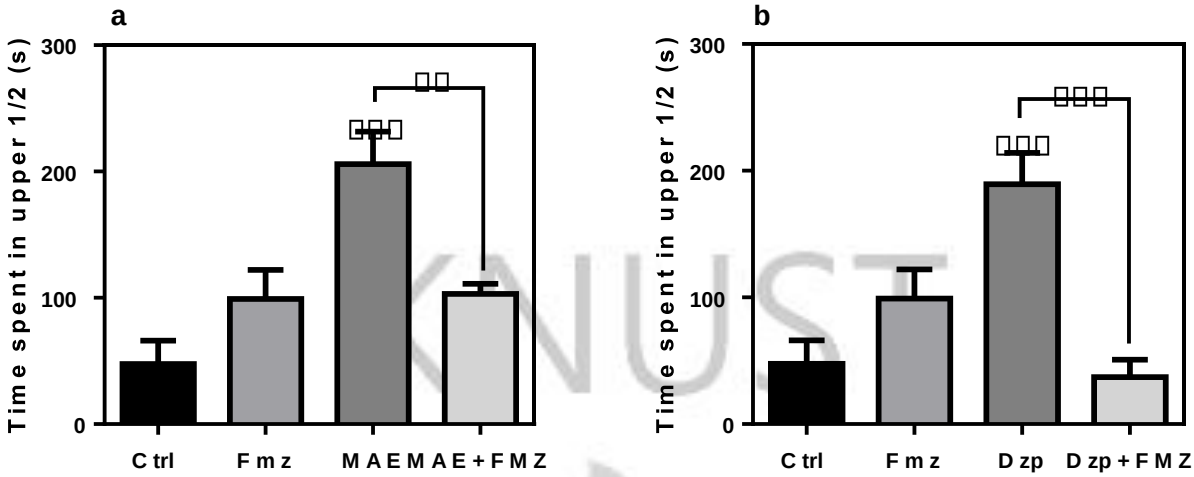


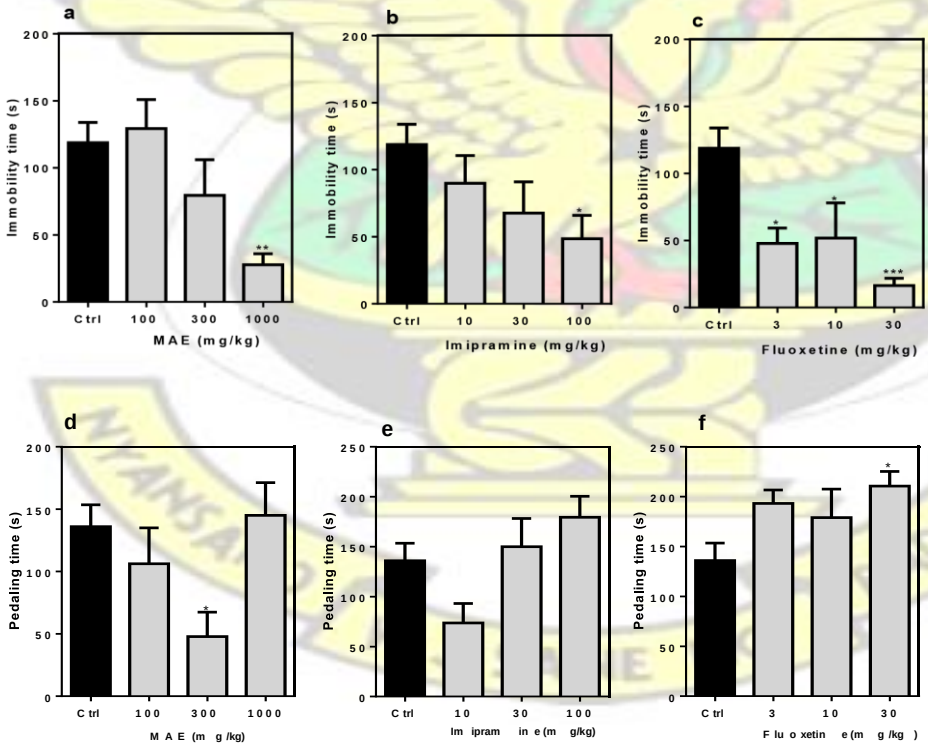
Figure 37: Effects of acute adminstration of acute administration of (a) MAE (1 mg mL^{1}) or (b) diazepam (0.15 µg L^{-1}) after pretreatment with flumazenil (1 µg mL^{-1}) on the time spent in upper half of the novel tank. Data are expressed as group mean ± SEM. Significant difference: ***P*<0.01, ****P*<0.001 compared to control group (one-way ANOVA followed by Sidak *post hoc* test).

3.8 ACUTE ANTIDEPRESSANT ACTIVITY

3.8.1 Tail suspension test

Figure 38 represent the effect of acute administration of MAE (100-1000 mg kg^{-1} *p.o.*), imipramine (10-100 mg kg^{-1}) or fluoxetine (3-30 mg kg^{-1}) on mice behaviours in the tail suspension test. Administration of fluoxetine, MAE and imipramine reduced immobility time in a dose dependent manner by a maximum (E_{max}) of 56.07 ± 14.62 %, 82.06 ± 9.35 % and 86.19 ± 4.56 % respectively (Fig 39). The ED_{50} values shows the order of potency of the test compounds: fluoxetine < MAE < imipramine. Holm-Sidak post hoc test following one-way ANOVA test revealed the MAE at 1000 mg kg^{-1} significantly decreased the immobility time ($F_{3\ 20}$ = 5.744, P = 0.0053) when compared to the control

group. Similarly imipramine at 100 mg kg^{-1} ($F_{3, 20}$ = 2.412, P = 0.0969) and all selected doses of fluoxetine ($F_{3, 20}$ = 6.846, P = 0.0023) decreased immobility significantly. The duration of pedaling was significantly altered after MAE (300 mg kg^{-1}) ($F_{3, 20}$ = 3.493 P = 0.0347) and fluoxetine (30 mg kg^{-1}) ($F_{3, 20}$= 2.681 P = 0.0745) but not imipramine treatment. MAE ($F_{3, 20}$ = 0.6709 P = 0.5799) and imipramine ($F_{3, 20}$ = 1.962 P = 0.1523) treatment did not affect the swinging time, however fluoxetine ($F_{3, 20}$ = 10.68 P = 0.0002) at doses of 10 and 30 mg kg^{-1} caused a significant increase. The cumulative duration of curling for the 5 min test period was significantly increased only after 1000 mg kg^{-1} MAE ($F_{3, 20}$= 7.558 P = 0.0014) treatment (Fig 38g).



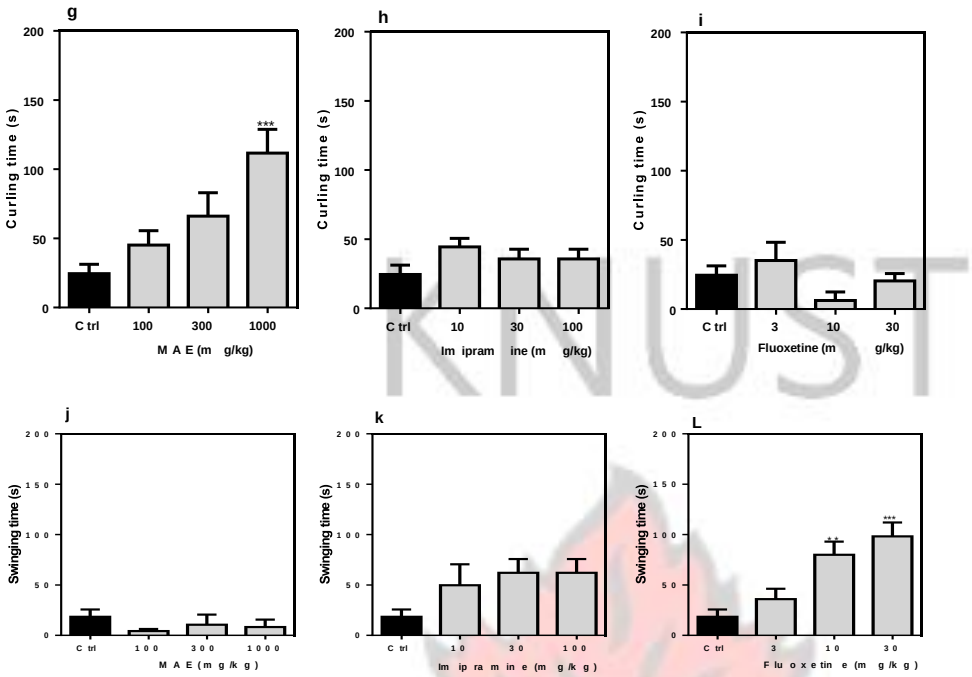


Figure 38: Performance of mice in the TST: behavioural assessment including duration of immobility (a, b, c), pedaling (d, e, f), curling (g, h, i) and swinging (j, k and l) after acute treatment of mice with MAE (100-1000 mg kg^{-1}), imipramine (10-100 mg kg^{-1}) and fluoxetine (3-30 mg kg^{-1}). Significantly different from control: **P*<0.05; ***P*<0.01; ****P* <0.001 (one-way ANOVA followed by Sidak *post hoc* test).

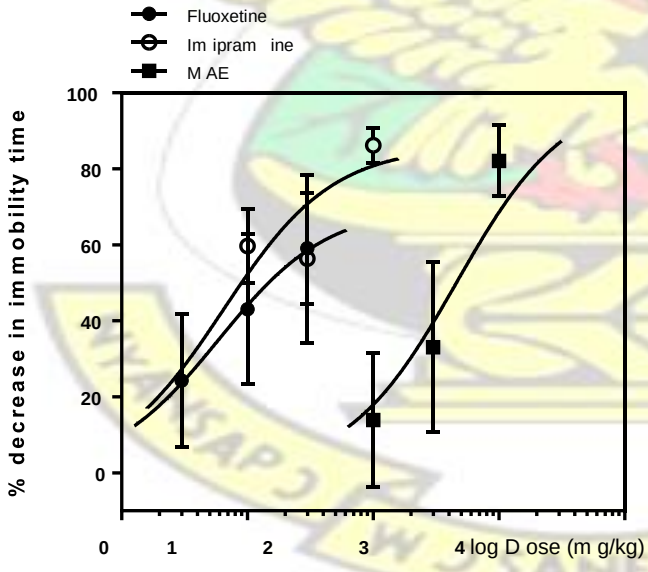


Figure 39: Dose-response curves for MAE (100-1000 mg kg^{-1}), fluoxetine (3-30 mg kg^{-1}) and imipramine (10-100 mg kg^{-1}) with respect to % decrease in immobility in the tail suspension test in mice. Each point represents the mean ± SEM (n=7)

3.8.2 Forced swim test

Figure 40 represent the effect of acute administration of MAE (100-1000 mg kg^{-1} *p.o.*), imipramine (10-100 mg kg^{-1} *p.o.*) or fluoxetine (3-30 mg kg^{-1} *p.o.*) on mice behaviours in the forced swim test.

From figure 41, it can be observed that administration of fluoxetine, MAE and imipramine reduced immobility time in a dose dependent manner by a maximum (E_{max}) of 44.11 ± 14.29 %, 71.72 ± 7.78 % and 91.47 ± 2.865 % respectively. The ED_{50} values shows the order of potency of the test compounds: MAE < imipramine < fluoxetine. A two-way ANOVA analysis revealed that 1000 mg kg^{-1} of MAE significantly decreased the immobility time and increased swimming time ($F_{3, 38}$=10.33, P<0.0001) of mice in the FST. *Post-hoc* analysis revealed statistical significance for the effect of MAE on climbing ($F_{3, 22}$=5.271, P=0.0068) at all doses used. ANOVA revealed that MAE significantly increased the latency to immobility at 100 and 1000 mg kg^{-1} ($F_{3, 20}$=3.718 P=0.0283). ANOVA revealed that fluoxetine at all tested doses significantly increased swimming time ($F_{3, 40}$=2.433, P=0.0002) of mice in the FST (Fig 40g). Similarly imipramine treatment increased the swimming time duration at all doses as well as decreased the immobility duration at 10 and 100 mg kg^{-1} ($F_{3,40}$=19.67, P<0.0001) (Fig 40d). Although the climbing duration ($F_{3, 20}$=2.325, P=0.1056) was not increased, the latency to immobility ($F_{3, 20}$=16.02, P<0.0001) was significantly increased after 100 mg kg^{-1} treatment with imipramine (Fig 40f).

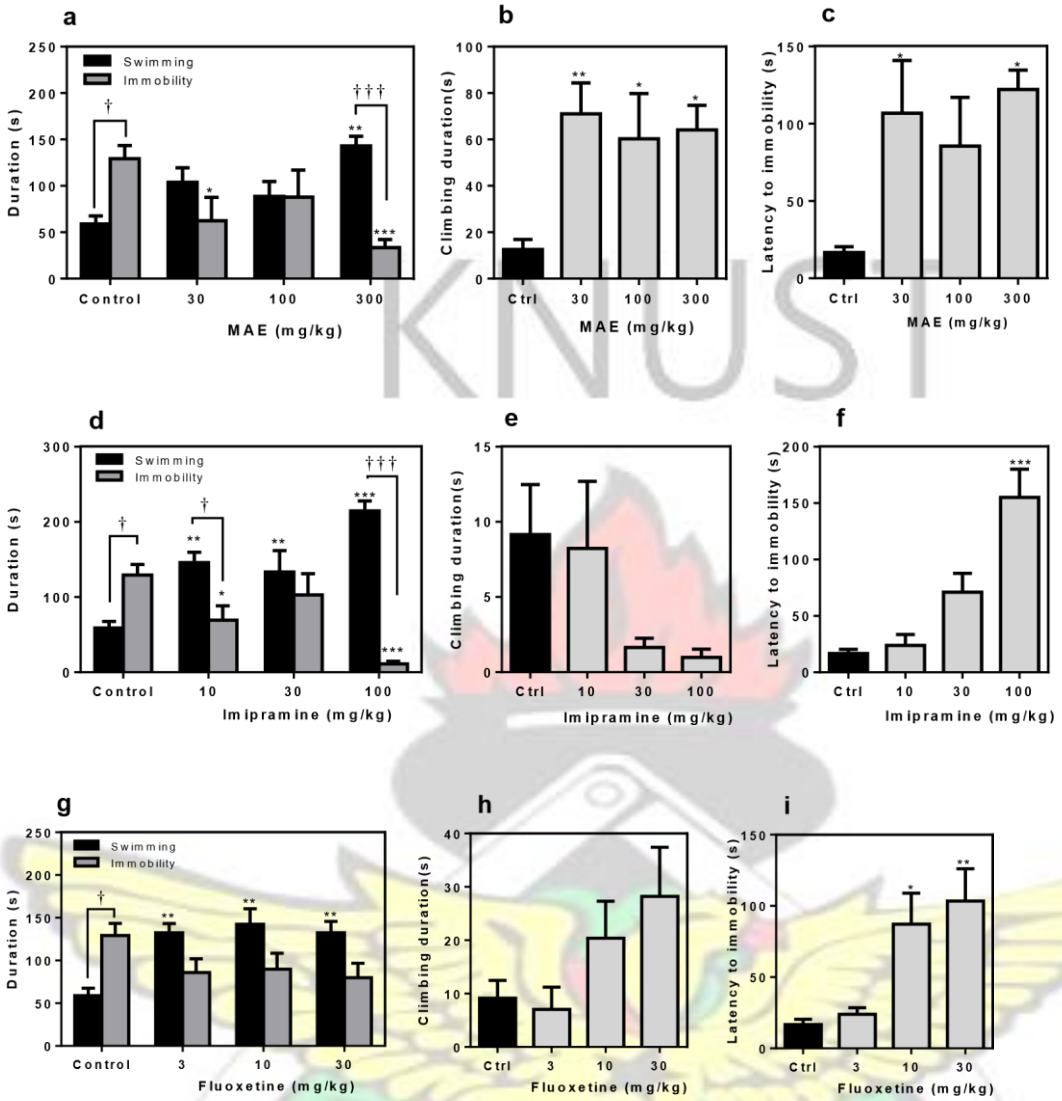


Figure 40 Performance of mice in the FST: behavioural assessment including immobility and swimming duration (a, d, g), climbing duration (b, e, h) and latency to immobility(c, f, i) after acute treatment with MAE (100-1000 mg kg^{-1}), imipramine (10-100 mg kg^{-1}) and fluoxetine (3-30 mg kg^{-1}).

Data are expressed as group mean ± SEM. Significantly different from control: **P*<0.05; ***P*<0.01; ****P* <0.001 (one-way ANOVA followed by Sidak *post hoc* test) and significant difference when immobility and swimming were compared to each other. †*P*<0.05, †††*P*<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

a **b**

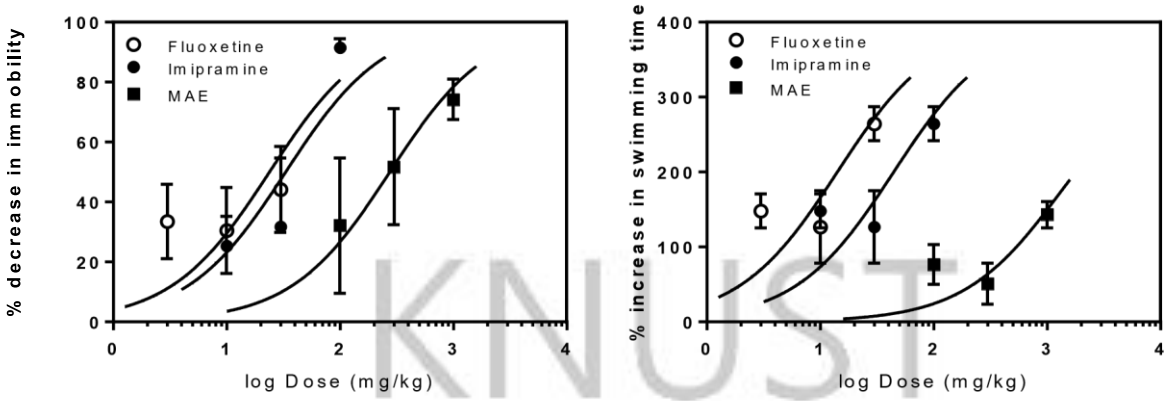


Figure 41: Dose response curves for MAE (100-1000 mg kg^{-1}), fluoxetine (3-30 mg kg^{-1}) and imipramine (10-100 mg kg^{-1}) with respect to (a) decrease in immobility and (b) increase in swimming time in the forced swim test. Each point represents the mean ± SEM (n=7).

3.9 IN-VITRO ANTIOXIDANT ASSAY

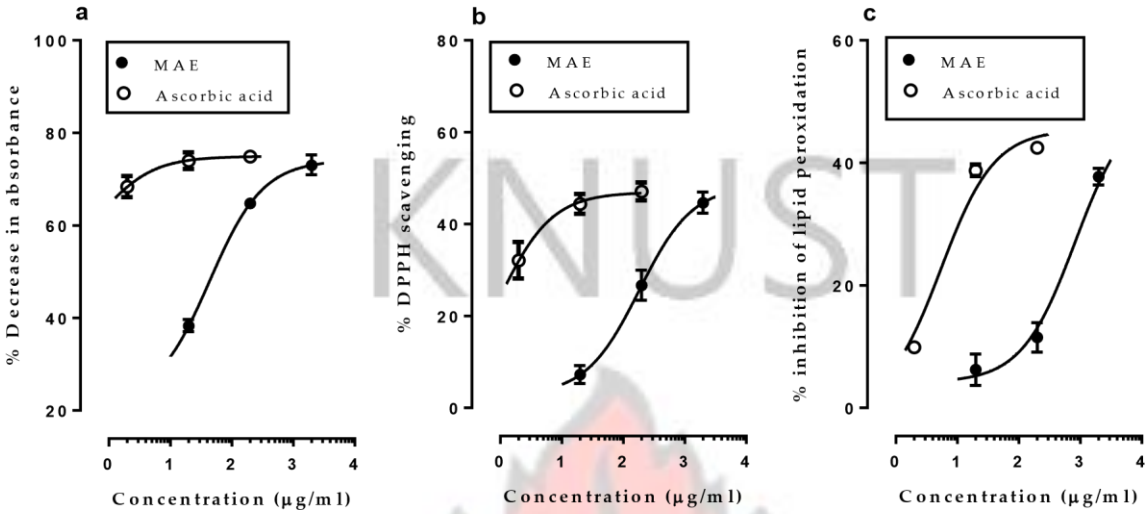


Figure 42: Dose response curves for MAE (20- 2000 µg mL^{-1}) and ascorbic acid (2-200 µg mL^{-1}) with respect to (a) superoxide scavenging activity (b) DPPH radical scavenging activity and (c) anti-lipid peroxidation activity. Each point represents the mean ± SEM, (n=3).

3.9.1 DPPH radical scavenging activity

MAE (20- 2000 µg mL^{-1}) and ascorbic acid (2-200 µg mL^{-1}) exerted a concentrationdependent scavenging activity. The E_{max} of MAE (48.46 ± 3.652 %) and ascorbic acid (46.95 ± 2.203 %) indicates similar efficacy at tested doses. However the IC_{50} (µg mL^{-1}) suggest that the potency of MAE (181.7 µg mL^{-1}) is lower than ascorbic acid (0.9325

µg mL^{-1}) (Fig 42b)

3.9.2 Superoxide anion scavenging activity

MAE (20- 2000 µg mL^{-1}) and ascorbic acid (2-200 µg mL^{-1}) exerted a concentrationdependent superoxide scavenging activity. The E_{max} of MAE (74.13 ± 8.199 %) and ascorbic acid (74.92 ± 1.029 %) indicates similar efficacy at tested doses.

However the IC_{50} (µg mL^{-1}) suggest that the potency of MAE (43.57 µg mL^{-1}) is lower than ascorbic acid (0.7181 µg mL^{-1}) (Fig 42a)

3.9.3 Estimation of lipid peroxidation

MAE (20- 2000 µg mL^{-1}) and ascorbic acid (2-200 µg mL^{-1}) exerted a concentrationdependent scavenging activity. The E_{max} of MAE (50 ± 0.00 %) and ascorbic acid (45.44 ± 1.174 %) indicates similar efficacy at tested doses. However the IC_{50} (mg mL^{1}) suggest that the potency of MAE (824.5 µg mL^{-1}) is lower than ascorbic acid (5.434 µg mL^{-1}) (Fig 42c)

3.10 PTZ-INDUCED OXIDATIVE STRESS IN THE BRAIN

Thirty minutes after subcutaneous administration of PTZ (65 mg kg^{-1}), the brain was excised and the oxidative status was assessed.

3.10.1 Catalase

MAE ($F_{4, 15}$=5.110, P=0.0084) and diazepam ($F_{4, 15}$=65.43, P<0.0001) pre-treatment dose-dependently increased catalase levels as compared to the PTZ treated group (Fig 43)

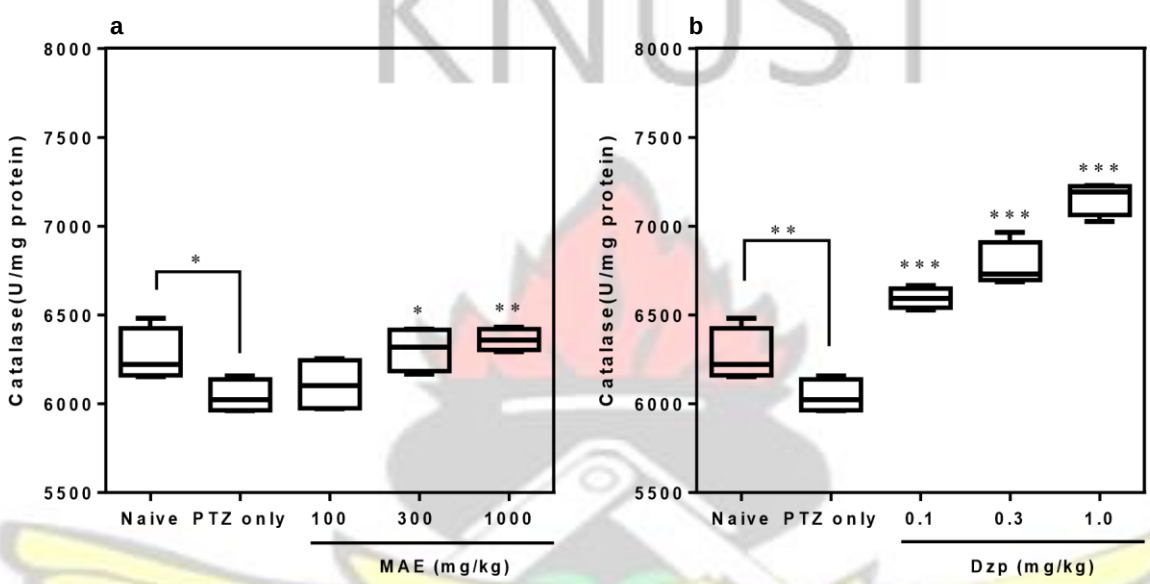


Figure 43: Effects of (a) MAE (100-1000 mg kg^{-1}) and (b) diazepam (0.1-1.0 mg kg^{-1}) treatment on whole brain catalase levels after PTZ induced seizures. The lower and upper margins of the boxes represent 25th and 75th percentiles with the extended arms representing the 10th and 90th percentiles respectively. Significantly different from control compared to PTZ group: *P<0.05; **P<0.01; ***P <0.001 (one-way ANOVA followed by Sidak *post hoc* test)

3.10.2 Superoxide dismutase

MAE ($F_{4, 13}$=68.99, P<0.0001) and diazepam ($F_{4, 13}$=34.35, P<0.0001) pre-treatment dose-dependently increased superoxide dismutase levels as compared to the stressed (PTZ treated) group (Fig 44).

a **b**

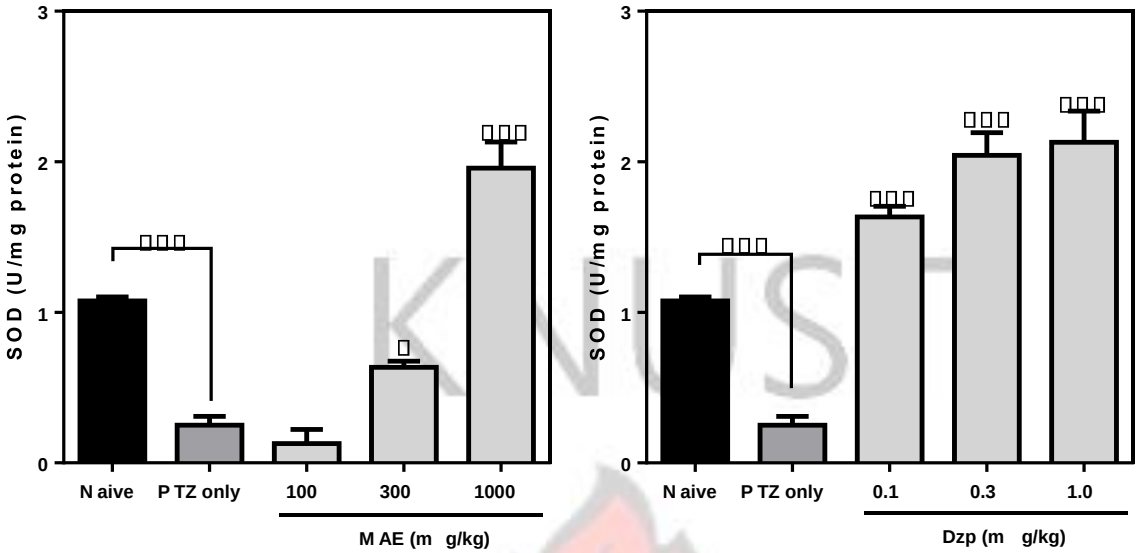


Figure 44: Effects of (a) MAE (100-1000 mg kg^{-1}) and (b) diazepam (0.1-1.0 mg kg^{-1}) treatment on whole brain superoxide dismutase levels after PTZ induced seizures. Data are expressed as group mean ± SEM. Significant difference: **P*<0.05, ****P*<0.001 compared to PTZ group (one-way ANOVA followed by Sidak *post hoc* test).

3.10.3 Reduced Glutathione

MAE ($F_{4, 13}$=168.4, *P*<0.0001) and diazepam ($F_{4, 14}$=38.48, *P*<0.0001) pre-treatment dose dependently reduced glutathione levels as compared to the PTZ treated group (Fig 44).

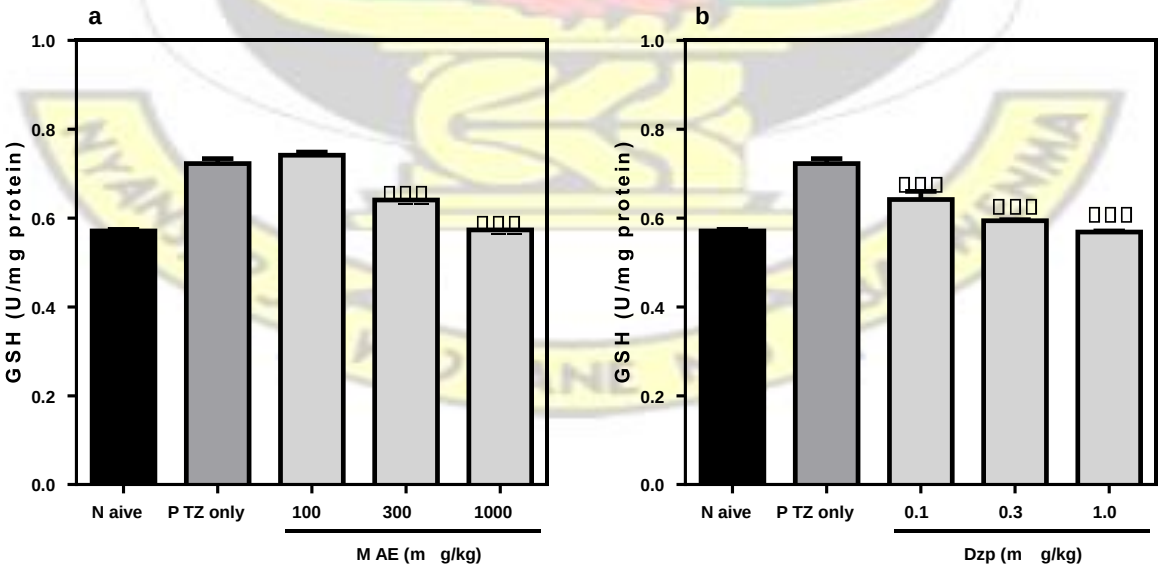


Figure 45: Effect of (a) MAE (100-1000 mg kg^{-1}) and (b) diazepam (0.1-1.0 mg kg^{-1}) treatment on whole brain reduced glutathione levels after PTZ induced seizures. Data are expressed as group mean ± SEM. Significant difference: ****P*<0.001 compared to PTZ group (one-way ANOVA followed by Sidak *post hoc* test).

3.10.4 Lipid peroxidation (thiobarbituric acid reactive substances assay).

MAE ($F_{4, 15}$=49.40, *P*<0.0001) and diazepam ($F_{4, 15}$=112.2, *P*<0.0001) pre-treatment dose-dependently reduced thiobarbituric acid reactive substance levels as compared to the PTZ treated group (Fig 46).

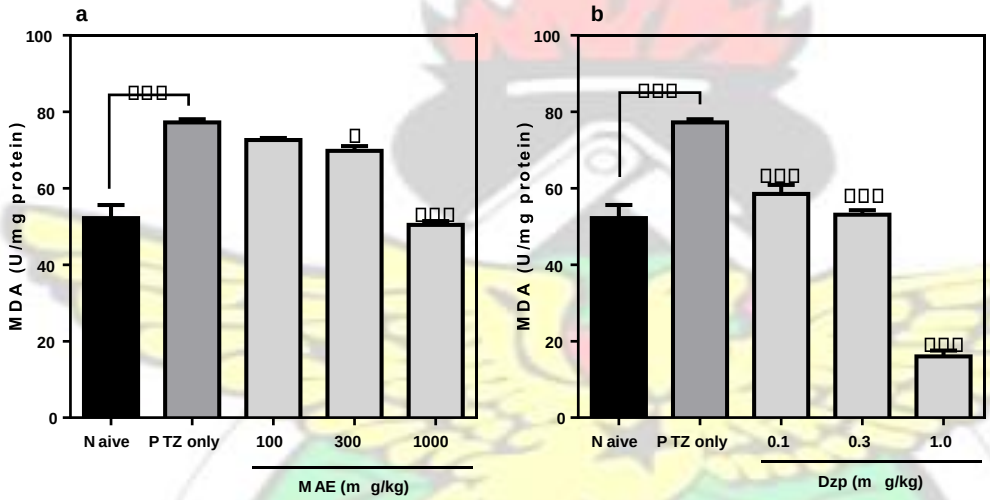


Figure 46: Effects of (a) MAE (100-1000 mg kg^{-1}) and (b) diazepam (0.1-1.0 mg kg^{-1}) treatment on whole brain lipid peroxidation after PTZ induced seizures. Data are expressed as group mean ± SEM. Significant difference: **P*<0.05, ****P*<0.001 compared to PTZ group (one-way ANOVA followed by Sidak *post hoc* test).

3.11 PTZ INDUCED CONVULSIONS IN RATS

3.11.1 Effect on $GABA_A$

PTZ administration after pre-treatment with flumazenil did not significantly affect the duration, frequency and latency to convulsion when compared to the saline-treated group (Fig 47a). However there was a significant reduction in the duration of convulsions after MAE and diazepam. Further comparison between the saline and flumazenil-treated groups also revealed a significant increase in duration after flumazenil treatment. The frequency of convulsions over the five minute period was reduced after both MAE and diazepam treatment (Fig 47b). Interestingly, a significant difference between the saline and flumazenil-treated groups was only revealed after MAE but not diazepam treatment. On the other hand, flumazenil pre-treatment did not alter the latency to convulsion after MAE treatment but there was a significant increase in the latency after diazepam treatment. Rats treated with flumazenil before diazepam administration showed a significant decrease in latency to convulsion compared to the saline treated group (Fig 47c).

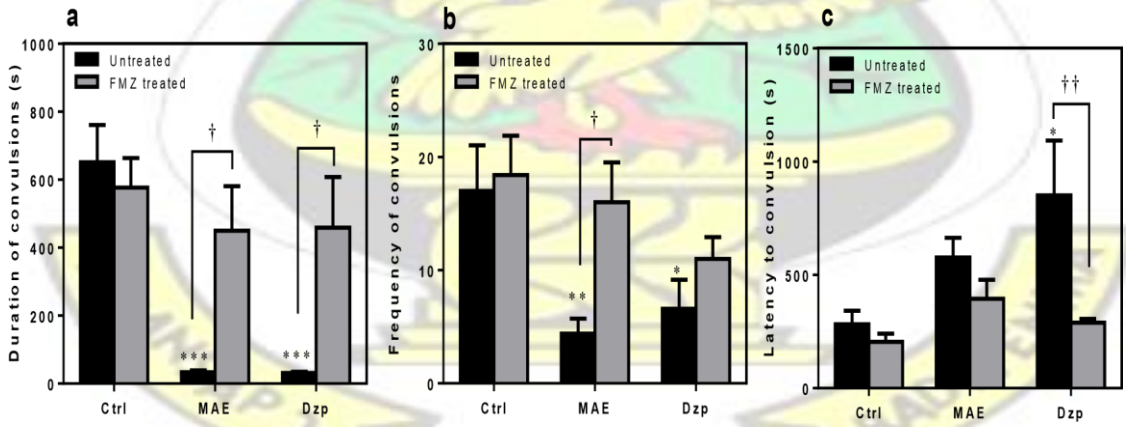


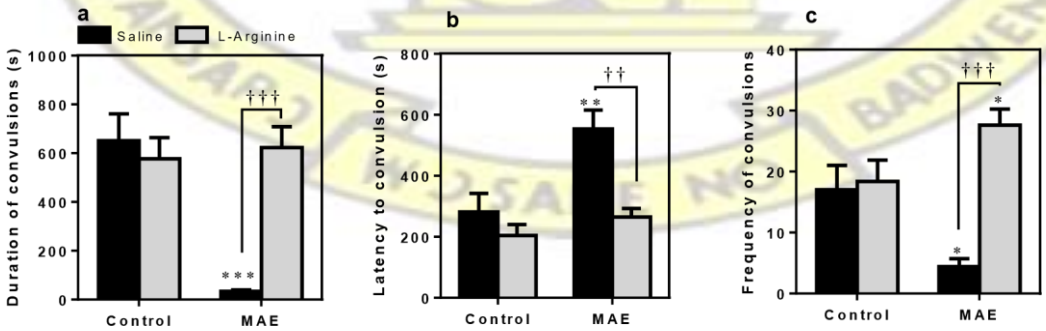
Figure 47: Effects of MAE (300 mg kg^{-1}) and diazepam (1.0 mg kg^{-1}) on the (a) duration (b) latency and (c) number of clonic convulsions for a in the PTZ-induced convulsions after flumazenil treatment.

Data are expressed as group mean ± SEM. Significant difference: **P*<0.05, ***P*<0.01, ****P*<0.001 compared to control (one-way ANOVA followed by Sidak *post hoc* test).

Significant difference when treated and untreated were compared: †*P*<0.05, ††*P*<0.01 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

3.11.2 Effect of MAE on L-arginine-NO-cGMP Pathway

Acute MAE (300 mg kg^{-1}, *p.o.*) treatment significantly increased latency and decreased both frequency and duration of clonic convulsions. Administration of L-arginine (150 mg kg^{-1}, i.p., a precursor of nitric oxide) had no anticonvulsant effects compared with saline treated control. However, pre-treatment with L-arginine significantly inhibited the anticonvulsant effect of MAE (300 mg kg^{-1}, *p.o.*) by decreasing latency (*P*<0.001), increasing frequency (*P*<0.0001) and increasing duration (*P*<0.0001) of clonic seizures as revealed by post hoc analysis (Figure 48, a-c). Figure 48 (d-e) shows the effect of pre-treatment with sildenafil, a phosphodiesterase 5 inhibitor on PTZ-induced clonic seizures. Concomitant administration of sildenafil (5 mg kg^{-1}, i.p.) significantly reversed the anti-convulsant action of MAE (300 mg kg^{-1}, *p.o.*). This is observed as a decrease in the onset (*P*<0.001) and increase in the frequency (*P*< 0.05) and duration (*P*<0.05) of clonic convulsions induced by PTZ



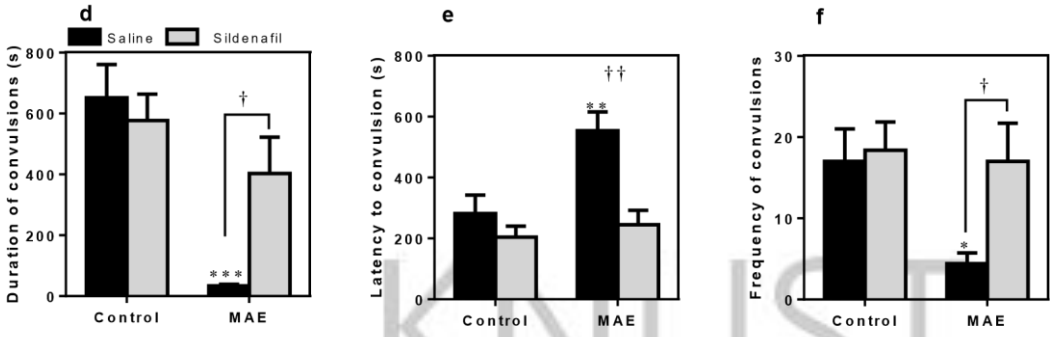


Figure 48 Effects of MAE (300 mg kg^{-1}) on the (a & d) duration (b & e) latency and (c & f) number of clonic convulsions for a 5 minute test period in a pentylenetetrazole- induced convulsions after pretreatment with L-arginine (upper panel) and sildenafil (lower panel).

Data are expressed as group mean ± SEM. Significant difference: **P*<0.05, ***P*<0.01, ****P*<0.001 compared to control (one-way ANOVA followed by Sidak *post hoc* test). Significant difference when treated and untreated were compared: †*P*<0.05, ††*P*<0.01, †††*P*<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

CHAPTER FOUR DISCUSSION

The present study demonstrates that the lipophilic fraction of the stem bark extract significantly reduced anxiety levels in the zebrafish due to interaction with the $GABA_A$ and 5-HT_{1-3} receptors. The stem bark extract also possess anticonvulsant effects; through the $GABA_A$ receptor and nitricoxidergic system, and antidepressant effects in murine models. The GABAergic mediation of anxiolysis is in agreement with earlier studies carried out with the ethanolic fraction of the stem bark (Malami *et al.*, 2014).

The Irwin test evaluates the qualitative effects of test substances on the behaviour, autonomic as well as the physiological function of a test animal (Irwin, 1968). Results from such test can give an approximate onset and duration of action of different measured effects. In the Irwin test, the extract showed analgesic effect and reduction to fear and touch response at doses of 100-5000 mg kg^{-1} *p.o.* The onset of action was increased with dose increments, with the fastest onset observed at 30 min. Based on

onset and duration of the effects registered in the Irwin test, further tests were carried 60 minutes after oral administration. Continuous observation for 48 hours after the test revealed no physical signs of toxicity or lethality at all tested doses. This suggests that the LD_{50} in mice is beyond 5000 mg kg^{-1}.

The activity meter test was then employed to assess quantitatively the spontaneous behaviour with respect to locomotion after oral administration of MAE. There was significant reduction in locomotor activity after 1000 mg kg^{-1} MAE administration. Locomotor activity can be reduced significantly after dosing test animals with a sedative dose of CNS depressants. Also, a reduction in locomotor activity could also be due to motor impairment induced by the test drug. Consequently, the effects observed after MAE administration can be attributed to the sedative or locomotor impairment potential of the extract. Caffeine, a CNS stimulant increased whilst diazepam, a CNS depressant decreased the locomotor activity in this test.

In the Irwin test, test drugs that have seizure induction potential can be identified by observing physical signs such as tonic and or clonic convulsions during the test.

However, the Irwin test is not sensitive at identifying pro-convulsant effects of drugs. Instead, the seizure liability effect is uncovered after treatment with chemoconvulsants such as pentylenetetrazole. Such treatments can additionally be used to screen potential anticonvulsants since drugs with anticonvulsant properties are known to reduce seizure parameters induced by agents such as pentylenetetrazole. In the convulsive threshold test in mice, only the highest dose of the MAE increased the latency to clonic convulsion and survival compared to the saline group. A Kaplan-Meier analysis of survival revealed no significant protection compared to vehicle control. This indicates

that the extract was not effective in delaying and preventing lethality induced by pentylenetetrazole (85 mg kg^{-1}) administration.

Barbiturates are general CNS depressants that induce a state of calm, sedation and hypnosis at high doses (Essman, 1983). In mice, acute administration of barbiturates induce a state of sleep which is indicated by a loss of righting reflex. The sedating effects of CNS depressants are usually unmasked by co-administration with pentobarbitone. This behaviour is known to be reversed by stimulants and enhanced general CNS depressants. A significant decrease in latency to sleep and increase in sleep duration after co-administration with pentobarbitone indicates that MAE possesses sedative effects — which is in agreement with results obtained from the activity meter test.

Anxiety studies were performed in the mice and zebrafish models. The amelioration of innate anxiety induced by novel environment was explored in the open field, elevated plus maze test and the regular suok test. The open field, elevated plus maze and Suok tests assess the behaviour of mice in a conflict situation. The open field, tests for the natural aversion of rodents to brightly lit areas and the elevated plus maze assesses the aversion to height and open spaces (Bourin *et al.*, 2007). The Suok test, however, possesses a unique advantage of assessing the anxiety state of rodents as well as the sensorimotor coordination on a horizontal rod (Kalueff *et al.*, 2008; Kalueff *et al.*, 2005). In general anxiolytics are known to increase affinity for the aversive stimuli or environment whilst anxiogenic agents are known to enhance the innate aversion to these stimuli or environment

Mice placed in an open brightly lit environment, as in the open field test, are known to show increased anxiety-like behaviours such as increased time spent in the periphery

and corner arenas with a correspondingly decreased time spent in the central arena. Lower anxiety states of mice are usually indicated by a significant increase in time spent in the center. The number of crossings as well as the time spent in each of the three designated areas is sensitive to anxiolytics and anxiogenic treatments (Prut & Belzung, 2003; Walsh & Cummins, 1976). Analysis of both the time and number of crossings did not reveal any significant change compared to the baseline behaviour of the solvent treated group. However, the percentage of the time spent and frequency revealed the anxiolytic effects of both diazepam and MAE.

In the EPM test, vehicle-treated mice exposed to the EPM made fewer entries and spent less time in the open unprotected arms compared to the closed arms, a behaviour which is consistent with literature that, mice generally avoid open unprotected arenas (Carobrez & Bertoglio, 2005; Lister, 1987). Pretreatment with MAE or diazepam significantly reversed this behaviour at all tested doses suggesting an anxiolytic property of MAE. Although time spent are key indicators of the anxiety state of the test animal, its measurement provided offers a scintilla of information relating to the actual behavioral measures whiles exploring the maze. It is therefore important to assess, describe and possibly measure the general behavioural repertoire of the animal of interest in such behavioral assays to offer more insights into the general behavior of the test animals after treatment with drugs of interest.

Whiles the open arm entries as well as % open arm entries have been found to be a consistent measure of anxiety in rodents, the total head-dips (HD) and total stretch attend posture (SAP) is a measure of exploration. Additionally, the total SAP provides an additional measure of risk assessment of the test organism. Over the past 25 years, several workers have developed protocols that allow a comprehensive profiling of

behaviour of mice in the elevated-plus maze based on the defensive behaviours exhibited in the test. Behaviours such as freezing, stretch attend postures and head-dips are some of the ethological parameters that can give an indication of the anxiety and behavioural state of the test mouse (Rodgers *et al.*, 1997).

Mice in general tend to move freely in the closed arms with an increased tendency to freeze in the open arm of the EPM. Anxiolytics reduce this freezing behaviour whiles a converse occurs after giving an appropriate dose of anxiogenic agent. Based on the above facts, it was realized that a single ethological parameter could increase or decrease depending on whether it occurs in the open or closed arms of the EPM. Hence the designation, ―protected‖ and ―unprotected‖ is ascribed to each ethological parameter occurring in the closed or opened arms respectively (Cole & Rodgers, 1994; Rodgers *et al.*, 1997). Anxiogenic agents increase the duration and frequency of protected behaviours, such as head dips and stretch attend postures, with a corresponding decrease in unprotected ethological parameters. The converse is true for agents that possess anxiolytic properties in mice.

Similar to diazepam, MAE administration increased the number of head dips. An increase in the number of head dips is an indication of low anxiety states whilst a decrease indicates high anxiety states (Rodgers & Johnson, 1995). The duration of stretch attend postures have been recorded to decrease after the administration of anxiolytic agent. Consistent with literature, diazepam was able to reduce the SAP parameters. Similarly, MAE also reduced protected SAP parameters suggesting an anxiolytic effect.

To assess the effect on sensorimotor coordination and further establish the anxiolytic potential, the regular Suok test was employed. This test combines many aspects of the

EPM, OFT and beam walk tests. Behaviours such as head-dips, side-looks, and frequency and duration of freezing bouts are used as endpoints for assessing of the anxiety state of mice whilst the number of falls and missteps are known to predict the degree of sensorimotor coordination. Diazepam is known to exhibit anxiolytic effects at lower doses in several test paradigms including the Suok test (Kalueff *et al.*, 2008). However at relatively higher doses, diazepam induces a state of impaired motor coordination. This makes it an ideal positive control in the Suok test which explores both behaviours. Similar to diazepam, MAE increased both duration and frequency of side looks; a behaviour which is increased by anxiolytics. MAE similar to diazepam reduced the number of freezing events suggestive of a possible anxiolytic effect since increased freezing bouts are indicative of a heightened anxiety state. The sensorimotor coordination was impaired at the highest dose of diazepam which was reflected in the increased number of leg slips. However the anxiolytic doses of MAE did not affect the number of leg slips. Taken together, it suggests that MAE exhibits anxiolytic behaviour at doses that does not affect motor coordination although further tests will be required to corroborate this evidence.

Central to the generation and mediation of anxiety responses is the amygdala and its neural projections into areas such as the ventral tegmental area, locus coerulus and hippocampus (Davis, 1992). Evidence suggests that both conditioned and unconditioned fear stimuli excite the amygdala and the amygdaloid projections leading to a constellation of behaviours such as increased heart rate (lateral hypothalamus), freezing (central grey), respiratory distress (parabrachial nucleus) and increased vigilance/arousal (ventral tegmental area, locus coerulus). In zebrafish, similar to mammals, regulation of anxiety and fear occur at several brain regions (Davis, 1992).

The presence of analogous structures that mediate fear and anxiety have been identified in zebrafish. Structures such as the habenula, locus coerulus, periaqueductal grey area and raphe nuclei have been identified to be involved in anxiety responses in zebrafish (Mathuru & Jesuthasan, 2013). The amygdala in mammals corresponds to the medial region of the dorsal pallium (Dm) (Mueller *et al.*, 2011). Therefore zebrafish may exhibit anxiety/fear responses through the activation of analogous brain structures, however further characterization of the inherent neural connections involved in these responses is required. The benzodiazepine class of anxiolytics are known to cause a general decrease in basal neuronal firing (through the activation of $GABA_A$ receptor) in the dorsal raphe (5-HT), substantia nigra compacta (DA) and locus coeruleus (NE) (Davis, 1992; Shephard, 1987). Buspirone

(a selective 5-HT_{1A} receptor agonist) on the other hand is known to reduce neuronal firing at the dorsal raphe but increase the firing rate at the locus coerulus (Davis, 1992). This accounts for its anxiolytic as well as non-sedating and non-amnestic effect profile. Similar anxiolytic effects have been shown in zebrafish after exposure to diazepam (a benzodiazepine) and buspirone, in the novel tank test, further suggesting similarities at the psychopharmacological level (Bencan *et al.*, 2009; Gebauer *et al.*, 2011).

The novel tank and the light dark tests are robust screening paradigms employed in anxiety and stress research (Blaser & Rosemberg, 2012). The novel tank test explores the innate avoidance behaviour of adult zebrafish to avoid the upper section of a novel environment. Several studies suggest that, naïve zebrafish will spend a higher proportion of time , within a five minute test period, in the lower section of a novel tank (Blaser & Rosemberg, 2012; Sackerman *et al.*, 2010)**.** This behaviour has been found

to be sensitive to both anxiolytic drugs; increased time spent in upper section, and anxiogenic agents; increased the bottom dwelling behaviour. A similar avoidance behaviour has been demonstrated in the light-dark test where the aversive stimuli is a brightly lit environment as opposed to a darker one. Zebrafish in their natural state tend to have an increased affinity for the dark section i.e. scototaxis behaviour. Also naïve fishes usually demonstrate a higher degree of thigmotaxis in the light environment. The time spent in the light section is known to be increased by anxiolytic agents such as diazepam, ethanol, and chlordiazepoxide. Decreased latency to enter into the upper half of the novel tank and the light section of the light-dark test are also parameters usually associated with anxiolytic agents.

Both tests have the inherent property of detecting anxiolytic/anxiogenic behaviour based on the preference for light/dark or lower/upper regions of the test system (Bencan *et al.,* 2009). Since the behaviour in these paradigms depends on a combination of motivational as well as locomotion, an additional assessment of ―intank‖ movement velocity to rule out the possibility of locomotor impairment and or sedative effects that may confound findings are necessary. The reversal of the bottom dwelling and scototaxis behaviour by MAE, in the preliminary anxiety study, is suggestive of an anxiolytic-like behaviour. Acute administration of desipramine (Uptake 1 blocker) and fluoxetine (SSRI) exhibited similar anxiolytic behaviours upon dosing whilst higher doses of diazepam ($GABA_A$ agonist) showed opposite effects which might be attributed to its sedative and or locomotor impairment. Also, decreased latency to enter into the upper half of the novel tank and the light section of the light-dark test is also a parameter usually associated with anxiolytic agents.

Chronic models are considered more naturalistic in the induction of a depressive-like state. Such models are suggested to have better potential homology to the human

affective disorders (Duman, 2010). There is a growing need to develop a robust zebrafish model to study affective behaviours such as depression. Chronic unpredictable stress (CUS) is currently the only robust model that studies the effect of chronic stress on anxiety and related mood disorders in zebrafish. Although a few authors have suggested the use of behaviour phenotypes such as tail drooping as an indicator of a depressive state. Such measures still remain largely qualitative.

Assessment of anxiety and mood-related disorders by Chakravarty *et al.* (2013) in the CUS revealed decreased neurogenesis as well as alterations in CRF, calcineurin, pCREB which are common molecular markers of rodent affective disorders. In this study, signs of depressed behaviour such as reduced shoal cohesion, tail drooping, reduced motivation to escape and reduced aversion to human subjects, were observed.

The two latter points may be either due to reduced affect and or habituation to human contact in general, however the droopy tail cannot be accounted for by reason of habituation alone. It was consequently hypothesized that agent with both antidepressant and anxiolytic effects will reduce these behavioural signs as well as the quantitative indicators of anxiety.

Interestingly, acute treatment with previously established anxiolytic doses of *Maerua angolensis* extract reversed the increased anxiety state in the CUS-stressed animals. However, a 20 minute exposure of fluoxetine and MAE for three continuous days was also able to reverse anxiety and related mood disorders including shoal cohesion. This evidence suggests that MAE possess anti-depressant effects.

Another robust way of testing and screening for anxiolytic agents is to test it in an induced state of anxiety (Cachat *et al.*, 2010). This induced state has been achieved in

ethanol withdrawal-induced and chronic unpredictable stress paradigms (Chakravarty *et al.*, 2013; Holcombe *et al.*, 2013; Piato *et al.*, 2011). Ethanol withdrawal-induced anxiety can be suppressed by GABAergic and serotonergic mediated anxiolytics. Activation of the dopaminergic system (Ventral tegmental area-nucleus accumbensprefontal cortex) is largely known to mediate the reward pathway of most abused drugs, including ethanol (Pierce & Kumaresan, 2006). The need to restore this pathway after activation is known to be the underlying mechanism for physical and psychological dependence, of which heightened anxiety is a major component. Restoring the agent of abuse has been found to reduce the accompanying symptoms induced by withdrawal —an effect which was not observed in our study, possibly due to the acute nature of ethanol administration. This effect has been attributed largely to the re-activation of the dopaminergic reward pathway. However, the activation of this system might not account entirely for the abating signs of withdrawal, since dopaminergic activation by D1/D2 agonist have proved less useful in the management of withdrawal symptoms.

The amelioration of ethanol withdrawal-induced anxiety after *M. angolensis* extract administration may be suggestive of a synergistic agonistic interaction between the GABAergic, serotonergic and dopaminergic (reward) system.

Serotonin (5-HT) plays an essential role in the modulation of anxiety, aggression and depression (Lisboa *et al.*, 2007). In the zebrafish, 5-HT levels have been correlated with movement disorders and anxiety. Depletion of 5-HT levels, have been associated with induction of anxiogenic behaviours in zebrafish (Maximino *et al.*, 2013). Conversely, exposure to serotoninergic agents, reverses bottom dwelling and scototactic behaviour (Stewart *et al.*, 2013). Recent works by Nowicki *et al.* (2014) have revealed that

blockade of different serotonin receptor subtypes results in both anxiogenic and anxiolytic effects. Data derived from their work suggest that blockade of 5-$HT_{1B/D}$ causes anxiolysis whiles blockade of 5-HT_{1A}, 5-HT_2, and 5-HT_3 induced anxiogenic-like behaviours**.** This is in agreement with works done by Maximino *et al* in 2013 where they explored the serotonin receptor blockade and depletion to investigate the role of 5-HT levels in anxiety (Maximino et al., 2013). In the present study, pretreatment with methysergide (5-$HT_{2C/2B}$ antagonist), pizotifen (5-HT_1 and $5HT2_{A/2C}$ antagonist) and granisetron (5-$HT_{3A/3B}$ antagonist) did not induce significant anxiogenic behaviour both in the light dark and novel tank test. The reversal of the extract's anxiolytic effect, after pretreatment with the above antagonist, is suggestive of a possible activation of the serotonergic system in its anxiolytic effects. The interaction with the 5-HT system may be, at least in part, due to a possible blockade of the serotonin transporter (SERT/5-HTT) which increases the downstream concentration of 5-HT leading to activation of 5-HT (1-3) receptors. On the other hand, MAE may exhibit the anxiolytic effects by acting as an agonist at the 5-HT (13) receptors**.** However whether the activation is direct and/or as a result of decreased serotonin reuptake cannot be speculated based on the data obtained from these experiments. Similar effects were obtained after fluoxetine administration following pretreatment with antagonists. Fluoxetine is an SSRI with the principal mechanism of inhibiting of 5-HT reuptake, leading to increased concentrations of 5-HT in the synaptic cleft and ultimately, to greater postsynaptic neuronal activity (Fuller *et al.*, 1991). The reversal of the anxiolytic effect of fluoxetine may be due to reduced interaction with the 5-HT receptor due to blockade by 5-HT_{1-3} antagonists.

Flumazenil, a specific antagonist at the gamma-aminobutyric acid (GABA) type A receptor complex is known to antagonize the effects of benzodiazepines including the anxiolytic, sedative and hypnotic effects and hence provides an excellent tool for $GABA_A$ receptor studies (Mizuno, 2013; tue-Ferrer *et al.*, 1996). Reversal of anxiolysis by pre-treatment with flumazenil suggest a possible involvement of the $GABA_A$ receptor in the anxiolytic effects of MAE. The interaction on the GABAergic and serotonergic system may not be an exhaustive list of the potential site of interaction and hence further characterization may be needed in the future. However, the current evidence identified in this research gives some credence to the plants use in the management of anxiety.

Anxiety and depression are intimately linked and usually appear as comorbid states and treatment of both states positively affect the outcome of therapy (Outhoff, 2010).

Selective serotonin reuptake inhibitors are usually considered first-line treatment for patients with these disorders (Pollack, 2005). Several classes of drugs that modify serotonin (5-HT) neurotransmission have previously been explored for their possible role in depression, and schizophrenia (Levy & Van de Kar, 1992). Based on the above premise, the potential antidepressant effects of MAE was assessed in two acute depression models in mice: tail suspension and forced swim test. These models work on the principle that when mice are subjected to unavoidable inescapable stress, they assume escape oriented behaviours with intermittent moments of despair usually in the form of immobility (Steru *et al.*, 1985). Periods of immobility is known to model some aspects of depressive symptoms and hence most antidepressants are known to decrease

the duration of immobility. Consequently, these tests have been employed in the screening of potential antidepressant drugs.

In the TST, significant decrease in immobility duration was achieved after MAE, imipramine and fluoxetine treatment suggesting antidepressant activity. Antidepressants that inhibit serotonin and/or NA reuptake decrease immobility and increase swinging behaviour of mice in the TST —a behaviour that was not significantly altered in MAE- treated mice. Opioids, are known to decrease immobility whilst increasing curling behaviour (Berrocoso *et al.*, 2013; Berrocoso & Mico, 2009). Hence significant increase in the curling duration after MAE administration can be attributed to a possible interaction with the μ-opioid receptors.

Similarly MAE achieved antidepressant activity comparable to fluoxetine and imipramine in the FST. Antidepressants acting through the serotonergic system, including fluoxetine, selectively increase swimming behaviour. In addition, the FST differentiates between antidepressants that work through serotonergic mechanisms or noradrenergic mechanisms, as noradrenergic compounds selectively increase climbing behaviour (Detke *et al.*, 1995) and drugs with dual effects increased both swimming and climbing (Rénéric *et al.*, 1998). In this study, MAE caused a dose-dependent reduction in the immobility time, increase in the swimming behaviour as well as increase climbing duration. This behavioural profile may suggest that the mechanism of the antidepressant-like activity of MAE may be due to an interaction with both noradrenergic and serotonergic system.

M. angolensis, amongst other things, is purported to be traditionally used in the management of epilepsy (Chhabra et al., 1989). Preliminary investigations in mice indicated that the petroleum ether/ethyl acetate fraction of the stem bark offered some

degree of protection in delaying convulsion and reducing lethality only at 1000 mg kg^{-1}. However, at 300 mg kg^{-1}, MAE decreased the frequency and duration of convulsions after induction of PTZ-induced seizure in rats. This seeming contradiction can be attributed to the relatively lower dose of pentylenetetrazole used in the latter experiment. Generally a higher dose of PTZ, 85 mg kg^{-1}, is employed in the core battery test with the aim of identifying pro-convulsants whiles a lower does, 65 mg kg^{-1}, is usually employed in order to assess the anti-seizure properties, such as duration and frequency of convulsions, of at test compound. The GABAergic system is implicated in epilepsy since enhancement and inhibition of the neurotransmission of GABA will attenuate and enhance convulsion respectively (Meldrum, 2000; Quintans-Júnior *et al.*, 2008). PTZ blocks GABA-mediated Cl^- influx through an allosteric interaction in the Cl^- channel, thus leading to induction of convulsions in animals (Kubová, 2009; Velisek, 2006). Since the effect of PTZ is through GABA-mediated antagonism, it was posited that the protection may be due to an agonistic interaction with the $GABA_A$ receptor/system.

In this study, acute administration of MAE and diazepam, exhibited anticonvulsant activity against PTZ induced seizures by significantly delaying the occurrence of clonic seizures. In addition, MAE decreased the frequency and duration of the clonic seizures. The effect of MAE in PTZ-induced seizure models suggest its anticonvulsant action may be due to its interference with GABAergic system. To further confirm the possible contribution of GABAergic system in the anticonvulsant activity of MAE, flumazenil; a specific antagonist of the $GABA_A$ receptor complex (File *et al.*, 1986), was used in antiseizure mechanistic studies. Pretreatment with flumazenil reversed the

anticonvulsant effect of MAE suggesting involvement of $GABA_A$ receptor in its anticonvulsant effects. This further confirms the GABA enhancing activity of the MAE.

Current evidence suggests the involvement of nitric oxide in the onset, intensity and progression of seizures, including PTZ-induced seizures in mice (Tsuda *et al.,* 1997; Van Leeuwen *et al.,* 1995). Although some conflicting data exists, several works suggest decreased nitric oxide production leads to anticonvulsant effects whilst increased NO levels produce pro-convulsant effects in PTZ-induced seizures (Osonoe *et al.,* 1994). These effects were further corroborated by works done by Akula *et al.* (2008). The authors observed a potentiating effects of anticonvulsant activity after pretreatment with L-NAME (a non-selective nitric oxide synthase inhibitor) and a reversal of effect after pretreating with sildenafil (a phosphodiesterase 5 enzyme inhibitor) or L-Arginine (a precursor of nitric oxide).

Administration of L-arginine causes an increase in the release of NO. Consequently, large doses of L-arginine is also known to induce excitotoxicity due to excessive release NO and activation of the nitricoxidergic pathway (Garthwaite, 1991). In this present study administration of L-arginine alone did not alter the convulsion parameters significantly compared to the saline-treated group. However, pretreatment with Larginine attenuated the anticonvulsant effects of MAE.

Sildenafil is an inhibitor of PDE5 enzyme. An enzyme that actively breaks down cGMP to yield GMP (Boolell *et al.,* 1996), and hence increases the cGMP levels further potentiating nitric oxide-mediated effects, since a downstream effect of nitric oxide is the production of cGMP via Guanylate cyclase (Bredt & Snyder, 1992). The anticonvulsant effects was significantly reversed by a pre-treatment with sildenafil

further suggesting that MAE may interact with the nitricoxidergic pathway by increasing cGMP levels either directly or indirectly.

Oxidative stress is gaining grounds as a possible contributor to dysregulation underlying various pathologies such as anxiety and epilepsy. Under oxidative stress, the lipid rich constitution of the brain can undergo lipid peroxidation which can result in decreased membrane fluidity and altered neurotransmission. In oxidative stress, free radical are generated and overwhelm the normal protective mechanisms of the body. This offset in the normal oxidative state of the body have been implicated in several pathologies such as chronic inflammatory and neurodegenerative diseases. Surprisingly, there is still a debate whether oxidative stress is either a cause or consequence of these disease states. Irrespective of these uncertainties, it is well established that antioxidants or drugs with antioxidant potential offer a significant degree of protection with respect to the onset and/or progression of conditions such as epilepsy and depression. Several antiinflammatory agents have been shown to possess significant antioxidant properties which might account for their effectiveness in conditions such as arthritis where increased free radicals is a key component. Previous works done indicate that fractions of *Maerua angolensis* possess significant anti-inflammatory and *in vitro* antioxidant properties (Azi *et al.*, 2014; Meda *et al.*, 2013).

DPPH radical scavenging activity is widely used to assess hydrogen donating ability (antioxidant activity) of crude plants. DPPH is a stable free radical at room temperature which acts as a hydrogen radical acceptor to become a stable diamagnetic molecule (Soares *et al.*, 1997). In oxidative stress, free radicals including superoxide anions also contribute to the detrimental effects of dysfunctional oxidative states. The ability of MAE to scavenge superoxide anions generated *in vitro*, by a non-enzymatic phenazine

methosulfate nicotinamide adenine dinucleotide (PMS/NADH) coupling system using nitro blue tetrazolium (NBT) as the substrate dye, was also assessed. The decrease in absorbance of the purple formazan at 560 nm was considered as an indicator of superoxide anion scavenging activity. Similarly, lipid peroxidation damages tissues specifically membrane lipids. A simulation of lipid peroxidation in a lipid rich medium was employed in this study. The absorbance of the complex between malondialdehyde (product of the lipid peroxidation) and thiobarbituric acid (TBA-MDA). A reduction in the formation of the above complex was considered as an indicator of protection against lipid peroxidation.

In this study, it was observed that the petroleum ether/ethyl acetate fraction of *Maerua angolensis* possessed significant *in vitro* activity in the DPPH radical scavenging activity, superoxide anion-scavenging activity and thiobarbituric acid-reactive species assay (lipid peroxidation). The antioxidant property of MAE was further confirmed *in vivo* by assessing the degree of protection of the brain after administration of PTZ in rats. It has been established that PTZ administration increases reactive oxygen species and indicators of oxidative stress including lipid peroxidation (Bashkatova *et al.*, 2003; Ilhan *et al.*, 2005).

The human brain consumes ~20 % of the oxygen utilized by the body with a corresponding increase in reactive oxygen species (ROS). The lipid rich nature and the relatively lower level of SOD, catalase and GSH make the brain exceptionally vulnerable to oxidative stress making detoxification of ROS is an essential task in the brain (Dringen, 2000). In this aspect of the study, PTZ administration significantly reduced the levels of catalase and superoxide dismutase whilst reducing the level of reduced glutathione and degree of lipid peroxidation. This is indicative of significant

oxidative stress in the brain tissue. MAE or diazepam pretreatment resulted in decrease in glutathione and MDA levels in brain tissue. The activities of superoxide dismutase (SOD) and catalase (CAT) were also increased.

SOD is a potent protective enzyme that can selectively scavenge the $O_2^{\bullet}$ into H_2O_2 which is further converted by CAT to harmless H_2O and O_2 molecules. Under ineffective antioxidant enzyme status, lipid peroxidation in the cellular and subcellular membranes is the inevitable outcome of ROS injury. A decrease in the activity of superoxide dismutase and catalase could result in the decreased removal of superoxide and hydrogen peroxide radicals, which brings about harmful events that can lead to the neuronal tissue damage. Pretreatment with MAE showed increased activity of these enzymes, which suggests that MAE may have ability to prevent the deleterious effects induced by free radicals. Glutathione redox cycling is an extremely important system in cellular free radical detoxification. The increase in reduced glutathione (GSH) levels is usually indicative of significant oxidative stress in the tissue (Dringen, 2000). In the present study, the reduction in GSSG levels in MAE and diazepam-treated rats suggested significant protection against ROS insults. Lipid peroxidation is widely used to indicate oxidative injury in diseases (Halliwell & Chirico, 1993). A significant decrease in lipid peroxidation in the brains of MAE and diazepam treated animals.

Significant alteration in these oxidative parameters may be due to antagonistic effects of MAE and diazepam against PTZ. An additional antioxidant activity may also account for the neuroprotective effect of MAE treatment. This study demonstrates that coadministration of MAE protects the brain from oxidative stress resulting from PTZ administration. Taken together, the data support a role for MAE in attenuation of

neuronal damage after seizure attacks, in part at least, by inhibition of oxidative stress injuries.

CONCLUSIONS

Results from this study indicates that the petroleum ether/ethyl acetate fraction of *Maerua angolensis* stem bark possesses anxiolytic, antidepressant and anti-convulsant effects. The anxiolytic effects is mediated at least in part by the GABAergic and serotonergic system. *Maerua angolensis* extract interacts with the GABAergic system and L-arginine-NO-cGMP pathways to produce its anti-convulsant effects. The extract also exhibits significant in-vivo and in-vitro anti-oxidant effects which may account for part of its CNS effects.

Data gathered from this research supports the plants use for management of epilepsy and provide further evidence for alternative uses of the plant.

PROPOSAL FOR FURTHER RESEARCH

The crude extract obtained from the stem bark of *Maerua angolensis* have showed preliminary promise in amelioration of anxiety, depression and chemo-convulsant induced seizures. The next logical step will be to isolate and test the effectiveness of the isolates that might be responsible for the biological effects registered in the crude extract. The effective isolate (s) in the preliminary antidepressant and anti-convulsant models will be used for further studies.

Identification of mechanism(s) of antidepressant activity.

Initially, the anti-depressant effects of the isolates will be explored using both acute and chronic (chronic mild stress) paradigms. Further, discriminatory and non-selective depletion; using either pCPA , AMPT, reserpine or methyldopa, will be employed in the zebrafish and mice to assess the possible role of serotonin, dopamine and norepinephrine in the possible mechanism of action. Corresponding brain monoamine levels will be assessed either with spectrofluorometric methods or high performance liquid chromatography methods to assess the level of depletion and the effects of the extract/isolates on brain monoamines.

Identification of anticonvulsant effects

The potential for the isolate to protect against convulsions induced by chemoconvulsants with different mechanism of action will also be assessed in PTZ (60 mg/kg), picrotoxin (3 mg/kg), isoniazid (300 mg/kg), strychnine (0.5 mg/kg) and

4Aminopyridine (12 mg/kg) induced seizures. In the CNS, NMDA receptor activation contributes to many aspects of neuronal signaling and excitability; NMDA receptor

antagonists blocked or delayed seizure activity in rats and mice. The project will seek to identify the involvement of NMDA receptors in the protection provided by the isolates in PTZ- induced seizure.

To further evaluate the antiepileptic (versus anticonvulsant) potential of the test compounds the kindled rat assay described by Stöhr *et al.* (2007) will be employed making room for slight modifications.

The cobalt/homocysteine assay will be employed to assess the efficacy of the isolated compounds in status epilepticus induced in Sprague-Dawley rats. The acetylcholinesterase activity will be subsequently measured using Amplite™ Colorimetric Acetylcholinesterase Assay Kit to give an indirect measure of cognitive impairment or protection induced by pre-treatment with the isolate(s).

Assessment of toxicity

Toxicity assay in mice and zebrafish will be conducted to assess their potential for altering normal physiology and biochemical processes.

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