ANTICONVULSANT AND ANTIDEPRESSANT EFFECTS OF AN ETHANOLIC EXTRACT OF THE LEAVES OF PSEUDOSPONDIAS MICROCARPA (ENGL.) A. RICH. (ANACARDIACEAE) IN ANIMAL MODELS

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by

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DECLARATION

I hereby declare that I am the sole author of this thesis. 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. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

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ABSTRACT

The search for novel pharmacotherapy from medicinal plants for central nervous system (CNS) disorders has become of importance since new agents with improved efficacy for more effective therapy are required. *Pseudospondias microcapa* has been extensively used in Ghana and other parts of Africa as medication for various diseases including CNS

disorders. The present study examined the anticonvulsant, antidepressant, as well as some neurobehavioural properties of an ethanolic extract of the leaves of *Pseudospondias microcarpa* in animal models. Preliminary phytochemical screening of *Pseudospondias microcarpa* extract (PME) revealed the presence of saponins, tannins, glycosides, terpenoids, flavonoids and alkaloids. Neuropharmacological activities of the extract in mice was investigated. The extract produced sedation and analgesia in the Irwin test with an LD_{50} above 3000 mg kg^{-1}. PME potentiated pentobarbitone sleeping time and was metabolised by hepatic enzymes. It however showed no effect on locomotor activity or motor coordination. Furthermore, the extract blocked convulsions induced by PTZ and showed analgesic activity in the tail withdrawal test. Based on the findings from the preliminary studies, the anticonvulsant activity of PME and possible mechanism(s) in animal models was further explored. The extract significantly delayed the onset as well as decreased the duration and frequency of pentylenetetrazole (PTZ)-, picrotoxin (PTX)- and strychnine (STN)-induced seizures. In addition, pretreatment of mice with PME before administration of 4-aminopyridine (4-AP) or isoniazid (INH) significantly increased the latency to seizures and reduced the incidence of mortality.The $GABA_A$ receptor antagonist, flumazenil, reversed the anticonvulsant effect of PME, further suggesting the possible involvement of the GABAergic system in its action. Furthermore, the extract protected against 6-Hz psychomotor seizures but had no effect in the MES test. Moreover, the anticonvulsant effect of PME (100 mg kg^{-1}, *p.o.*) was prevented by pre-treatment with L-arginine or sildenafil. However, N-nitro-L-arginine methyl ester (L-NAME) or methylene blue (MB) augmented the anticonvulsant effect of PME. Acute treatment with PME reduced immobility of mice in the TST and FST. The antidepressant-like effect of PME (100 mg kg^{-1}, *p.o.*) in the TST was blocked by *p*-Chlorophenylalanine and cyproheptadine but not prazosin, propranolol or yohimbine, thus suggesting 5-HT pathway may be involved in the action of PME. This is further confirmed by the potentiation of 5-hydroxytryptophaninduced head-twitch response by PME in mice. Pretreatment with a combination of reserpine and α-methyl-p-tyrosine (AMPT) completely prevented the behavioural effects of PME, fluoxetine and desipramine. Concomitant administration of D-cycloserine and the extract potentiated the anti-immobility effect. In contrast, D-serine a full agonist of glycine/NMDA receptors abolished the effects. In the repeated open-space swim test, mice showed a progressive decrease of active swimming and a corresponding increase in immobility that persisted for several days. However, the increased inactivity was reversed selectively by the extract and the classical antidepressant drugs, in that they increased distance swum and decreased immobility. Moreover, the depressive-behaviour

induced by the repeated open-space swim test impaired spatial learning and memory performance in the Morris water maze (MWM) test. This was however reversed by the extract. The longterm effects of chronic mild stress (CMS) and PME treatment on depressive, anxiety-like behaviour and cognitive function were also investigated. Exposure of mice to the CMS paradigm displayed decreased sucrose intake, poor coat state, decreased grooming frequency (splash test), increased immobility, increased anxiety and impaired cognitive function. These effects were however reversed by chronic PME treatment. Anxiolytic-like effects of the extract in behavioural paradigms of anxiety was also evaluated. *P. microcarpa* treated mice (30-300 mg kg^{-1}, *p.o.*) exhibited anxiolytic-like activity similar to diazepam in all the anxiety models used. PME increased open arm activity in the elevated plus maze (EPM) as well as increasing the time spent in the lit area in relation to the time spent in the dark area of the light/dark box. The extract also increased the number of central entries and time spent in the center of the open field. In addition, an increase in social interaction and decreased stress-induced hyperthermia was obseverd for PME-treated mice. Acute and subacute toxicity in rats did not show deaths after 14 days treatment with the extract (30– 3000 mg kg^{-1}). Haematological or serum biochemical parameters were not affected except decrease in lymphocytes (100−3000 mg kg^{-1}), triglycerides (100 mg kg^{-1}) and very low density lipoproteins (100 mg kg^{-1}). Histopathological examination did not reveal toxic effect on the stomach, heart, liver, brain, kidney and spleen. Results of the present study suggests that PME possesses sedative, analgesic, anticonvulsant, antidepressant and
anxiolytic effects without affecting motor coordination.

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ABBREVIATIONS

4-AP	4-aminopyridine
5-HT	5-hydroxytryptamin, serotonin
5-HTP	5-hydroxytryptamine
AEDs	Antiepileptic drugs
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid
AMPT	α-methyl-p-tyrosine
ANOVA	Analysis of variance
BDZs	Benzodiazepines
cAMP	Cyclic adenosine monophosphate
CBZ	Carbamazepine
CFN	Caffeine
cGMP	Cyclic guanosine monophosphate
CMS	Chronic mild stress
CNS	Central nervous system
CSF	Cerebrospinal fluid
DA	Dopamine
DCS	D-cycloserine
DES	Desipramine
DS	D-seine
DZP	Diazepam
EAA	Excitatory amino acid
EPM	Elevated plus maze
EPSPs	Excitatory postsynaptic potentials

EST	Ethosuximide
FBM	Felbamate
FLX	Fluoxetine
FST	Forced swimming test
GABA	Gamma aminobutyric acid
GAD	Glutamic acid decarboxylase
GAFCO	Ghana Agro food Company
GBP	Gabapentin
GMP	Guanosine monophosphate
HDs	Head dips
HTR	Head-twitch response
HVACC	High-voltage activated calcium channel
KNUST	Kwame Nkrumah University of Science and Technology
INH	Isoniazid
i.p.	Intraperitoneal
LDB	Light dark/box
LEV	Levetiracetam
L-NAME	N-nitro-L-arginine methyl ester
LTG	Lamotrigine
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MB	Methylene blue
MDD	Major depressive disorder
MES	Maximal electroshock seizure
mGluR	Metabotropic glutamate receptors

MOR	Morphine hydrochloride
MPE	Maximal possible effect
MWM	Morris water maze
NA	Noradrenaline
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NSF	Novelty suppressed feeding
OCBZ	Oxcarbazepine
OFT	Open field test
PHB	Phenobarbitone
PBT	Pentobarbitone
*p*CPA	Para-chlorophenylalanine
PDE5	Phosphodiesterase 5
PGB	Pregabalin
PHE	Phenobarbitone
PHT	Phenytoin
PME	*Pseudospondias microcarpa* extract
p.o.	Per os
PTX	Picrotoxin
PTZ	Pentylenetetrazole
SAPs	Stretch-attend postures
s.c.	Subcutaneous
sGC	Soluble guanylate cyclase
SNRIs	Serotonin noradrenaline reuptake inhibitors

SSRI	Selective serotonin reuptake inhibitor
STN	Strychnine
TCA	Tricyclic antidepressant
TGB	Tiagabine
TL	Transfer latency
TPM	Topiramate
TST	Tail suspension test
IEPSPs	Inhibitory excitatory postsynaptic potentials
IPSPs	Inhibitory postsynaptic potentials
U/PHDs	Unprotected/Protected head dips
U/PSAPs	Unprotected/Protected stretch-attend postures
VMAT	Vesicular monoamine transporter
VPA	Valproate
ZNS	Zonisamide

Chapter 1 INTRODUCTION

1.1 GENERAL INTRODUCTION

For centuries people have used plants for healing. Natural products have been used with varying success to cure and prevent diseases throughout history, and continue to provide mankind with new remedies (Raskin *et al.*, 2002). The first records, written on clay tablets in cuneiform, are from Mesopotamia and date from about 2600 BC; among the substances that were used were oils of *Cedrus* species (Cedar) and *Cupressus sempervirens* (Cypress), *Glycyrrhiza glabra* (Licorice), *Commiphora* species (Myrrh) and *Papaver somniferum* (Poppy juice), all of which are still in use today for the treatment of ailments ranging from coughs and colds to parasitic infections and inflammation (Gurib-Fakim, 2006).

The interest in nature as a source of potential chemotherapeutic agents continues. Natural products and their derivatives represent more than 50 % of all the drugs in clinical use in the world. During the last few decades, at least a dozen potent drugs have been derived from flowering plants: reserpine and other anti-hypertensive and tranquilizing alkaloids from *Rauwolfia* species; pilocarpine to treat glaucoma and dry mouth, derived from a group of South American trees (*Pilocarpus* sp.) in the citrus family and laxative agents from *Cassia* species (Gurib-Fakim, 2006).

The World Health Organization (WHO) estimates that about 75 % of the world population—primarily those of developing countries—depend on traditional remedies (mainly herbs) for the healthcare of its people (Gilani and Atta ur, 2005). For instance, in Ghana, available estimates show that between 60 and 70 % of people rely on traditional medical systems for their health needs (Addo-Fordjour *et al.*, 2011). Patients with a variety of chronic illnesses, including epilepsy and depression, take herbal therapies for many reasons. For example, patients in developed countries may view herbal therapies as natural and time-tested and therefore safe compared with what are perceived as synthetic drugs. Also, in developing countries, there may be access to herbal therapies but not to pharmaceuticals, because of cultural and economic factors (Schachter, 2009). Considering the great reliance on traditional medicinal plants for treatment of diseases and the potential for drug discovery, it becomes relevant to search for potent, effective and relatively safe plant medicines as well as to scientifically validate success claims about plants already in use by traditional medicine practitioners.

Pseudospondias microcarpa, also known as the African grape tree in the West African subregion, is one of the plants used for managing various diseases including CNS disorders (Burkill, 1985). However, despite the wide use of the plant in traditional medical practice, there is no scientific data in literature on its pharmacological activity. Thus, this work seeks to investigate the pharmacological activity as proposed in traditional medicine.

1.2 THE PLANT *PSEUDOSPONDIAS MICROCARPA*

1.2.1 Name

Botanical name: *Pseudospondias microcarpa* (Engl.) A. Rich.

Family: Anacardiaceae

Local name: *Katawani*

1.2.2 Description

It is a tall tree usually up to 35 m high with the main stems stout and spreading. The bough is short, 3-18 m tall, up to 2 m in diameter, twisted and strongly buttressed. The bark is greyish-yellow, falling off in large flakes. Leaves 5-17-foliolate; petiole and rachis 10-50 cm long, striate, glabrous, rarely pubescent. The fruits, nearly 2.5 cm long, are red or bluish black when ripe (Burkill, 1985).

Figure 1.1 Leaves of *Pseudospondias microcarpa* plant

1.2.3 **Ecological and Geographical Distribution**

It is common and widespread in damp sites often on stream-banks, occurring throughout tropical Africa including Ghana, Gabon, Cote d'Ivoire, Liberia, Sierra Leone, Congo, Cameroon and Nigeria (Burkill, 1985).

1.2.4 **Traditional Uses**

1.2.4.1 ***Medicinal Uses***

Various parts of the plant are used extensively in African traditional medicine and these uses may be broadly classified as follows:

Disorders of the central nervous system: The tree is suspected of having a sedative effect on those who sit or sleep under it, hence the Akan name meaning 'close your eyes'. The plant is therefore used in Ghana as a sedative and for treating general CNS disorders (Burkill, 1985).

Infections, infestations and skin disorders: The bark contains a little reddish resin, which is used in Liberia to treat diseases affecting the eyes (Burkill, 1985). A bark-decoction is also used in Gabon as a diuretic for treating urethral discharge and in Congo the powdered bark is eaten for the treatment of gonococcal complications (Burkill, 1985). It is also held to be good for ulcers on the soles of the feet when compounded with the heartwood of *Pterocarpus soyauxii* (Leguminosae: Papilionoiideae), the feet having been previously washed in the sap of *Aframomum giganteum* (Oliv. & Hanb.) K. Schum. (Zingiberaceae) (Burkill, 1985).

Pain and inflammation: A bark-decoction is used in Gabon for toothache and in Congo the bark is put into vapour baths and the lees used in frictions for persistent rheumatic pain (Burkill, 1985).

Gastrointestinal disorders: In Congo, a bark-decoction is taken for stomach complaints. The bark is also used in Congo and Cote d'Ivoire for its purgative and diuretic properties in the treatment of jaundice (Burkill, 1985).

Respiratory disorders: The bark is used in Cote d'Ivoire for its purgative properties in the treatment of cough and in Gabon for febrile lumbago, pains in the ribs, cough and asthenia (Burkill, 1985).

1.2.4.2 *Non-Medicinal Uses*

Fruits of *P. microcarpa* are eaten in various parts of Africa and in Gabon they are used as fish-bait. In Ghana, the seeds are used as beads. The wood is used across Africa for construction works and sometimes used for poles and planks (Burkill, 1985).

1.2.5 Previous Work on *Pseudospondias microcarpa*

Although the plant has been widely used in Africa, there is little data in established scientific literature on its pharmacological activities.

1.2.5.1 *Phytochemical Constituents*

Yondo *et al.* (2009), reported on a survey conducted to evaluate the antioxidant potential and phytochemical constituents of two different stem bark extracts (methanol-methylene chloride and aqueous) of three Cameroonian medicinal plants used to manage parasitic diseases among which was the plant *Pseudospondias microcarpa*. In the study, saponins,

phenols, terpenoids, flavonoids, cardiac glycosides and coumarines were positive in both the methanol-methylene chloride and aqueous extracts of *P. microcarpa*. The leaves of *P. microcarpa* has also been reported to contain alkaloids, tannins, terpenoids and steroids (Akpona *et al.*, 2009).

1.2.5.2 ***Antioxidant Activity***

The methanol-methylene chloride and aqueous stem bark extracts of the plant possess potent antioxidant activity (Yondo *et al.*, 2009).

1.2.5.3 ***Antimicrobial Activity***

Crude extracts of the leaves of *P. microcarpa* (aqueous, petroleum ether and dichloromethane) possess antimicrobial activity against some microorganisms including *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* in the disc diffusion and agar well assays (Kisangau *et al.*, 2008).

1.3 EPILEPSY

1.3.1 Background

Epilepsy is a brain disorder characterized by occurrence of more than one epileptic seizure with a continuing predisposition to generate more epileptic seizures associated with neurobiologic, cognitive, psychological, and social disturbances (Raol and Brooks-Kayal, 2012). Epilepsy is the most common neurological disorder after stroke, with 0.5 % prevalence, and a 2–3 % life time risk of being given a diagnosis of epilepsy (Browne and Holmes, 2001; Löscher, 2002a). Recent studies both in the developing and in the developed world revealed that if properly treated up to 70 % of people with this condition could live productive and fulfilling lives, free from seizures. It has to be acknowledged that more than 80 % of people with epilepsy live in developing countries, where the condition remains largely untreated (de Boer *et al.*, 2008). The reasons for the unavailability of treatment include: inadequate health delivery systems, lack of trained personnel, lack of essential drugs, and traditional beliefs and practices that often do not consider epilepsy as a treatable condition (de Boer *et al.*, 2008). It often carries with it a high economic and social burden resulting from high healthcare costs and from loss of earnings, productivity, social interaction, self-esteem and independence (Gilliam, 2005). The unpredictable nature of

epileptic seizures, usually involving loss of consciousness, tends to impose an intense psychosocial burden and leads to restrictions in normal daily activities (Gilliam, 2005). The epileptic seizure is an event consisting of a sudden and transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain (Raol and Brooks-Kayal, 2012). An epileptic seizure can be as subtle as a momentary lapse of attention to very obvious involving violent and involuntary contractions of muscles (Elger and Schmidt, 2008; Raol and Brooks-Kayal, 2012). The phenotype of each seizure is determined by the point of origin of the hyperexcitability and its degree of spread in the brain. By convention, the diagnosis of epilepsy requires that the patient has had at least two unprovoked seizures (Elger and Schmidt, 2008). However, a person with isolated nonrecurrent, externally provoked seizures that are also caused by excessive discharge of cerebral neurons is not thought to have epilepsy as long as the seizures are not recurrent and each seizure is preceded by a provocation (e.g., substance abuse, fever, exposure to alcohol combined with lack of sleep) (Elger and Schmidt, 2008). In only one of three patients, the cause of epilepsy is known and includes brain tumors, CNS infections, traumatic head injuries, developmental malformations, perinatal insults, cerebrovascular disease, febrile seizures, and status epilepticus (Browne and Holmes, 2001). Approximately 10-25 % of epilepsies have a presumed genetic base, these are called idiopathic. In the remaining patients, the cause of epilepsy is currently unknown (Schmidt, 2002).

1.3.2 **Classification of Seizures**

Seizures may be classified by their semiology as partial or generalized (ILAE, 1981; Berg *et al.*, 2010). In partial seizures, the excess neuronal discharge is thought to originate within one region of the cerebral cortex. Partial seizures, also referred to as focal seizures can be simple or complex and result from a localized brain disturbance. The site of dysfunction determines the clinical manifestation of partial seizures (Elger and Schmidt, 2008).

Symptoms that patients with partial seizures experience and remember are called simple partial seizures – "simple" means that consciousness is not impaired. Patients often refer to these as auras. Typical symptoms of simple partial seizures are fear, nausea, jerking of the arm and leg on one side of the body, though many other symptoms may occur, and usually in a stereotyped fashion for each patient. Other patients may not be consciously aware of their partial seizures because they abruptly lose consciousness, which is called a complex

partial seizure ("complex" means that consciousness is impaired). During complex partial seizures, patients appear to stare off into space and either remain still or exhibit repetitive non-purposeful behaviours called automatisms such as chewing motions, lip smacking, repeating words or phrases, aimless walking or running, or undressing. Complex partial seizures typically last less than 3 minutes and may be immediately preceded by a simple partial seizure or followed by a generalized tonic–clonic seizure. Because their consciousness is impaired during complex partial seizures, patients have no or incomplete memory of what takes place during their occurrence (Klitgaard *et al.*, 2008).

In generalized seizures, the discharge bilaterally and diffusely involves the entire cortex. Generalized seizures may impair consciousness and cause bilateral motor manifestations from the onset. Such attacks often have a genetic or metabolic cause. Common types of generalized seizures include absence, tonic–clonic, and myoclonic seizures (Elger and Schmidt, 2008).

1.3.3 **Pathophysiology of Epilepsy**

Several neurotransmitters or neuromodulators have been shown to play a role in neuronal excitation. However, L-glutamate and gamma-amino butyric acid (GABA) are the major excitatory and inhibitory neurotransmitters in the brain respectively. An abnormal function of either of these could result in a seizure (Avanzini and Franceschetti, 2003).

1.3.3.1 ***GABA Receptors***

Gamma-amino butyric acid is the main inhibitory neurotransmitter in the central nervous system (Olsen and Avoli, 1997), and impairment of its function is widely recognized to provoke seizures, while facilitation has an anticonvulsant effect (Löscher, 1999). The two main types of ionotropic GABA receptors ($GABA_A$ and $GABA_B$) are coupled to chloride and potassium ionophores, respectively. A third receptor subtype, $GABA_C$, has also been reported but its physiological function is unclear at present. The inflow of chloride ions and outflow of potassium ions prompted by binding of GABA to its receptors leads to membrane hyperpolarisation and inhibitory postsynaptic potentials (IPSPs) (Avanzini and Franceschetti, 2003). Considerable evidence suggests that impaired GABA function can cause seizures and may be implicated in some types of epilepsies. $GABA_A$ blockers (pentylenetetrazole, bicuculline, penicillin, and picrotoxin) are well-known epileptogenic

agents and are commonly used in experimental studies. Altered $GABA_A$ receptor function may contribute to inherited or acquired epilepsies (Avanzini and Franceschetti, 2003). Epilepsy may result from genetic predisposition that leads to a decrease in GABA-mediated inhibition (Najm *et al.*, 2001). A low number of GABAergic neurons has been found in brain tissue resected from patients with refractory epilepsies (Avanzini and Franceschetti, 2003).

1.3.3.2 ***Excitatory Amino Acid Receptors***

Glutamate is the predominant excitatory neurotransmitter in the mammalian brain and exerts its pharmacological effects on several receptors, classified into ionotropic and metabotropic families (Kwan *et al.*, 2001). Ionotropic glutamate receptors are classified into three specific subtypes namely α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA), which form ligand-gated ion channels, permeable to Na^+ and, depending on subtype and subunit composition, Ca^{2+} ions (Trist, 2000). The NMDA receptor is further distinguished by having glycine as a coagonist. The AMPA and kainate subtypes of the glutamate receptor are involved in fast excitatory neurotransmission, while the NMDA receptor is recruited during periods of prolonged depolarization (Meldrum, 2000). The sodium- and calcium-dependent components of the NMDA receptor are powerful enough to sustain membrane
depolarisation and can significantly increase and prolong excitatory postsynaptic potentials (EPSPs). Excitatory amino acid (EAA) receptor agonists, such as kainic acid and NMDA, induce various types of epilepsies in animals, thus illustrating the epileptogenic potential of EAA systems in the brain. Changes in excitatory amino acid receptors that notably increase NMDA-dependent excitability have been found in surgical specimens of dysplastic human cortex (Najm *et al.*, 2000). Moreover, rearrangements of circuitry that cause the selective facilitation of the NMDA-dependent EPSP have been shown in temporal-lobe epilepsy (Avanzini and Franceschetti, 2003).

Metabotropic glutamate receptors (mGluRs) are second-messenger coupled receptors that have diverse effects on the cellular and synaptic properties of nerve cells. Recent evidence suggests that mGluRs may play a role in the production of neuronal plasticity and epilepsy. The mGluRs include at least eight receptor subtypes. Various studies show evidences that mGluR I subtypes are proconvulsants and that mGluR II and III subtypes are

anticonvulsants (Cartmell *et al.*, 1993; Taylor *et al.*, 1995; Merlin *et al.*, 1995; Najm *et al.*, 2001).

1.3.4 **Pharmacological Management of Epilepsy**

For the vast majority of people with epilepsy, initial therapy consists of pharmacological treatment with one or more of the established antiepileptic drugs (AEDs). These medications include phenytoin (PHT), carbamazepine (CBZ), and valproate (VPA, valproic acid). Barbiturates such as phenobarbital , certain benzodiazepines (BZDs), and ethosuximide (ESM), are now used infrequently. Others are the second-generation AEDs introduced into the US market since 1993 (i.e., felbamate, FBM; gabapentin, GBP; lamotrigine, LTG; topiramate, TPM; pregabalin, PGB; tiagabine, TGB; oxcarbazepine, OCBZ; levetiracetam, LEV; and zonisamide, ZNS) (White *et al.*, 2007).

1.3.5 **Mechanisms of Action of Antiepileptic drugs**

1.3.5.1 ***Modulation of Voltage-Gated Ion Channels***

Actions at both the voltage-gated sodium and calcium channels stabilize neuronal membranes, block action potential firing and propagation, reduce neurotransmitter release, and prevent seizure spread. Interestingly, the vast majority of clinically available AEDs are thought to exert their anticonvulsant effects at voltage-gated ion channels including sodium (PHT, CBZ, VPA, FBM, LTG, TPM, OCBZ, and ZNS), calcium (VPA, ESM, FBM, LTG, TPM, and GBP), and potassium (TPM) channels (Guerrini and Parmeggiani, 2006; White, 1999; 2007). However, VPA, ESM, and perhaps ZNS are unique in that they appear to attenuate voltage-dependent, low-threshold T-type calcium currents in thalamocortical neurons, thereby interrupting the thalamic oscillatory firing patterns associated with spikewave seizures. In contrast to VPA, ESM, and ZNS, several other AEDs or their active metabolites modulate N-type (LTG and OCBZ), L-type (FBM and TPM), and P-type (LTG) calcium currents (Stefani *et al.*, 1997; Zhang *et al.*, 2000). In addition, LEV has been found to modulate high-voltage activated calcium current (HVACC) mainly the N-type and to a lesser extent the P/Q type (Pisani *et al.*, 2004). The ability of TPM to activate potassium currents is somewhat unique among the AEDs and this effect may contribute to membrane hyperpolarization (Herrero *et al.*, 2002; White *et al.*, 2007).

1.3.5.2 ***Enhanced Inhibition***

Once released from presynaptic nerve terminals, the inhibitory neurotransmitter γaminobutyric acid binds to both $GABA_A$ and $GABA_B$ receptors (Macdonald and Olsen, 1994). The $GABA_A$ receptor complex is a multimeric, macromolecular protein that forms a chloride-selective ion pore (Macdonald and Olsen, 1994). Several of the available AEDs have been demonstrated to potentiate GABAergic neurotransmission mediated by the $GABA_A$ receptor complex (e.g., BZDs, barbiturates, FBM, and TPM) (Macdonald and

Kelly, 1995; White *et al.*, 2007). Furthermore, several AEDs have been found to increase GABAergic tone by decreasing GABA metabolism (i.e., VPA) , preventing GABA reuptake (i.e., TGB) (During *et al.*, 1992), or increasing GABA synthesis (i.e., VPA and GBP) (Löscher, 1981; Löscher *et al.*, 1991).

1.3.5.3 ***Glutamate-Mediated Excitation***

Inhibition of the neuronal release of glutamate and blockade of its receptors has received substantial attention in the search for novel AEDs (Meldrum, 2000; Kwan *et al.*, 2001). Although none of the frequently used AEDs exert their pharmacological effects solely by an action on the glutamatergic system, blockade of ionotropic glutamate receptors is believed to contribute to the antiepileptic activity of many compounds (Kwan *et al.*, 2001). Furthermore, several AEDs have been reported to inhibit glutamate release, although this effect may be more indicative of their actions on neuronal Ca^{2+} channels than a direct effect on the glutamatergic system (Stefani *et al.*, 1997; Kwan *et al.*, 2001). Among the available AEDs, FBM and TPM are novel in that they possess an ability to reduce glutamatemediated excitatory neurotransmission by modulation of NMDA (FBM), AMPA (TPM), and kainate (TPM) Receptors (White *et al.*, 2007).

1.3.5.4 ***Carbonic Anhydrase Inhibition***

Several AEDs, including acetazolamide, topiramate and zonisamide, act either principally or in part by inhibiting certain carbonic anhydrase isoforms (Simeone and Rho, 2009). The clinical use of carbonic anhydrase inhibitors covers the last half century, but it is unclear how anticonvulsant effects are achieved with these agents. Inhibition of brain carbonic anhydrase results in an increase in total CO_2 concentrations in neurons, and increased pH and a decreased HCO_3^- concentration in neuroglia (Simeone and Rho, 2009). The potassium

ions shift to the extracellular compartment to buffer the acid-base status. It is well known that a reduction in extracellular K^+ leads to diminished neuronal excitability, but the impact of decreased extracellular pH is less clear, despite the fact that – depending on subunit composition – extracellular protons can block NMDA receptor-mediated excitation and enhance $GABA_A$ receptor function (Simeone and Rho, 2009).

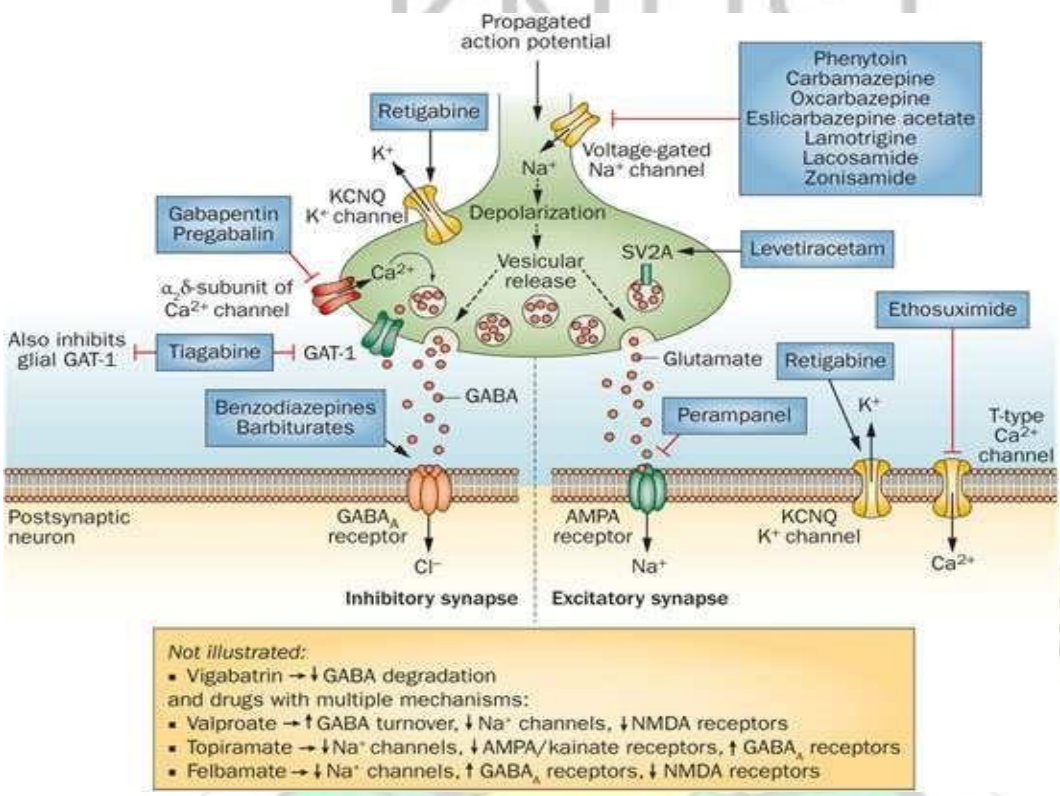


Figure 1.2 Mehanism of action of antiepileptic drugs (Adopted from Epilepsy: Perampanel-new promise for refractory epilepsy?)

1.3.6 **Unmet Medical Needs**

Despite unprecedented growth in the number of approved pharmacotherapies, seizures in approximately 30 % of patients with epilepsy remain refractory, and many others have unacceptable medication-related side effects (Brodie, 2005). Moreover, because only a small fraction of these patients are candidates for brain surgery, there continues to be an unmet medical need for effective and tolerable treatments for epilepsy (Klitgaard *et al.*, 2008). Currently available AEDs suppress seizures, but do not resolve the pathophysiological process underlying a patient's epilepsy, nor do they prevent the development of seizures in patients without epilepsy but at high risk, such as those who

suffer a serious brain injury. There is therefore a need for drugs that are antiepileptogenic (Schachter, 2002; Klitgaard *et al.*, 2008).

1.3.7 **Experimental Models of Epilepsy**

For AED discovery, which necessitates screening of large numbers of compounds, animal models should be easy-to perform, time- and cost-efficient, and predictive of clinical activity. Apart from the bromides and phenobarbitone, the anticonvulsant effect of all old and new AEDs was first investigated in animal models, such as the maximal electroshock seizure (MES) or the pentylenetetrazole (PTZ) seizure tests in mice or rats, confirming that clinical activity can be predicted by employing these animal models (Löscher, 2002a).

Drugs interacting with the GABAergic system are probably the most frequently used type of chemoconvulsant. This group of drugs involves $GABA_A$ receptor antagonists and also drugs that block GABA. The antagonists bind to different binding sites of $GABA_A$ receptors, but despite these differences, they tend to induce similar seizures (Kubova, 2009).

Pentylenetetrazole noncompetitively antagonizes the $GABA_A$ receptor, likely through a benzodiazepine binding site and an allosteric interaction in the chloride channel (Kubova, 2009). PTZ induces all four behavioural phenomena: freezing, myclonic twitches, clonic seizures, and tonic-clonic seizures (Velíšek, 2006). It has been suggested that PTZ-induced clonic seizures model myoclonic seizures and generalized tonic–clonic seizures induced by PTZ represent a model of primary or secondary generalized seizures in humans (Kubova, 2009).

The $GABA_A$ receptor antagonist bicuculline, and the chloride ion-channel blocker picrotoxin, induce similar seizure phenomena as seen with PTZ. Bicuculline is believed to exert its epileptogenic effect through blocking GABAergic neurotransmission by competing with GABA for its binding site (De Deyn *et al.*, 1992; Kubova, 2009). Picrotoxin (PTX) seizures develop at a slower pace than PTZ and bicuculline seizures, and they also occur less reliably. However, the very specific mechanism of action (even compared with bicuculline) and the very good solubility provide a small advantage over bicucullineinduced seizures (Velíšek, 2006).

The suppression of glycinergic inhibition, by the alkaloid strychnine, induces generalized clonic or tonic–clonic convulsions. Typically, the tonic seizure phase is accompanied by apnotic pause and high mortality (Velíšek, 2006). The mechanism of the convulsant action of strychnine has been defined as a blockade of chloride channel associated with glycine receptors (Velíšek, 2006).

1.4 DEPRESSION

1.4.1 Background

Depression is a common psychiatric disorder characterized by change in mood, lack of interest in the surroundings, and psychosocial and physical impairment (Girish *et al.*, 2012; Nakajima *et al.*, 2010). Cognitive symptoms include a sense of hopelessness and helplessness, worthlessness, and guilt. Physiological symptoms include changes in appetite and sleep, fatigue, and concerns about aches and pains. In additon, diminished sexual interest is commonly reported in depressed individuals. Behavioural symptoms include decreased activity, often the result of anhedonia, which is the loss of interest in and an inability to derive pleasure from activities that previously were interesting and pleasurable (Cardemil, 2002).

Depression is an important global public health issue, both because of the relatively high lifetime prevalence ranging from 2 % to 15 % and because it is associated with substantial disability (Moussavi *et al.*, 2007; Dhingra *et al.*, 2012). The World Health Organization (WHO) estimates that by 2020, depression will become the second largest cause of global disease problems in the world, only behind ischemic heart disease (Lopez and Murray, 1998; Mao *et al.*, 2008). It is highly heritable, with roughly 40–50 % of the risk for depression being genetic, although the specific genes that underlie this risk have not yet been identified. The remaining 50–60 % of the non-genetic risk also remains poorly defined, with suggestions that early childhood trauma, emotional stress and physical illness (Berton and Nestler, 2006).

Depression represents one of the most prevalent comorbid disorders in patients with epilepsy (Kanner and Balabanov, 2002; Harden, 2002). Although psychosocial aspects of experiencing a seizure may contribute to depression associated with epilepsy, there is a growing consent that this condition has neurobiological basis (Kanner, 2005). For instance,

both clinical and experimental evidence suggest that imbalances in neurotransmitters as GABA, glutamate, norepinephrine and serotonin, which are commonly observed in epilepsy patients, may simultaneously contribute to the evolvement of depression (Kanner and Balabanov, 2002; Jobe, 2003; Kondziella *et al.*, 2007; Kanner, 2005).

1.4.2 **Neurobiology of Depression**

Great enthusiasm initially surrounded the monoamine hypothesis of mood disorders. This theory ultimately led to the successful development of antidepressants such as selective serotonin reuptake inhibitors (SSRIs) (Heninger *et al.*, 1996). These agents significantly improved the treatment of mood disorders; however, the effect they provide is often delayed and unsatisfactory. This is best highlighted by the fact that over 40 % patients fail to achieve full remission following treatment with conventional antidepressants (Berman *et al.*, 1997). The existing monoamine theory does not fully explain these issues, raising the possibility that other neurotransmitter systems may also contribute to the pathophysiology of mood disorders and the mechanism of antidepressant action (Farvolden *et al.*, 2003). The exploration of alternative neurotransmitter systems may provide novel targets to develop better treatments. There is evidence suggesting that the glutamatergic system as well as the GABAergic system are altered in mood disorders and contribute to the mechanism of antidepressant action for several treatment modalities (Kugaya and Sanacora, 2005).

1.4.3 **Monoamine Theory of Depression**

The monoamines as neurotransmitters are believed to be involved in the pathogenesis of several mental disorders. According to the most accepted hypothesis of depression, the monoamine theory, the major neurochemical process in depression is the impairment of monoaminergic neurotransmission and the concomitant decrease of extracellular concentration of noradrenaline (NA) and/or serotonin (5-HT) (Schildkraut, 1965; Doris *et al.*, 1999; Kiss, 2008). The theory was originally based on the observations that two classes of compounds serendipitously found to be effective in the treatment of depression were both able to enhance the monoaminergic neurotransmission. The tricyclic drugs (like desipramine) inhibited the reuptake of NA, whereas the monoamine oxidase inhibitors (like iproniazide) inhibited the major catabolic enzyme of monoamine transmitters (Nestler *et al.*, 2002). Although in the last few years a number of alternative hypotheses have been

suggested for the possible etiology of depression (Hindmarch, 2002; Wong and Licinio, 2004), the majority of currently used antidepressants works on the basis of monoamine hypothesis, i.e. enhances the monoaminergic neurotransmission through different mechanisms. Monoamines are synthesized in the presynaptic nerve terminal and

concentrated in vesicles at the nerve terminal by a specific vesicular monoamine transporter (VMAT) (Erickson and Varoqui, 2000). VMAT-1 is primarily present in endocrine and paracrine cells of peripheral organs. On the other hand, VMAT-2 is the predominant monoamine vesicular transporter in the central nervous system (CNS) (Masson *et al.*, 1999). The monoamines are released by Ca^{2+}–dependent exocytosis (Vizi, 2000). The released monoamine will act on specific receptors located either on postsynaptic or presynaptic membranes. Stimulation of the postsynaptic receptors results in changes in the properties of the postsynaptic membrane with either a shift in membrane potential when the receptors are coupled to ion channels (known as ionotropic receptors), or biochemical changes in intracellular cyclic nucleotides, protein kinase activity, and related substrate proteins when the receptors are coupled to G-proteins (known as metabotropic receptors) (Elhwuegi, 2004). On the other hand, stimulation of the presynaptic receptors located on the nerve terminal will regulate the monoamine release triggered by action potential, i.e. vesicular release, thereby providing a feedback mechanism that controls the concentration of the transmitter in the synaptic cleft (Boehm and Kubista, 2002).

The acute effect of antidepressants on the monoamine system is (1) inhibition of neuronal MAO, (2) blockade of the presynaptic α_2 receptors, or (3) inhibition of the reuptake of the monoamines. These three mechanisms will increase acutely the level of the monoamines at the synapse (Elhwuegi, 2004).

1.4.4 **Role of Glutamate in the Pathophysiology of Depression**

A number of papers have reported alterations in the glutamate levels of blood and cerebrospinal fluid (CSF) in patients with major depressive disorder (MDD). Kim *et al.* (1982) reported that serum levels of glutamate in patients with depression were significantly higher than those of healthy controls. Furthermore, Altamura *et al.* (1993) reported that plasma concentrations of glutamate in patients with mood disorders were significantly higher than those in the control group. In addition, there is a positive

correlation between plasma glutamate levels and severity of depressive symptoms in patients with MDD (Mitani *et al.*, 2006). Interestingly, 5-week treatment with antidepressants has been shown to significantly decrease the levels of glutamate in sera (Maes *et al.*, 1998), suggesting the possible role of glutamate in the action of antidepressants. Taken together, these findings suggest an abnormality of the brain glialneuronal glutamine/glutamate cycle associated with glutamate receptor systems in patients with MDD, and that the determination of blood (or CSF) levels of glutamate may be a possible biomarker for MDD (Hashimoto, 2009). Postmortem human brains are critical for examining molecular and cellular changes associated with the pathophysiology of MDD. Using postmortem brain samples from the Stanley Foundation Brain Collection, increased levels of glutamate in the frontal cortex have been reported, suggesting an abnormality of glutamatergic neurotransmission in the pathophysiological features of MDD (Hashimoto *et al.*, 2007; Hashimoto, 2009).

Mood disorders such as MDD are known to be complex disorders with multiple genetic and environmental factors that contribute to the risk of manifestation of these disorders (Levinson, 2006; Shyn and Hamilton, 2010). It has been reported that variations in the GAD1 gene may contribute to susceptibility across MDD and anxiety disorders (Hettema *et al.*, 2006). The Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study demonstrated that genetic variation in the GRIK4 gene, a subtype of kainate receptor, is reproducibly associated with response to the antidepressant citalopram (Paddock *et al.*, 2007; Lekman *et al.*, 2008). Taken together, it is possible that the genes associated with glutamatergic systems may be susceptibility to the risk of developing MDD (Hashimoto, 2011).

1.4.5 **Pharmacological Treatment of Depression**

A myriad of controlled trials have established the antidepressant efficacy of medications that modulate monoaminergic neurotransmission, primarily the serotonin and norepinephrine systems. These medications typically act by inhibiting reuptake of norepinephrine and/or serotonin from the synapse, inhibiting monoamine oxidase (which degrades these neurotransmitters) or acting at receptors that modulate monoaminergic transmission. Monoaminergic modulators generally include the tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), monoamine oxidase inhibitors

(MAOIs), serotonin/norepinephrine reuptake inhibitors (SNRIs) and various atypical antidepressants with less well-described mechanisms of action (Holtzheimer III and Nemeroff, 2006).

Tricyclic antidepressants (imipramine, trimipramine, doxepin, clomipramine, desipramine and amoxapine) are potent inhibitors of norepinephrine and/or serotonin reuptake. These medications also tend to block muscarinic cholinergic receptors and, as a result, are associated with anticholinergic side effects including dry mouth, dry eyes, constipation, blurred vision, urinary hesitancy, mild cognitive disturbance, and tachycardia. They also have antihistamine effects resulting in sedation and weight gain. Because of these side effects (and the considerable cardiac toxicity observed after TCA overdose), the TCAs have largely been replaced by other antidepressants as first-line agents in the treatment of depression (Holtzheimer III and Nemeroff, 2006).

The MAOIs prevent the degradation of all monoamines within the presynaptic terminal. These agents are clearly efficacious in depression. MAOIs can interact with other agents that affect the monoaminergic systems to produce synergistic—and dangerous—effects. Examples include isocarboxazid, phenelzine, selegiline, and tranylcypromine (Holtzheimer III and Nemeroff, 2006).

The SSRIs have largely replaced the TCAs and MAOIs as first-line agents in the treatment of depression, because of their relatively benign side effect profile and limited drug-drug interactions. Examples include citalopram, escitalopram, fluoxetine, paroxetine and fluvoxamine (Anderson, 2000; Holtzheimer III and Nemeroff, 2006; Nash and Nutt, 2007). SSRIs strongly and selectively inhibit the reuptake of serotonin into the presynaptic terminal. As a class, they have little effect on norepinephrine reuptake and little to no effect on cholinergic or histaminergic function. However, their robust proserotonergic activity results in several, relatively unique, side effects including gastrointestinal distress (nausea, diarrhea, constipation), insomnia, nervousness and agitation, and sexual dysfunction (Nash and Nutt, 2007).

Atypical antidepressants (bupropion, mirtazapine, nefazodone, and trazodone) are also widely used in the treatment of depression. In general, the mechanisms of action for these medications are poorly understood but still suggest modulation of monoaminergic systems (Holtzheimer III and Nemeroff, 2006).

1.4.6 **Animal Models of Depression**

Animal models of depression have been utilized vigorously to screen novel compounds with antidepressant potential (Bourin, 1990). Currently used animal models for depression research vary considerably in the extent to which they produce features that resemble a depressive-like state, and models that include stress exposure are widely used. Paradigms that employ acute or sub-chronic stress exposure include learned helplessness, forced swim test, and tail suspension test, which employ relatively short-term exposure to inescapable or uncontrollable stress and can reliably detect antidepressant drug response. Longer-term models include chronic mild stress models, repeated open-space model, early-life stress models, and social conflict models, which may more accurately simulate processes that lead to depression. The chronic models are considered more pragmatic in the induction of a depressive-like state and are suggested to have better potential correlation to the human situation (Duman, 2010). The models are usually evaluated for their reliability or reproducibility, their ability to accurately predict outcome in humans (predictive validity), their ability to reproduce in animals aspects of the illness in humans (face validity), and the extent to which they model the true disease process or its etiology in humans (construct or etiologic validity) (Willner, 1984; McKinney, 2001).

1.5 **EPILEPSY AND ANXIETY**

1.5.1 **Background**

Anxiety is the subjective experience of anticipating a future aversive event (or state) (Steckler *et al.*, 2008). Anxiety disorders represent a frequent and clinically important comorbid disorder in epilepsy patients (Vazquez and Devinsky, 2003).

Available body of evidence strongly suggests that epilepsy patients have a higher prevalence of anxiety disorders than controls, in both hospital and community samples (Scicutella and Ettinger, 2002; Vazquez and Devinsky, 2003). Moreover, a number of epidemiological studies indicate that anxiety disorders are common in people with epilepsy than in the general population. A Canadian study showed that lifetime prevalence of anxiety in people above 15 years was 22.8 % in patients with epilepsy compared to 11.2 % in the control group (Tellez-Zenteno *et al.*, 2007). In another study, prevalence of specific subtypes of anxiety disorders—social phobia, panic disorder, generalised anxiety disorder

and specific phobia— in adult patients with refractory focal epilepsy, were greater than in the general population (Brandt *et al.*, 2010; Kimiskidis and Valeta, 2012).

Although numerous brain regions are likely to be involved, the amygdala and the hippocampus play key roles in the neurobiology of both epilepsy and anxiety. The amygdala is determinant in the experience of fear and its autonomic and endocrine responses (Stahl, 2003; Hamid *et al.*, 2011). Furthermore, the hippocampus is important in the re-experiencing of fear. Activation of fear circuits is a major hypothesis for explaining symptoms in anxiety disorders, and reduction of the excessive output from these neurons may theoretically improve the clinical picture (Stahl, 2003; Rogawski and Loscher, 2004). Such a mechanism has a number of similarities to the excessive outburst typical of epileptic neurons, explaining the effects of antiepileptic agents (such as benzodiazepines [BDZs] and antiepileptic drugs) in the treatment of anxiety (Mula *et al.*, 2007; Hamid *et al.*, 2011).

GABA is the principal inhibitory neurotransmitter of the brain and, along with serotonin and norepinephrine, is one of several neurotransmitters that appear to be involved in the pathogenesis of anxiety (Stahl, 2003). The $GABA_A$ receptor subtype regulates excitability and rapid changes in fear arousal, such as anxiety, panic, and the acute stress response. Drugs that stimulate $GABA_A$ receptors, such as BDZs and barbiturates, have anxiolytic and antiseizure effects via $GABA_A$-mediated reduction of neuronal excitability, which effectively raises the seizure threshold (Mula *et al.*, 2007). This hypothesis is supported by the observation that some substances, including the GABAergic antiepileptic drugs gabapentin, vigabatrin, tiagabine, valproate, and pregabalin (Blanco *et al.*, 2003; Carta *et al.*, 2003; Kinrys *et al.*, 2003; Lauria-Horner and Pohl, 2003; Pande *et al.*, 2003), as well as barbiturates, benzodiazepines, and neuroactive steroids, have antiepileptic as well as anxiolytic properties (Beyenburg *et al.*, 2001; Beyenburg *et al.*, 2005).

1.5.2 **Animal Models of Anxiety**

A basic assumption when using animals to study anxiety is that neural and behavioural processes analogous to human anxiety can be evoked in situations that are a real threat to the animal's survival (Darwin, 1867). For nocturnal small prey animals like rats and mice, open and well-lit spaces are putatively dangerous but, on the other hand, must be explored in order to obtain food, and find a better home or a potential mate (Rodgers, 1997). This ethological premise has underpinned the development of the so-called approach–avoidance

conflict tests of rodent "anxiety" that has become the most widely used assays for "anxietylike behaviour" and anxiolytic-like activity (Rodgers, 1997). For example, in the popular elevated plus-maze test the rat or mouse is faced with a conflict between remaining in protected enclosed arms or venturing out into exposed arms, with relatively high open arm avoidance being the readout of relatively high anxiety-like behaviour. Along similar lines, conflict between approach and avoidance in various forms underlies most of the frequently used rodent tests of anxiety-related behaviour (e.g., novel open field, light/dark exploration, Vogel conflict, Geller-Seifter, social interaction, hyponeophagia, defensive burying, marble burying); although with exceptions (e.g., stress-induced hyperthermia) (Steckler *et al.*, 2008).

1.6 JUSTIFICATION OF STUDY

Despite the availability of many antiepileptic drugs (AEDs), nearly one in three patients with epilepsy who have access to AEDs continue to have seizures, and a similar proportion experience unacceptable AED-related adverse effects (Brodie, 2005; Löscher, 2002b). In addition, the large majority of people with epilepsy around the world are not under treatment with AEDs, largely because of their lack of access to physicians, the costs of AEDs, and cultural attitudes toward modern treatments (Meinardi *et al.*, 2001; Schachter, 2009). A major limitation of antidepressant therapy is its side effects such as sedation, blurred vision, constipation, seizures, sexual dysfunction, anxiety and weight gain (Dhingra *et al.*, 2012; Hyman and Nestler, 1996; Andreasen *et al.*, 2009). Furthermore, although the classical antidepressants are effective in treating most depressive episodes, a significant proportion of depressed patients do not display signs of mood improvement until 2–3 weeks after the start of the treatment (Poleszak *et al.*, 2011). It is therefore desirable to research and develop more effective antiepileptics and antidepressants with fewer adverse effects.

Plant extracts are some of the most attractive sources of new drugs, and have been shown to produce promising results for the treatment of epilepsy and depression (Schulz, 2006; Nassiri-Asl *et al.*, 2007). The plant *Pseudospondias microcapa* has been extensively used in Ghana and other parts of Africa as medication for various diseases including CNS disorders (Burkill, 1985). However, all claims of the therapeutic success of the plant in the treatment of these ailments are based on its traditional uses and have not been verified scientifically. Thus, this study aims to investigate the anticonvulsant, antidepressant and

other neuropharmacological effects of the ethanolic leaf extract of *Pseudospondias microcapa*.

1.7 AIMS AND OJECTIVES OF STUDY

The core aim of this study was therefore to provide substantial pharmacological evidence for the traditional use of the leaves in the management of some CNS disorders including epilepsy and depression.

The objective of this study was to evaluate the anticonvulsant and antidepressant activities of the hydroethanolic leaf extract of *Pseudospondias microcarpa* in various animal models.

Specific objectives included evaluating the extract for:

- Presence of some secondary metabolites
- Prelimary CNS activity
- Anticonvulsant activity in pentylenetetrazole-, picrotoxin-, maximal electroshock-, 6-Hz-, isoniazid-, strychnine-, 4-aminopyridine-induced seizures and possible mechanism(s) of action
- Antidepressant activity in the tail suspension and forced swimming tests as well as possible mechanism(s) of action
- Its effect in chronic models of depression (chronic mild stress and repeated openspace swim test)
- Anxiolytic activity in various classical models of anxiety including the elevated plus-maze, light/dark exploration, open field, social interaction and stress-induced hyperthermia tests.
- General CNS depressant and neuromuscular effects
- Acute and sub-acute toxicity studies.

Chapter 2 PLANT COLLECTION, EXTRACTION AND PHYTOCHEMICAL ANALYSIS

2.1 PLANT COLLECTION AND EXTRACTION

2.1.1 Collection of Plant Material

Fresh leaves of *P. microcarpa* were collected from the campus of Kwame Nkrumah

University of Science and Technology (KNUST), Kumasi near the Department of Agricultural Engineering (6º 40.626'N, 1º 34.041'W) during the month of August, 2010 and authenticated at the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, KNUST, Kumasi, Ghana. A voucher specimen (KNUST/HM1/2013/L005) was kept at the herbarium of the Faculty.

2.1.2 **Plant Extraction**

Leaves of the plant were room-dried for seven days and pulverised into fine powder. The powder was extracted by cold percolation with 70 % (v/v) ethanol in water over a period of 72 h and the resulting extract concentrated into a syrupy mass under reduced pressure at 60 °C in a rotary evaporator. It was further dried in a hot air oven at 50 °C for a week and kept in a refrigerator and used when required. The yield was 20.5 % (w/w). In this study the crude extract is subsequently referred to as PME or extract.

2.2 PHYTOCHEMICAL TESTS

The freshly prepared extract was analyzed for phytochemical constituents for the detection of alkaloids, saponins, reducing sugars, flavonoids, terpenoids, tannins and steroids.

2.2.1 **Alkaloids**

An amount of 0.5 g of PME was boiled with 10 ml of dilute hydrochloric acid in a test tube for 5 minutes. The supernatant liquid was filtered into another test tube and 1 ml of the filtrate was taken. Three drops of Dragendorff's reagent (potassium bismuth iodide solution) was then added, shaken and observed for the appearance of an orange-red spot and precipitate formation (Sofowora, 1993).

2.2.2 **Saponins**

A small amount (0.2 g) of the extract was shaken with a few millilitres of water in a test tube and the mixture observed for the presence of a froth which does not break readily upon standing (Sofowora, 1993).

2.2.3 **Flavonoids**

Five millilitres of dilute ammonia solution was added to a portion of the aqueous filtrate of PME followed by addition of concentrated H_2SO_4 and observed for yellow coloration (Ayoola *et al.*, 2008).

2.2.4 **Tannins**

An amount of 0.5 g of the extract was boiled with 25 ml of water for 5 minutes, cooled and filtered. The volume of the filtrate was adjusted to 25 ml with water. To 1 ml of the filtrate was added 10 ml of water and 5 drops of 1 % ferric chloride and observed for a blue-black or green precipitate formation. The procedure was repeated using 5 drops of 1 % lead acetate and observed for any change in colour or formation of precipitate (Sofowora, 1993).

2.2.5 **General test for Glycosides (Reducing Sugars)**

An amount of 0.2 g of PME was boiled in 5 ml dilute H_2SO_4 on a water bath for 2 minutes. The mixture was cooled, filtered and rendered distinctly alkaline with 2 to 5 drops of 20 % NaOH. 1 ml each of Fehling's A and B solutions were added to the filtrate, heated on a water bath for 2 minutes and observed for a red-brown precipitate (Sofowora, 1993).

2.2.6 **Terpenoids**

An amount of 0.5 g of PME was extracted with 2 ml of chloroform in a test tube followed by addition of 1 ml of concentrated sulphuric acid. The reddish-brown coloration at interface shows the presence of terpenoids (Sofowora, 1993).

2.2.7 **Steroids**

An amount of 0.5 g of PME was extracted with 2 ml of chloroform in a test tube. 2 ml acetic anhydride was added to the extract. Concentrated sulphuric acid was carefully added at the side of the test tube. A blue colour that appeared at the interface suggested the presence of. steroids (Sofowora, 1993).

2.3 **RESULTS**

Phytochemical analysis of the extract revealed the presence of alkaloids, saponins, reducing sugars, tannins, terpenoids and flavonoids. Steroids were however absent (Table 2.1).

Table 2.1 Phytochemical constituents of the ethanolic extract of the leaves of *P. microcarpa*

CONSTITUENT	**INFERENCE**
Tannins	+
Alkaloids	+

Saponins	+
Reducing sugars	+
Terpenoids	+
Flavonoids	+
Steroids	–

+ =present – =absent

2.4 DISCUSSION

The phytochemical screening indicated the presence of flavonoids, tannins, saponins, glycosides, terpenoids and alkaloids. Presence of alkaloids, tannins and terpenoids in PME confirms preliminary phytochemical tests done on the leaves of *P. microcarpa* (Yondo *et al.*, 2009). As has been reported in several studies, the presence of many biologically active phytochemicals such as flavonoids, triterpenes, alkaloids, steroids, tannins and glycosides in various plant extracts may be responsible for their respective pharmacological properties (Liu *et al.*, 2010; Ibarra *et al.*, 2010; Maganha *et al.*, 2010; Gomes *et al.*, 2009).

Flavonoids possess a broad range of biological activities including a multiplicity of neuroprotective actions (Vafeiadou *et al.*, 2009). For example, fruit and vegetable flavonoids improve general cognitive ability through modulation of neurotransmitter release (Joseph *et al.*, 1998; 1999) and stimulation of hippocampal neurogenesis (Casadesus *et al.*, 2004). Numerous flavonoids, including apigenin, chrysin, wogonin, baicalein and baicalin, have been shown to have anxiolytic effects in the elevated plus-maze (EPM) and Vogel conflict test (VCT) with a potency comparable to that of typical benzodiazepine (BDZ) agents (Zanoli *et al.*, 2000; Dhawan *et al.*, 2001; Rocha *et al.*, 2002; Liao *et al.*, 2003). Flavonoids, also exhibit sedative, anticonvulsant and antidepressant effects (Wolfman *et al.*, 1998; Cho *et al.*, 2012; Herrera-Ruiz *et al.*, 2011; Rodrigues *et al.*, 2008).

Aside their usefulness as astringents, tannins, have anti-inflammatory, antioxidant, antinociceptive, antiulcer, antimicrobial and antiviral properties (Mota *et al.*, 1985; Lin *et al.*, 2001; Souza *et al.*, 2007; Koleckar *et al.*, 2008). Saponins also exhibit various pharmacological activities: anticonvulsant (Gurib-Fakim, 2006), antidepressant (Xiang *et*

al., 2011), anxiolytic (Wei *et al.*, 2007), sedative (Jiang *et al.*, 2007) and analgesic (Nemmani and Ramarao, 2002) . Alkaloids have a remarkable range of pharmacological activities including antidepressant (Li *et al.*, 2007), anxiolytic (Flausino *et al.*, 2007), anticonvulsant (Bhutada *et al.*, 2010), sedative (Xu *et al.*, 2007) and analgesic (Reanmongkol *et al.*, 1994) activities .

The presence of these phytochemicals (flavonoids, tannins, saponins, glycosides, terpenoids and alkaloids) could therefore be responsible for the sedative, anxiolytic, analgesic, antidepressant, and anticonvulsant effects of the extract in the various animal models used.

2.5 CONCLUSION

The hydroethanolic leaf extract of *P. microcarpa* contain alkaloids, saponins, reducing sugars, tannins, terpenoids and flavonoids which could be responsible for its effects in the management of depression, anxiety and epilepsy.

Chapter 3 PRELIMINARY CNS SCREENING

3.1 INTRODUCTION

Core battery CNS procedures are typically simple tests employed in safety assessment, and are frequently applied at the very beginning of the discovery process as a screen to eliminate substances with a potential for CNS risk. These include protocols for measuring general behavioural signs induced by test substances (Irwin test), effects on neuromuscular coordination (Rotarod test), effects on spontaneous locomotion (Activity meter test), effects on the convulsive threshold (PTZ seizure test), interaction with hypnotics (Barbiturate interaction test) and effects on the pain threshold (Tail immersion test). Because of their use early in the safety evaluation process, such studies are conducted almost exclusively in the rodent (Porsolt *et al.*, 2002).

As part of the continuing search for plants that act on the CNS, this work focuses on the screening of *Pseudospondias microcarpa* (A. Rich.) Engl. (Anacardiaceae). *Pseudospondias microcarpa* is one of such plants used for managing various diseases including CNS disorders. In Ghana it is locally known as *katawani* literally meaning ‘close your eyes’ because the tree supposedly has a sedative effect on those who sit or sleep under it. The plant is therefore used in Ghana as a sedative and for treatment of general central

nervous system disorders (Burkill, 1985). Other medicinal uses of the bark and leaves are treatment of arthritis, rheumatism, eye problems, kidney disorders, naso-pharyngeal infections, stomach complaints, malaria and jaundice (Burkill, 1985).

Despite the wide use of the plant, there is no data in literature on its probable CNS activity. The core aim of this study was therefore to provide evidence of possible neuropharmacological properties in experimental animal models.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Male ICR mice (20-25 g) were purchased from the Noguchi Memorial Institute for Medical Research, Accra, Ghana and kept in 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 cm × 47 cm × 18 cm) 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). A period of at least one week for adaptation to the laboratory facilities was allowed. The studies were conducted in accordance with accepted principles for laboratory animal use and care (NRC, 2010).
Approval for this study was obtained from the faculty Ethics Committee.

3.2.2 Drugs and Chemicals

Caffeine (CFN), Diazepam (DZP), Pentobarbitone (PBT), Pentylenetetrazole (PTZ) and Phenobarbitone (PHB) were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA.
Morphine hydrochloride (MOR) was obtained from Phyto-Riker Pharmaceuticals Limited, Accra, Ghana.

3.2.3 General Pharmacological Observation (Irwin Test)

Male ICR mice were orally treated with the extract (30, 100, 300, 1000 and 3000 mg kg^{-1}) and placed in observation cages (20 cm × 40 cm × 22 cm). The mice were evaluated for general pharmacological and physiological behaviours as well as mortality at 0, 15, 30, 60, 120, 180 min, up to 24 h after treatment as described by Irwin (1968).

3.2.4 **Activity Meter Test**

Locomotor activity of PME was evaluated with the Ugo Basile activity cage (model 7401, Comerio, VA, Italy). Mice were pretreated orally with extract (as described above), diazepam (8 mg kg^{-1}), caffeine (16 mg kg^{-1}) or saline. After 1 h, the animals were individually placed in the activity meter cage and their activities scored every 5 min for 30 min. Diazepam and caffeine were used as CNS depressant and stimulant agents respectively.

3.2.5 **Rotarod Test**

Effect of PME on motor co-ordination was assessed with a rotarod apparatus (Ugo Basile, model 7600, Cormerio, Milan, Italy). The rotarod consisted of a rotating rod (diameter: 3 cm) with individual compartments for each mouse. Mice were trained for 3 days before the test to stay on the rotating rod (speed 20 rpm) for at least 3 min. On the test day, mice were randomly divided into seven groups (n=8): saline-treated control group, PME group (30, 100, 300, 1000 or 3000 mg kg^{-1}, *p.o.*) and diazepam group (8 mg kg^{-1}, *p.o.*). After oral administration of the test compounds, mice were put on the rotarod and the latency until fall during a 3-min session was recorded at 0, 0.5, 1, 1.5 and 2 h.

3.2.6 **Pentobarbitone-Induced Sleeping Time**

Animals were randomly divided into eight groups (n=8): saline-treated control, PME (30, 100, 300, 1000 or 3000 mg kg^{-1}, *p.o.*), diazepam (8 mg kg^{-1}, *p.o.*) or caffeine (16 mg kg^{-1}, *p.o.*). Sodium pentobarbitone (50 mg kg^{-1}) was intraperitoneally administered 60 min after administration of test drugs. Two parameters were recorded: time elapsed since the application of pentobarbitone until the loss of the righting reflex (latency/onset of action) and the time elapsed from the loss to regaining of the righting reflex (duration of sleep).

3.2.7 **Barbiturate Interaction Test**

This was done to assess the influence of hepatic enzyme induction on pentobarbitone sleeping time. Mice were pre-treated with phenobarbitone (25 mg kg^{-1}, i.p.) for two consecutive days. On the third day, administration of test compounds was repeated as described above and sleeping time determined.

3.2.8 **Convulsive Threshold Test (PTZ Seizure Test)**

Mice were divided into seven groups (n=10) and received PME (30, 100, 300, 1000 or 3000 mg kg^{-1}, *p.o.*), vehicle or the standard drug diazepam (16 mg kg^{-1}, *p.o.*). One hour after administration of test compounds, animals were injected subcutaneously with a single dose of PTZ (100 mg kg^{-1}). Thereafter, mice were observed 60 min for both clonic and tonic seizures. Clonic seizures were characterized as appearance of facial myoclonus, forepaw myoclonus and forelimb clonus and tonic seizures were characterized as explosive clonic seizures with wild running and tonic forelimb and hind limb extension. The latency for the onset, frequency and duration of the convulsive episodes (clonic or tonic) were recorded as indicators of pro or anticonvulsive effect of compounds.

3.2.9 **Tail Immersion Test**

Tail immersion test, a measure of analgesia, was carried out as described by Janssen *et al.* (1963) with modifications. Male ICR mice were divided into 7 groups of 7 animals each: control, PME (30-3000 mg kg^{-1}, *p.o.*) and morphine (10 mg kg^{-1}, i.p.). The tail (up to 3.5 cm) was then dipped into a water bath maintained at 48±0.5 °C. The time in seconds to withdraw the tail out of water was taken as the reaction time (T). A cut-off latency of 10 s was adopted to avoid tissue damage. Withdrawal latency was taken after 0.5, 1, 2, and 3 h intervals after 30 min (i.p.) or 1 h (*p.o.*) following administration of the test drugs.

The percentage maximal possible effect (% MPE) was calculated from the reaction times using the formula:

$$\% \text{ MPE} = \frac{\quad}{\quad} \times 100\ \%$$

where $\mathbf{T_1}$ and $\mathbf{T_2}$ are the pre- and post-drug reaction times and $\mathbf{T_0}$ is the cut-off time.

3.2.10 **Statistical Analysis**

In all experiments, a sample size of seven to ten (n=7-10) was used. Data are presented as mean±SEM. To compare differences between groups, one-way ANOVA was performed with Newman-Keuls' test as *post hoc.* The time-course curves were subjected to two-way (*treatment* × *time*) repeated measures analysis of variance (ANOVA) with Bonferroni's *post hoc* test. GraphPad® Prism for Windows Version 5.0 (GraphPad Software, San Diego, CA,

USA) was used for all statistical analysis. $P<0.05$ was considered statistically significant for
all test.

3.3 RESULTS

3.3.1 Irwin Test

Treatment of mice with the extract produced sedation and analgesia at all doses used (Table 3.1). No deaths were recorded over the 24 h observation period, indicating an LD_{50} above 3000 mg kg^{-1}.

Table 3.1 Effects of *Pseudospondias microcarpa* hydroethanolic leaf extract (PME) in the primary observation test in mice

Dose (mg kg^{-1})	Mortality D/T	Effects
0	0/7	no change
30	0/7	sedation, analgesia
100	0/7	sedation, analgesia
300	0/7	sedation, analgesia
1000	0/7	sedation, analgesia

3000	0/7	sedation, analgesia

D/T: Dead/Treated

3.3.2 **Activity Meter Test**

PME had no effect on locomotor activity ($F_{5,30}$=1.425, P=0.243; figure 3.1) at all the doses used (30-3000 mg kg^{-1}, *p.o.*). However, diazepam (8 mg kg^{-1}, *p.o.*), a CNS depressant significantly ($F_{2,15}$=24.90, P<0.0001; figure 3.1) reduced activity in mice whereas caffeine (16 mg kg^{-1}, *p.o.*), a CNS stimulant, increased it (P<0.05).

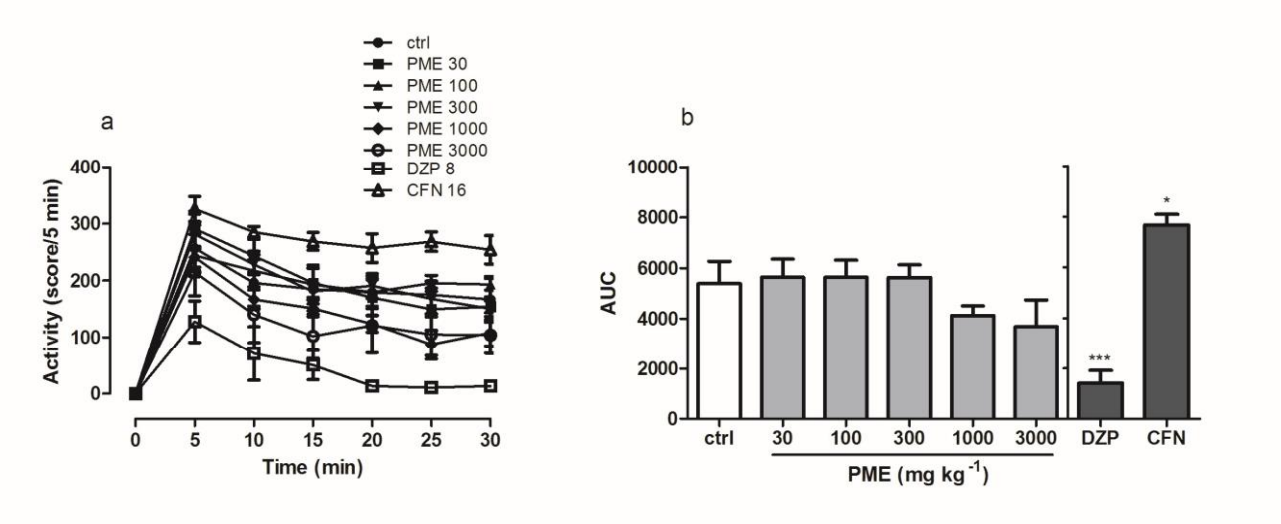


Figure 3.1 Effects of acute PME (30-3000 mg kg^{-1}, *p.o*), diazepam (8 mg kg^{-1}, *p.o*) and caffeine (16 mg kg^{1}, *p.o*) treatment in the activity meter test. Data are presented as group mean±SEM (n=7). Analysis by oneway ANOVA followed by Newman-Keuls' *post hoc* test. *P<0.05, ***P<0.001

3.3.3 **Rotarod Test**

In figure 3.2, the extract caused no significant effect on the latency to fall off the rotarod compared to the control at all the doses used (P>0.05 at 30-3000 mg kg^{-1}). However, at the dose used diazepam (8 mg kg^{-1}, *p.o.*), significantly decreased the latency to fall off the rotating rod (P<0.01).

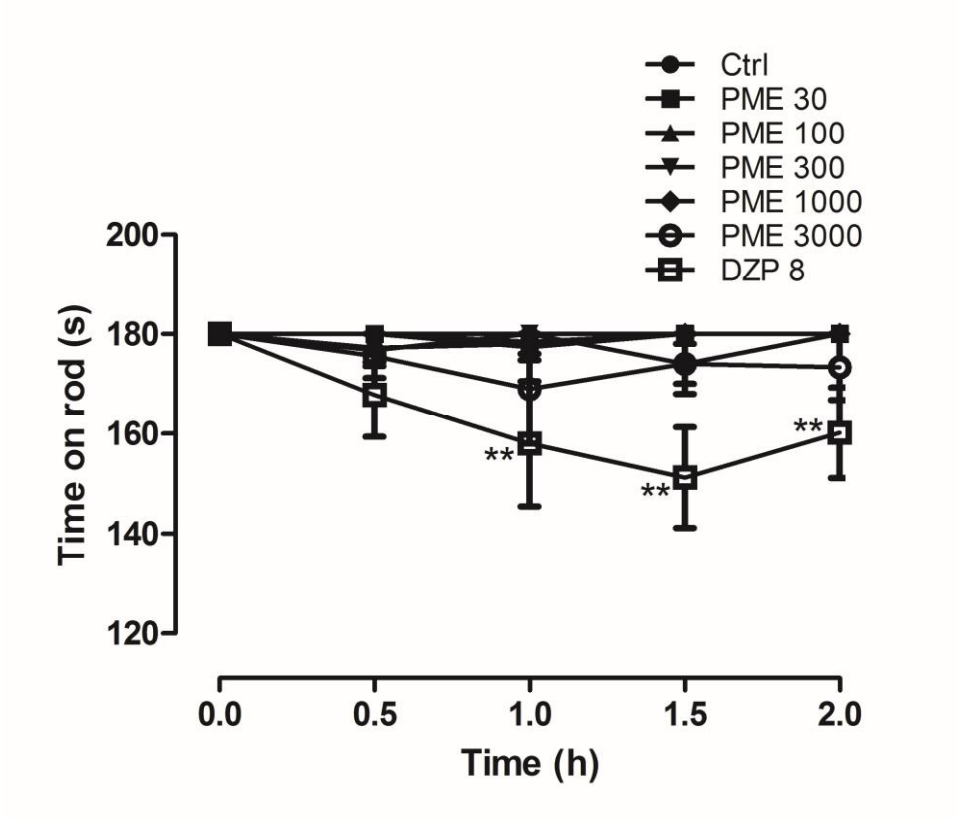


Figure 3.2 Effect of PME (30-3000 mg kg^{-1}, *p.o.*) and diazepam (8 mg kg^{-1}, *p.o.*) on the time course curve of the rotarod test in mice. Data are presented as mean±SEM (n=8). *P<0.05; **P<0.01; ***P<0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test).

3.3.4 **Pentobarbitone-Induced Sleeping Time**

As shown in figure 3.3, pre-treatment with PME (30-3000 mg kg^{-1}, *p.o.*) significantly decreased the latency to sleep ($F_{5,42}$=4.090, P=0.0041) and increased sleeping time ($F_{5,42}$=4.86, P=0.0013) induced by sodium pentobarbitone (50 mg kg^{-1}, i.p.). Similar to the extract, diazepam (8 mg kg^{-1}, *p.o.*), a CNS depressant, decreased latency to sleep ($F_{2,21}$=12.69, P=0.0002) and increased sleeping time ($F_{2,21}$=55.15, P<0.0001). In contrast to PME and diazepam, caffeine (16 mg kg^{-1}, *p.o.*), a CNS stimulant, delayed the onset and decreased duration of sleep (both at P<0.05).

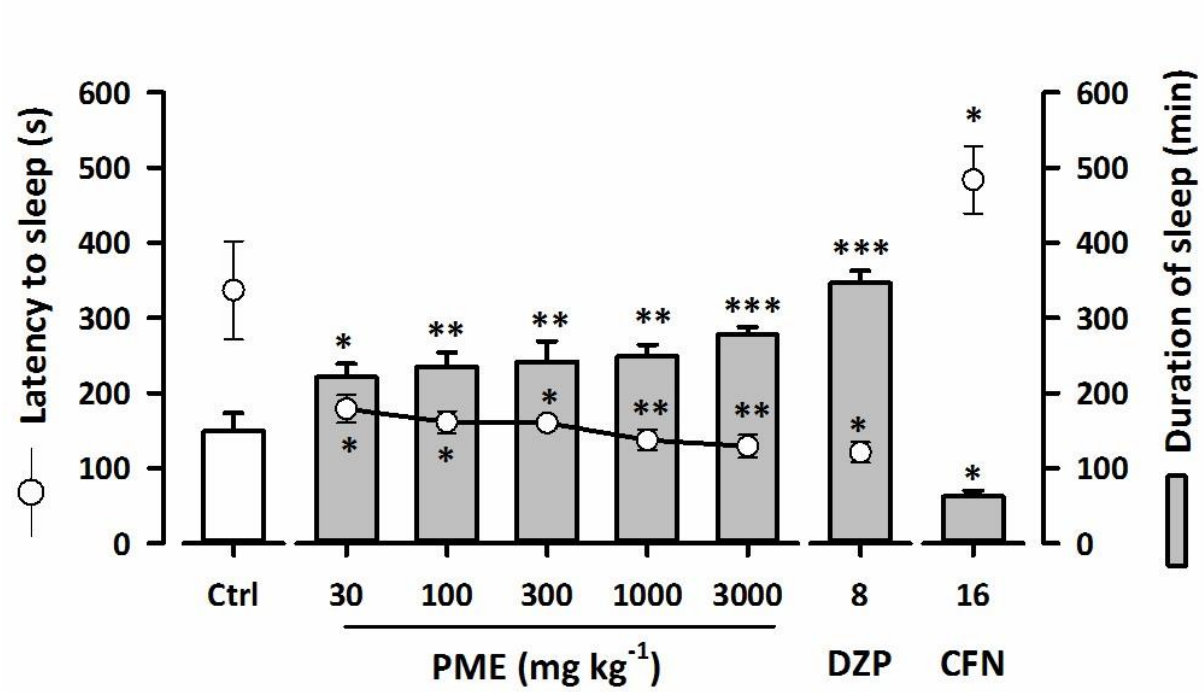


Figure 3.3 Effects of acute PME (30, 100, 300, 1000 and 3000 mg kg^{-1}, *p.o.*), diazepam (8 mg kg^{-1}, *p.o.*) and caffeine (16 mg kg^{-1}, *p.o.*) in the pentobarbitone-induced sleeping time. Data are presented as group mean±SEM (n=8). Analysis was done by one-way analysis of variance followed by Newman-Keuls' *post hoc* test. Significantly different from control: *P<0.05, **P<0.01, ***P<0.001

3.3.5 **Barbiturate Interaction Test**

In the phenobarbitone-pretreated mice, PME, as revealed by ANOVA significantly increased duration of sleep ($F_{5,42}$=3.251, P=0.014; figure 3.4b). However, Newman-Keuls' *post hoc* analysis showed no statistical significance at all the doses used (P>0.05). Diazepam also increased duration of sleep in mice pretreated the phenobarbitone (P<0.01).

A two-way ANOVA showed a significant effect in the duration of sleep for PME when pentobarbitone treatment and phenobarbitone pretreatment were compared ($F_{5,84}$=6.705, P<0.0001; figure 3.4b). Similar results were obtained for diazepam ($F_{2,42}$=59.54, P<0.0001).

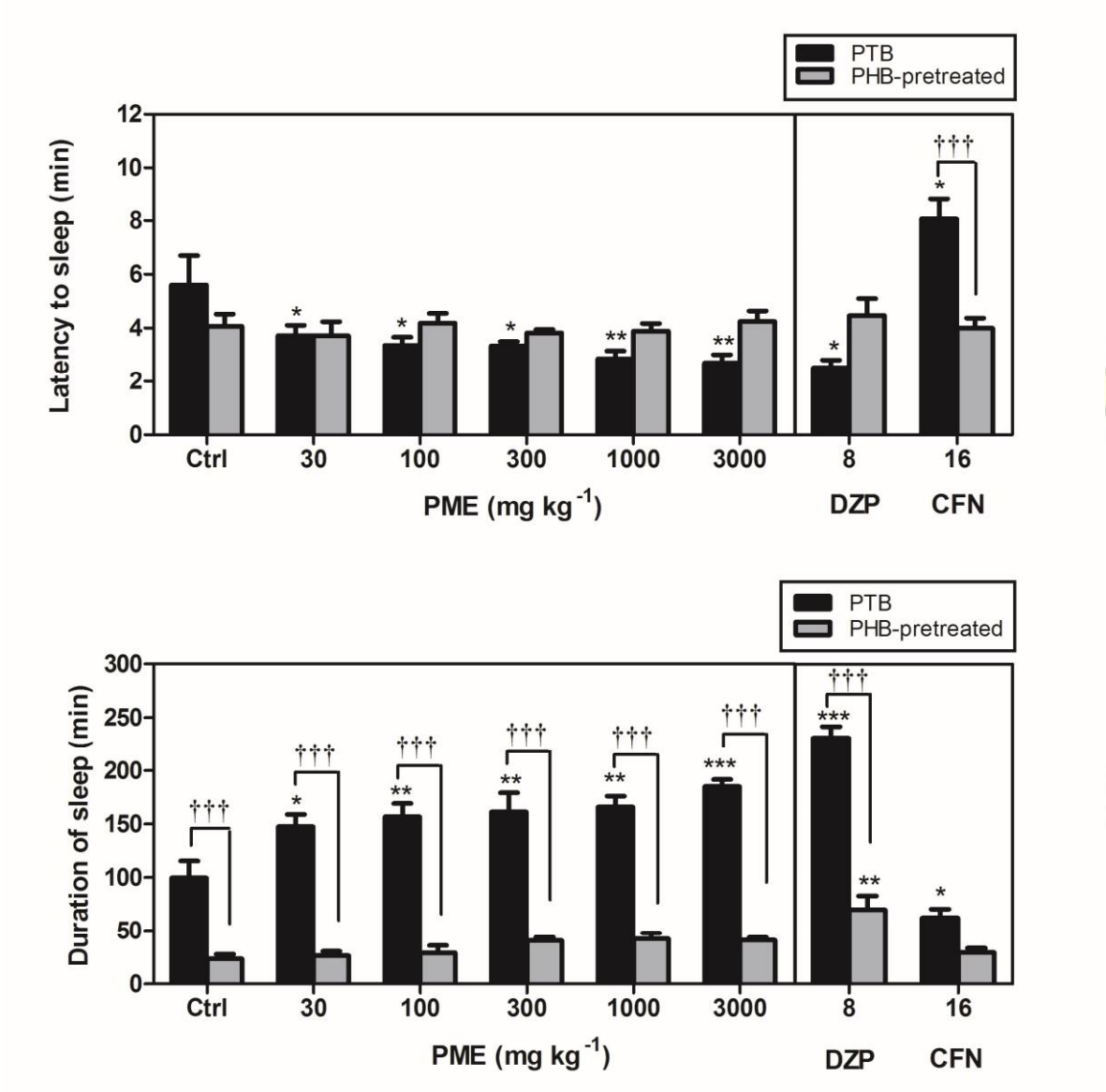


Figure 3.4 Effects of acute PME (30, 100, 300, 1000 and 3000 mg kg^{-1}, *p.o*), diazepam (8 mg kg^{-1}, *p.o.*) and caffeine (16 mg kg^{-1}, *p.o.*) in the barbiturate interaction test. Data are presented as group mean±SEM (n=8). Analysis was done by one-way analysis of variance followed by Newman-Keuls' *post hoc* test.

Significantly different from control: *P<0.05, **P<0.01, ***P<0.001 and two-way ANOVA followed by Bonferroni's test. ††† P<0.001.

3.3.6 **Convulsive Threshold Test (PTZ Seizure Test)**

The extract showed significant anticonvulsant activity against PTZ-induced seizures. Pretreatment of animals with PME (30-3000 mg kg^{-1}, *p.o.*) caused a significant delay in the latency to clonic ($F_{6,56}$=67.88, P<0.0001) and tonic ($F_{6,56}$=3.636, P=0.0041) convulsions. The frequencies of both clonic ($F_{6,56}$=7.761, P<0.0001) and tonic ($F_{6,56}$=8.598, P<0.0001) convulsions were also significantly reduced. ANOVA revealed that PME also significantly reduced the duration of both clonic ($F_{6,56}$=7.534, P<0.0001) and tonic ($F_{6,56}$=6.247, P<0.0001) convulsions. Furthermore, the extract provided 50 % (at 30 and 100 mg kg^{-1}) and 60 % (300, 1000 and 3000 mg kg^{-1}) protection against PTZ-induced tonic seizures in mice. The standard drug diazepam, at the dose used (16 mg kg^{-1}, *p.o.*) completely abolished convulsionsigure (figure 3.5).

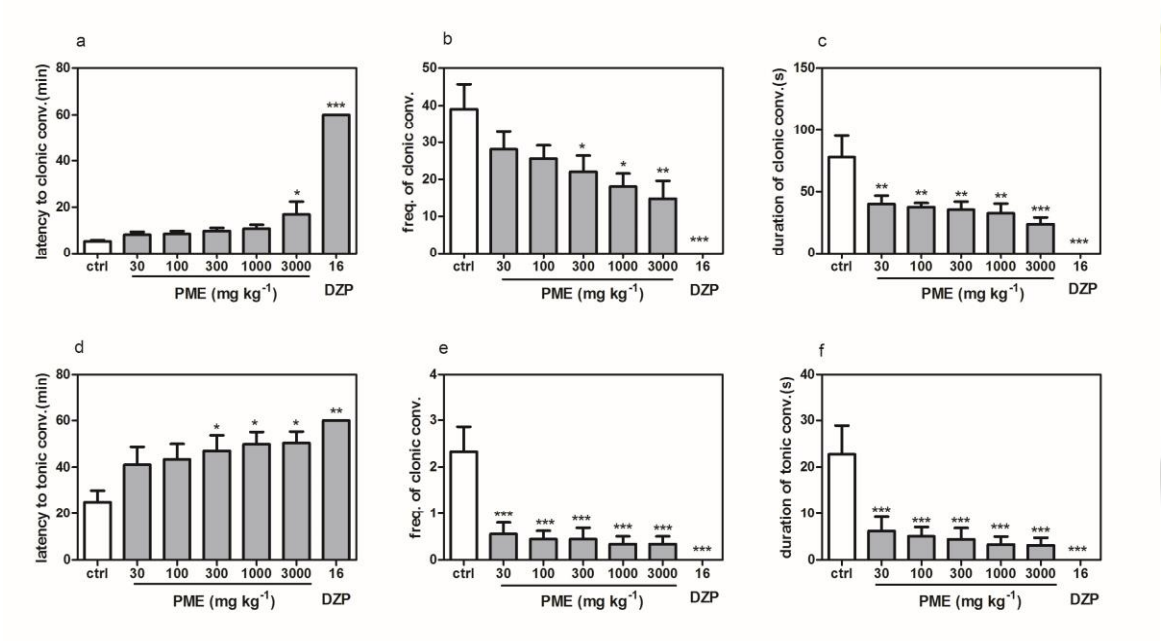


Figure 3.5 Effect of PME (30, 100, 300, 1000 and 3000 mg kg^{-1}, *p.o.*) and diazepam (16 mg kg^{-1}, *p.o.*) on the latency (a, d), frequency (b, e) and duration (c, f) of clonic and tonic seizures in mice. Data are presented as mean±SEM (n=10). Analysis was done by one-way analysis of variance followed by Newman-Keuls' *post hoc* test. Significantly different from control: *P<0.05, **P<0.01, ***P<0.001.

3.3.7 **Tail Immersion Test**

From the time-course curves in figure 3.6, two-way ANOVA (*treatment* x *time*) revealed a significant effect of drug treatments on the tail withdrawal latencies calculated as a percentage of the maximum possible effect (% MPE) ($F_{4,209}$=14.71, P<0.0001). PME (303000 mg kg^{-1}, *p.o.*) significantly increased tail withdrawal latency ($F_{6,42}$=4.182, P=0.0022), with a maximal effect at the dose of 1000 mg kg^{-1}. Morphine (10 mg kg^{-1}, i.p.), the standard analgesic drug used, also showed a significant increase in the withdrawal latency (P<0.001).

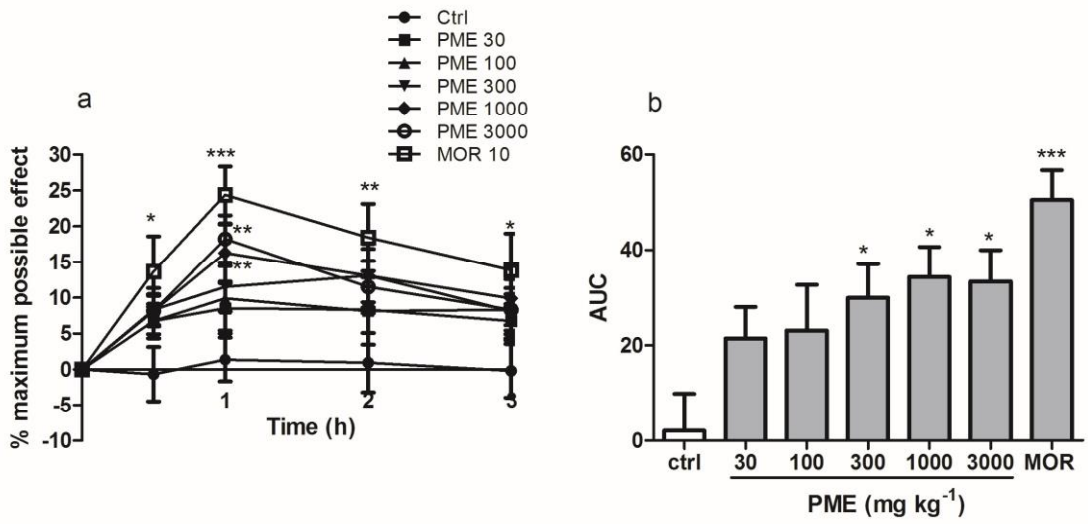


Figure 3.6 Effect of PME (30-3000 mg kg^{-1}, *p.o.*) and morphine (10 mg kg^{-1}, i.p.) on the time course curve (a) of the tail immersion test and the AUC (b) in mice. Data are presented as mean±SEM (n=7). *P<0.05; **P<0.01; ***P<0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test for time course curve or one-way ANOVA followed by Newman-Keuls' *post hoc* test for AUC).

3.4 **DISCUSSION**

Results of the present study show that a hydroethanolic leaf extract of the plant possesses CNS depressant, anticonvulsant and analgesic activity without affecting motor coordination in the animal models used. The extract is also metabolized by hepatic enzymes.

Before investigating a new substance in a specific test, substances are usually evaluated in the primary observation procedure originally described by Irwin (1968). This test is used to estimate the general effects of a drug or drug candidate on central nervous system (CNS)

activity, minimum lethal dose of a test substance and the primary effects on behaviour and physiological functions. Data from this test are also used to assess the safety pharmacology of drugs (Irwin, 1968; Porsolt *et al.*, 2002; Roux *et al.*, 2005). In this study, mice treated with PME showed signs of sedation and analgesia, suggesting possible central depressant and analgesic effects. Furthermore, presence of sedation in the Irwin test suggests possible anxiolytic, antipsychotic, or anticonvulsant activity (Roux *et al.*, 2005) and thus confirming the traditional use of the plant. In drug discovery and evaluation, it is important to assess the toxicity (minimum lethal dose) of test compounds. In this experiment, the LD_{50} of the plant extract, given orally, was found to be above 3000 mg kg^{-1}. At the relatively high doses used, the plant extract caused no mortality and appeared to cause no apparent toxicity. This suggests that PME is relatively non-toxic since substances with an LD_{50} value of 1000 mg kg^{-1} by the oral route are regarded as being safe or of low toxicity (Obici *et al.*, 2008).

Barbiturates are sedatives inducing sleep in human beings and animals by depressing the CNS (Tripathi, 1999). Pentobarbitone potentiates the effect of GABA, acting at the GABA receptor–ionophore complex (Olsen, 1981; Ticku and Maksay, 1983). Activation of $GABA_A$ receptors depresses the CNS and favours sleep. Thus, increase or decrease in pentobarbitone-induced sleeping time can be a useful tool for examining influences on the GABAergic system (Liao *et al.*, 1998; Ma *et al.*, 2009). In this test, a decrease in sleep latency and an increase in sleeping time are classically related to central nervous system (CNS) depressant drugs (Willianson *et al.*, 1996; Gomes *et al.*, 2008; Venancio *et al.*, 2011). In the present study, it was demonstrated that PME, similar to diazepam, potentiated pentobarbitone-induced sleeping behaviour in mice indicative of a CNS depressant effect. This finding is consistent with the sedation observed in the Irwin test.

In addition to the pentobarbitone-induced sleeping time, pre-treatment with phenobarbitone for two consecutive days shortened the duration of sleep in PME treated mice. This reduction in sleeping time suggests that PME is metabolised by cytochrome P450 enzymes (Maronpot *et al.*, 2010; Kushikata *et al.*, 2003). Drug metabolism through the cytochrome P450 enzymes has emerged as one important mechanism in the occurrence of herbal-drug or herbal-herbal interactions, which can result in drug or herbal toxicities (Gonzalez, 1990; Ogu and Maxa, 2000). Thus, the possibility of interactions between PME and other drugs may exist as it is metabolised by hepatic enzymes.

Locomotor activity is required for many complex behavioural tasks and increases or decreases non-specifically affects performance in many behavioural tests (Karl *et al.*, 2003). The potential to impair psychomotor functions is one of the most common side effects of widely-used sedatives (Hattesohl *et al.*, 2008). In this regard, the influence of *P. microcarpa* extract on locomotor activity was assessed in the activity meter test. Generally, CNS stimulation increases, and CNS depression decreases the amount of activity (Antoniou *et al.*, 1998; Himmel, 2008; Uzbay *et al.*, 2007; Zhang *et al.*, 2011). PME, in contrast to diazepam had no effect on spontaneous motor activity. This indicates that the sedative effect of PME over the doses tested does not impair psychomotor function, giving it an advantage over most of the clinically used sedatives.

Performance of mice on a rotarod is a sensitive, widely used method for assessing balance and coordination aspects of motor function (Hamm *et al.*, 1994; Barlow *et al.*, 1996; Rogers *et al.*, 1997). Thus, the fore and hind limb motor coordination and balance can be analyzed. This task requires an intact cerebellar function and motor coordination (Carter *et al.*, 1999). Mice with severe motor coordination problems will have difficulties to remain on the rotating rod. The extract did not alter the time of permanence on the bar in the rotarod test suggesting the absence of impaired motor function. Furthermore, this suggests a possible absence of neurotoxicity and that the inhibitory effect of the extract might be elicited via central mechanisms, not by peripheral neuromuscular blockade (Perez *et al.*, 1998; Chindo *et al.*, 2003). However, in contrast to PME, DZP decreased this parameter significantly indicating impaired motor coordination. Data in literature suggests that benzodiazepines, such as diazepam, act as anxiolytics (at low doses) and anticonvulsants, producing also a myorelaxant effect at higher doses (Onaivi *et al.*, 1992; Wolffgramm *et al.*, 1994). This is therefore not surprising as diazepam produced a myorelaxant effect at the dose used.

The PTZ test is the most frequently used acute chemical experimental model employed in the search for new AEDs (Löscher, 2011). PTZ blocks GABA-mediated Cl^- influx through an allosteric interaction in the Cl^- channel, thus leading to induction of convulsions in animals (Velíšek, 2006; Kubova, 2009). The GABAergic system has long been implicated in epilepsy. The inhibition and enhancement of the GABAergic neurotransmission will enhance and attenuate convulsions respectively (Meldrum, 1981; Gale, 1992; QuintansJunior *et al.*, 2008). Defects in GABA neurotransmission are linked to epilepsy in

both experimental animal models and human syndromes (Velíšek, 2006; Ambavade *et al.*, 2009). The ability of an agent to prevent or delay the onset of clonic and tonic-clonic convulsions induced by PTZ in animals is an indication of anticonvulsant activity (Vellucci and Webster, 1984; Amabeoku and Chikuni, 1993). In this study, acute administration of PME and the benzodiazepine diazepam, exhibited anticonvulsant activity against PTZinduced seizures by significantly and dose-dependently delaying the occurrence of seizures. In addition, they decreased the frequency and duration of both clonic and tonic convulsions in mice. The potent effect of diazepam as evident in the PTZ-induced convulsions agrees with its enhancing effects in GABAergic neurotransmission (Patil *et al.*, 2011).

In the tail immersion test, PME caused a prolonged latency period, indicating an increase in the nociceptive threshold. This test is able to differentiate between central opioid-like analgesics and peripheral analgesics (de Mesquita Padilha *et al.*, 2009; Asongalem *et al.*, 2004). The response to the tail-immersion test is a spinal reflex, but may also involve higher neural structures (Jensen and Yaksh, 1986; Pavin *et al.*, 2011). The antinociceptive effect of PME in this test is a further confirmation of analgesia observed in the Irwin test.

3.5 CONCLUSION

The present study concludes that the extract possesses CNS depressant, analgesic and anticonvulsant activity without affecting motor function.

Chapter 4 ANTICONVULSANT ACTIVITY

4.1 INTRODUCTION

Epilepsy is a brain disorder characterized by occurrence of more than one epileptic seizure with a continuing predisposition to generate more epileptic seizures associated with neurobiologic, cognitive, psychological, and social disturbances (Raol and Brooks-Kayal, 2012). Epilepsy is the most common neurological disorder after stroke, with 0.5 % prevalence, and a 2–3 % life time risk of being given a diagnosis of epilepsy (Browne and Holmes, 2001; Löscher, 2002a). It has to be acknowledged that more than 80 % of people with epilepsy live in developing countries, where the condition remains largely untreated (de Boer *et al.*, 2008).

Despite the availability of many antiepileptic drugs (AEDs), nearly one in three patients with epilepsy who have access to AEDs continue to have seizures, and a similar proportion experience unacceptable AED-related adverse effects (Brodie, 2005; Löscher, 2002b).

Thus, there is need for the development of better and safer AEDs with improved clinical profiles. Plant extracts are some of the most attractive sources of new drugs, and have been shown to produce promising results for the treatment of epilepsy. Examples include *Passiflora incarnata*, *Berberis vulgaris*, *Butea monosperma* and *Cymbopogon winterianus* (Nassiri-Asl *et al.*, 2007; Bhutada *et al.*, 2010; Kasture *et al.*, 2002; Quintans-Junior *et al.*, 2008). *Pseudospondias microcarpa* has been extensively used in Ghana and other parts of Africa as medication for various diseases including CNS disorders. Preliminary studies from our laboratory indicate that the hydroethanolic leaf extract of *P. microcarpa* (PME) possesses anticonvulsant activity against PTZ-induced seizures. Therefore, this study further explored the anticonvulsant activity of PME and possible mechanism(s) in animal models.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Male ICR mice were used in this experiment and experimental conditions were as described in section 3.2.1.

4.2.2 **Drugs and Chemicals**

Pentylenetetrazole (PTZ), picrotoxin (PTX), 4-aminopyridine (4-AP), strychnine (STN), Isoniazid (INH), N-nitro-L-arginine methyl ester (L-NAME), L-arginine and methylene blue (MB) (Sigma-Aldrich Inc., St. Louis, MO, USA); diazepam (INTAS, Gujarat, India); sildefanil (Pfizer, U.S.A); carbamazepine (Tegretol®, Novartis, Basel, Switzerland); flumazenil (Anexate®, Roche products Ltd., Herts, England); Sodium valproate (Epilim®,Sonofi-Synthelabo Ltd-UK).

4.2.3 **Pentylenetetrazole-Induced Seizures**

Pentylenetetrazole (60 mg kg^{-1}, s.c.) was used to induce clonic convulsions (Oliveira *et al.*, 2001). Mice were divided into 7 groups (n=8) and received PME (30, 100 or 300 mg kg^{-1}, *p.o.*), diazepam (0.1, 0.3 or 1 mg kg^{-1}, i.p.) or vehicle (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 (made of Perspex of dimensions 15 cm x15 cm x15 cm). 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 (Everio™ model GZ-MG 130U, JVC, Tokyo, Japan) placed directly opposite to the mirror. Videos of each 30 min session was later scored using public domain software JWatcher™ Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sidney, Australia available at http://www.jwatcher.ucla.edu/.) for behavioural parameters including: latency, frequency and duration of clonic convulsions. The observed clonic seizures were characterized for the appearance of facial myoclonus, forepaw myoclonus and forelimb clonus. The ability of a drug/extract to prevent the seizures or delay/prolong the latency or onset of the clonic convulsions was considered as an indication of anticonvulsant activity.

4.2.4 **Picrotoxin-Induced Seizures**

Anticonvulsant testing method of Leewanich *et al*. (1996) was used with modifications. Briefly, mice were divided into 7 groups (n=8) and received PME (30, 100 or 300 mg kg^{-1}, *p.o.*), diazepam (0.1, 0.3 or 1 mg kg^{-1}, i.p.) or vehicle (normal saline; 10 mL kg^{-1} i.p.) 30 min (i.p.) or 1 h (*p.o.)* before the injection of PTX (3 mg kg^{-1} i.p.) respectively. The latency to, frequency and duration of clonic convulsions were recorded for 30 min.

4.2.5 **Isoniazid-Induced Seizures**

Mice were divided into seven groups (n=10) and received PME (30, 100 or 300 mg kg^{-1}, *p.o.*), vehicle or the standard drug diazepam (0.1, 0.3 or 1.0 mg kg^{-1}, i.p.). One hour (*p.o.*) or 30 min (i.p.) after administration of test compounds, animals were injected with isoniazid (300 mg kg^{-1}, s.c.). Thereafter, mice were observed for 120 min for characteristic behavioural signs, such as intermittent forelimb extension, clonic seizures, tonic seizures and death. The latencies to the onset of the convulsive episode (clonic or tonic) and death were recorded as indicators of pro- or anticonvulsive effect of compounds.

4.2.6 **Strychnine-Induced Seizures**

Method as described by Porter *et al*. (1984) was employed. Mice were divided into 7 groups (n=7) and received PME (30, 100 or 300 mg kg^{-1}, *p.o.*), diazepam (0.1, 0.3 or 1 mg kg^{-1}, i.p.) or vehicle (normal saline; 10 mL kg^{-1} i.p.) 30 min (i.p.) or 1 h (*p.o.)* before the injection of STN (0.5 mg kg^{-1}, i.p.), respectively. Latency, frequency and duration of clonic convulsions were assessed for 30 min.

4.2.7 **4-Aminopyridine-Induced Seizures**

The method adopted for this study was as described by Rogawski and Porter (1990) with modifications. Mice were divided into 7 groups (n=10) and received PME (30, 100 or 300 mg kg^{-1}, *p.o.*), vehicle or the standard drug carbamazepine (30, 100 or 300 mg kg^{-1}, *p.o.*). One hour after administration of test compounds, animals were treated with a single injection of 4-AP (12 mg kg^{-1}, i.p.). Thereafter, mice were observed 60 min for both clonic and tonic seizures. Clonic seizures were characterized as appearance of facial myoclonus, forepaw myoclonus and forelimb clonus and tonic seizures were characterized as explosive clonic seizures with wild running and tonic forelimb and hind limb extension. Latencies for the onset of convulsive episodes (clonic or tonic) and death were recorded as indicators of pro or anticonvulsive effect of compounds.

4.2.8 **Maximal Electroshock Seizure Test**

Electroconvulsions were produced by means of an electric current (60 Hz, 50 mA, 0.2 s) delivered via ear-clip electrodes with an ECT Unit 7801 (Ugo Basile, Comerio, Italy). This current intensity elicited complete tonic extension of the hind limbs in control mice. Mice

received PME (30, 100 or 300 mg kg^{-1}, *p.o.*), carbamazepine (3, 10 or 30 mg kg^{-1}, *p.o.*) or vehicle (normal saline; 10 mL kg^{-1}, *p.o.*) 60 min before tonic hind-limb convulsions were induced. Protection against tonic hind limb seizures was determined. An animal was considered to be protected if the characteristic electroshock convulsive seizure pattern was absent.

4.2.9 **6-Hz seizure test**

The 6-Hz seizure model was performed as previously described (Brown *et al.*, 1953; Luszczki *et al.*, 2012). Psychomotor (limbic) seizures were induced via transauricular stimulation (6 Hz, 0.2 ms rectangular pulse width, 32 mA, 3 s duration) delivered by an ECT Unit 5780 (Ugo Basile, Comerio, Italy). Animals were manually restrained and released immediately following the stimulation and observed for the presence or absence of seizure activity. Saline (0.9 %) was used to wet the electrodes immediately before testing to ensure good electrical contact. Immediately following stimulation, mice were placed separately in Plexiglas cages (25 cm × 15 cm × 10 cm) for behavioural observation. After stimulation, the animals exhibited behavioural signs of psychomotor seizures—behavioural arrest, forelimb clonus, twitching of the vibrissae, and Straub tail—that lasted from 60-120 s in untreated animals. Animals resumed their normal exploratory behaviour after the seizure. The experimental endpoint was protection against the seizure: an animal was considered to be protected if it resumed its normal exploratory behaviour within 10 s after stimulation. Protection in the 6-Hz model was defined as the absence of a seizure. Mice not experiencing seizures exhibited normal exploratory behaviour when placed in the cages. Mice were divided into 8 groups (n=10) and received PME (30, 100, 300 or 1000 mg kg^{-1}, *p.o.*), sodium valproate (100, 200 or 400 mg kg^{-1}, *p.o.*) or vehicle (normal saline; 10 mL kg^{-1},*p.o.*) 60 min before psychomotor seizures were induced.

4.2.10 **Effect on $GABA_A$**

To investigate the possible involvement of $GABA_A$ receptors in the anticonvulsant action of PME, mice were pre-treated with flumazenil (2 mg kg^{-1}, i.p.), a selective benzodiazepine receptor antagonist or vehicle 15 min before PME (100 mg kg^{-1}, *p.o.*) or DZP (0.3 mg kg^{-1}, i.p.) administration. After 45 min, mice were challenged subcutaneously with PTZ (65 mg kg^{-1}) and assessed 30 min for latency, frequency and duration of clonic convulsions.

4.2.11 **Effect of PME on L-arginine-NO-cGMP Pathway**

Doses of the modulators were chosen based on pilot experiments and previous reports (Riazi *et al.*, 2006; Akula *et al.*, 2008; Bahremand *et al.*, 2010). To investigate the possible involvement of the L-arginine-NO-cGMP pathway in the anticonvulsant action of PME, mice were pre-treated with sub-effective doses of L-arginine [150 mg kg^{-1}, i.p., a precursor of nitric oxide (NO)], L-NAME [30 mg kg^{-1}, i.p., a non-selective nitric oxide synthase (NOS) inhibitor], methylene blue [1 mg kg^{-1}, i.p., an inhibitor of NO synthase and an inhibitor of soluble guanylate cyclase (sGC)], sildenafil [5 mg kg^{-1}, i.p., a phosphodiesterase 5 (PDE5) inhibitor] or vehicle 15 min before PME (100 mg kg^{-1}, *p.o*) administration. After 45 min, mice were challenged subcutaneously with PTZ (65 mg kg^{-1}) and assessed 30 min for latency, frequency and duration of clonic convulsions.

4.2.12 **Grip-Strength Test**

The effects of PME and diazepam on skeletal muscular strength in mice were quantified by the grip-strength test of Meyer *et al*. (1979). The grip-strength apparatus (BioSeb, Chaville, France) comprised a wire grid (8 cm × 8 cm) connected to an isometric force transducer (dynamometer). Mice were randomly divided into eight groups (n=6): saline-treated control group; diazepam group (0.1, 0.3, 1 and 3 mg kg^{-1}, i.p.) and PME group (30, 100 and 300 mg kg^{-1}, *p.o*.).Thirty minutes after i.p. and 1 h after oral administration of the test compounds, mice were lifted by the tails so that their forepaws could grasp the grid. Mice were then gently pulled backward by the tail until the grid was released. The maximal force exerted by the mouse before losing grip was recorded. The mean of 4 measurements for each animal was calculated and subsequently, the mean maximal force was determined. Skeletal muscular strength in mice was expressed in Newton (N).

4.2.13 **Statistical Analysis**

In all experiments, a sample size of six to ten (n=6-10) was used. Data are presented as mean±SEM. To compare differences between groups, one-way ANOVA was performed with Newman-Keuls' test as *post hoc.* In analyzing the possible role of GABAergic and nitric oxide mechanisms in the anticonvulsant effect of the extract, two-way ANOVA with the Bonferroni's *post hoc* test (*treatment* × *dose*) was performed. In the 4-aminopyridine and isoniazid seizure tests, the Kaplan-Meier method was used in estimating survival

relative to time and survival differences were analyzed with the log-rank test. GraphPad® Prism Version 5.0 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis. $P<0.05$ was considered statistically significant for all test. Doses for 50 % of the maximal effect (ED_{50}) for each drug were determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation:

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

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.

4.3 RESULTS

4.3.1 Pentylenetetrazole-Induced Seizures

The extract significantly and dose-dependently delayed the onset of clonic convulsions ($F_{3,28}=3.009$, $P=0.0469$; figure 4.1a) which was significant at 300 mg kg^{-1} ($P<0.05$).

ANOVA revealed that PME also significantly reduced the frequency of clonic convulsions ($F_{3,28}=6.947$, $P=0.0012$) with statistical significance observed at all the doses used ($P<0.05$ at 30 mg kg^{-1}; $P<0.01$ at 100 and 300 mg kg^{-1}). Newman-Keuls' *post hoc* test indicated statistically significant reduction in the duration of clonic convulsions by the extract at all the doses used ($P<0.05$ at 30 mg kg^{-1}; $P<0.001$ at 100 and 300 mg kg^{-1}). Diazepam, the reference anticonvulsant used, delayed the onset of clonic convulsions ($F_{3,36}=24.76$, $P<0.001$; figure 4.1c) with statistical significance at 0.3 and 1.0 mg kg^{-1} (both $P<0.001$).

Also, diazepam caused significant and dose-dependent reduction in the frequency ($F_{3,36}=38.01$, $P<0.001$) and duration of clonic convulsions ($F_{3,36}=49.60$, $P<0.0001$; figure 4.1d). Diazepam ($ED_{50}=0.204$ mg kg^{-1}, $E_{max}=100$ %) was more potent and efficacious than the extract ($ED_{50}=416.4$ mg kg^{-1}, $E_{max}=80$ %) in increasing the % latency to clonic seizures (figure 4.2). A similar trend was also observed for % duration as PME produced $ED_{50}=23.86$ mg kg^{-1} and $E_{max}=80$ % while the standard diazepam achieved $ED_{50}=0.1234$ mg kg^{-1} and $E_{max}=100$ %. However, though less potent ($ED_{50}=16.27$ mg kg^{-1}), PME

achieved maximum efficacy (E_{max}=100 %) similar to diazepam (ED_{50}=0.1841 mg kg^{-1}, E_{max}=100 %) with respect to the % decrease in frequency of clonic convulsions (figure 4.2).

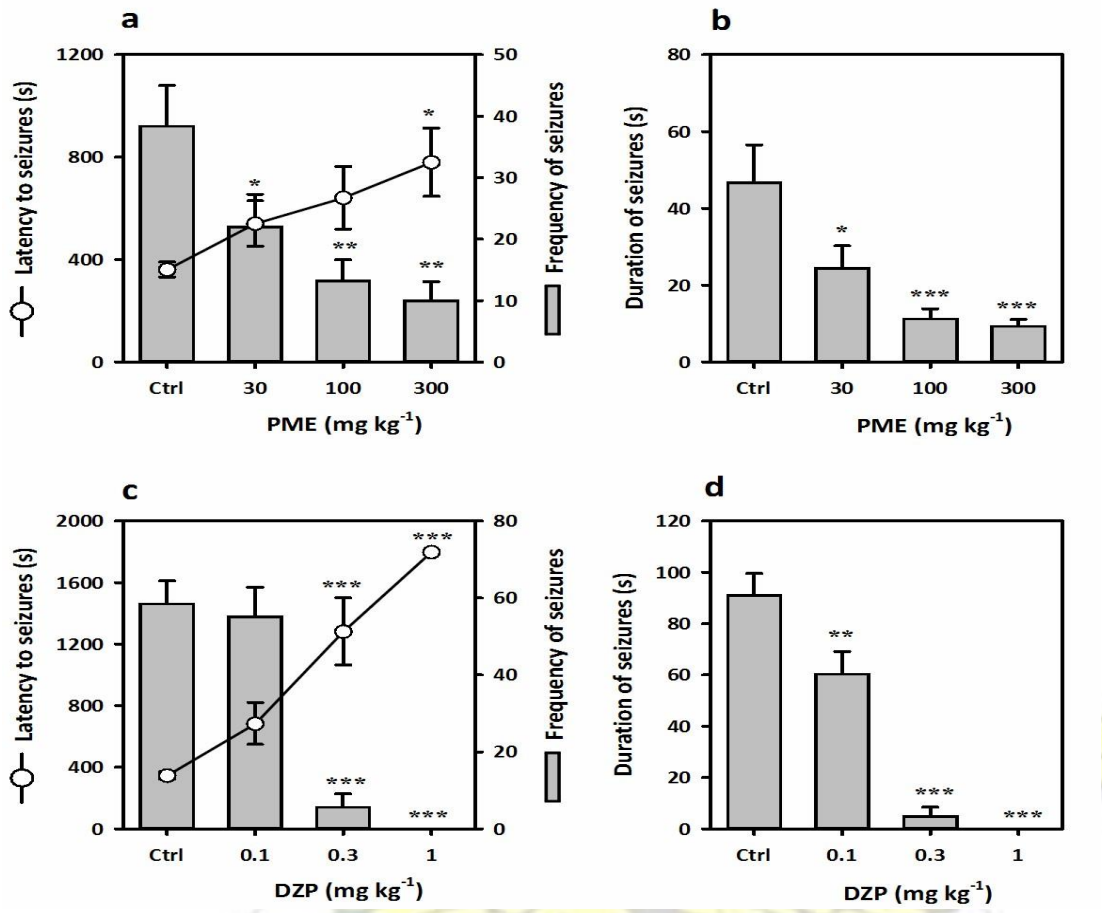


Figure 4.1 Effect of PME (30-300 mg kg^{-1}) and diazepam (0.1-1.0 mg kg^{-1}) on frequency (a, c), latency (a, c) and duration (b, d) of PTZ-induced clonic seizures in mice. Data are expressed as mean±SEM (n=8). Each group consisted of at least 10 mice. *P<0.05; **P<0.01; ***P <0.001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

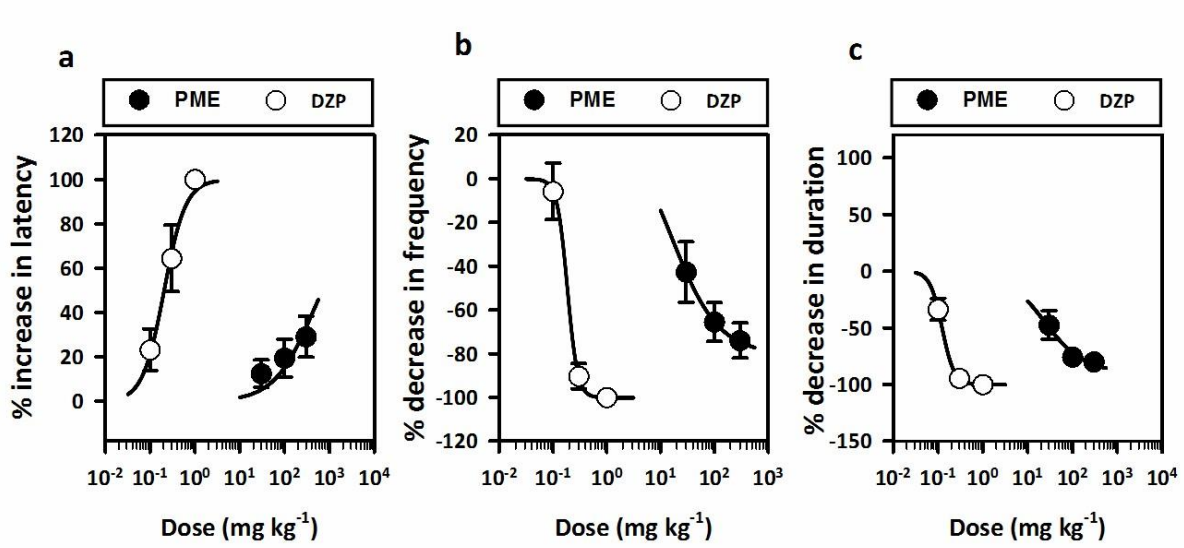


Figure 4.2 Dose-response curves of PME and diazepam on the % increase in latency to seizures (a), % decrease in frequency (b) and % decrease in duration of seizures (c) in PTZ-induced seizures. Each point represents mean±SEM (n=8).

4.3.2 **Picrotoxin-Induced Seizures**

PME exhibited anticonvulsant effect against picrotoxin-induced seizures by significantly and dose-dependently reducing the frequency ($F_{3,36}$=5.071, P=0.0063; figure 4.3a) and duration ($F_{3,36}$=6.117, P=0.0025; figure 4.3b) of clonic convulsions. PME also significantly delayed the onset of clonic convulsions ($F_{3,28}$=6.117, P=0.0028) with statistical significance observed at 300 mg kg^{-1} (P<0.01). PME, at the highest dose of 300 mg kg^{-1}, produced a 10 % protection against picrotoxin-induced convulsions. No mortality was observed for the entire duration of the experiment. Diazepam produced effects similar to that of the extract. It significantly delayed the onset of convulsions ($F_{3,36}$=9.7118, P<0.0001; figure 4.3c) and reduced the frequency ($F_{3,36}$=9.131, P<0.0001) and duration ($F_{3,36}$=15.63, P<0.0001; figure 4.3d) of convulsions. Dose response curves (figure 4.4) indicates that the extract achieved a maximum efficacy of 80 % for all the parameters measured (latency, frequency and duration of clonic convulsions) while showing a higher potency (ED_{50}=15.50 mg kg^{-1}) in reducing the duration of convulsions. It, however, was less potent and efficacious than diazepam in affecting the measured parameters.

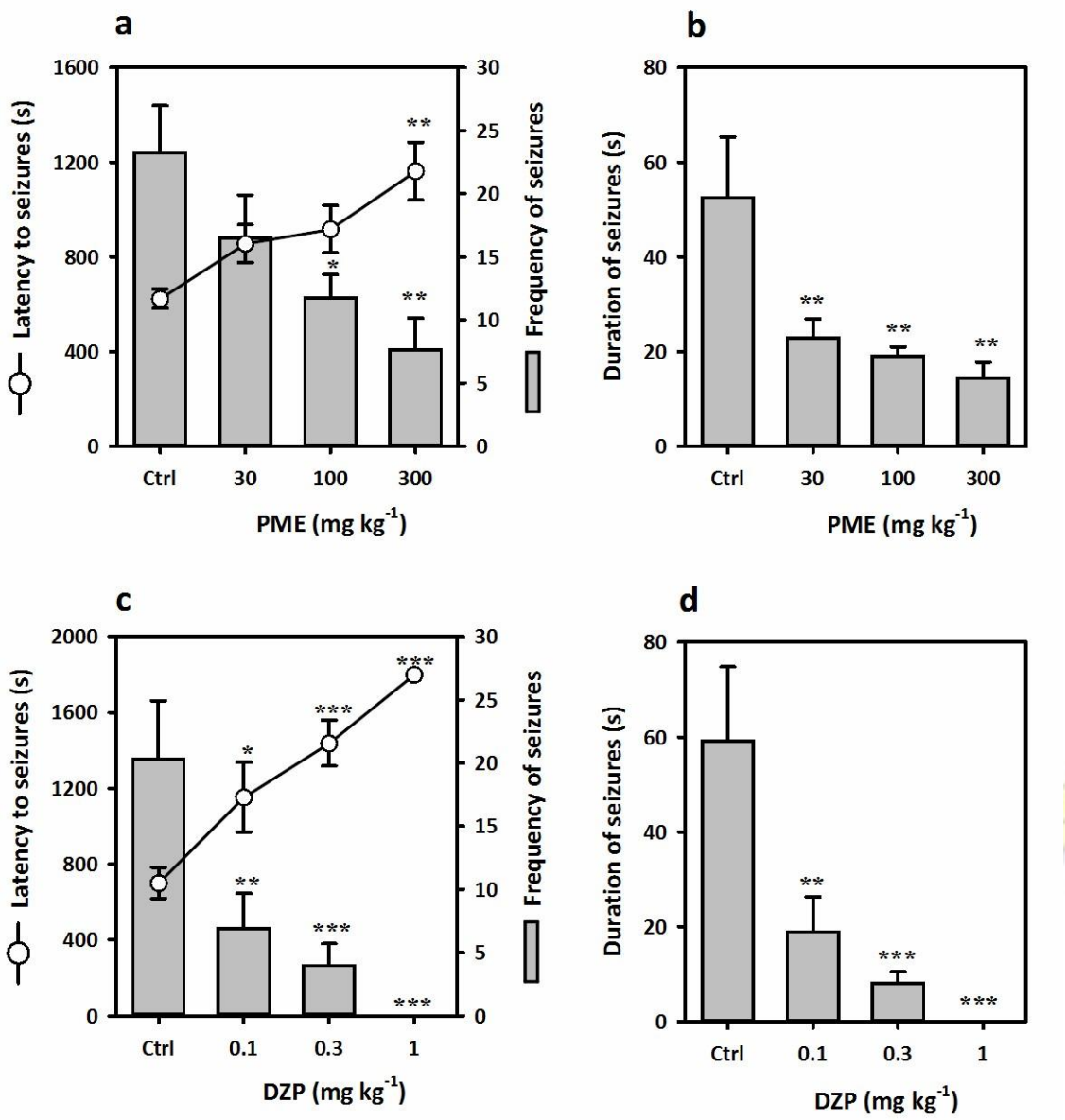


Figure 4.3 Effect of PME (30-300 mg kg^{-1}) and diazepam (0.1-1.0 mg kg^{-1}) on frequency (a, c), latency (a, c) and duration (b, d) of picrotoxin-induced clonic seizures in mice. Data are expressed as mean±SEM (n=8). Each group consisted of at least 10 mice. **P*<0.05; ***P*<0.01; ****P*<0.001 compared to vehicletreated group (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

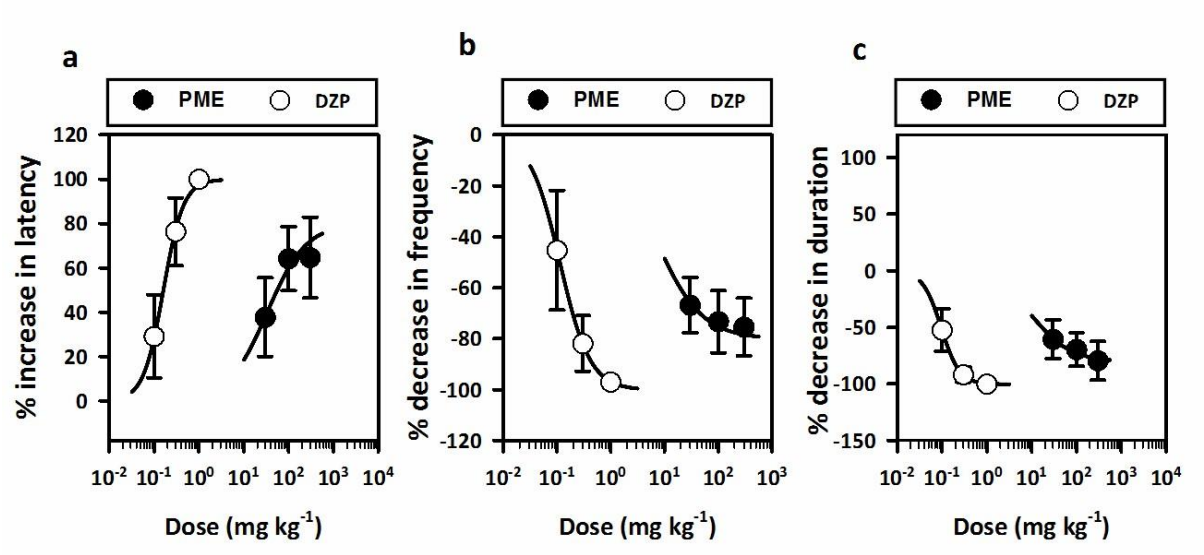


Figure 4.4 Dose-response curves of PME and diazepam on the % increase in latency to seizures (a), % decrease in frequency (b) and % decrease in duration of seizures (c) in picrotoxin-induced seizures. Each point represents mean±SEM (n=8).

4.3.3 **Isoniazid-Induced Seizures**

Isoniazid (300 mg kg^{-1}, s.c.) elicited clonic convulsions followed by tonic hind limb extension and mortality in mice. Treatment with PME (30-300 mg kg^{-1}, *p.o.*) significantly delayed the onset to both clonic ($F_{3,36}$=12.90, P<0.0001; figure 4.5) and tonic ($F_{3,36}$=15.63, P<0.0001; figure 4.5) convulsions as compared to vehicle treated mice. Furthermore, PME significantly (P=0.0005, $\chi 2$ (df =3) =17.65) improved survival of the animals after induction of convulsions (figure 4.6). As compared to vehicle control mice, diazepam (0.1-1.0 mg kg^{-1}, i.p.) treated mice showed significant protection against INH-induced mortality (P=0.003, $\chi 2$ (df =3) =13.90). Furthermore, in figure 4.5, it significantly delayed onset of convulsions [clonic ($F_{3,36}$=19.69, P<0.0001) and tonic ($F_{3,36}$=25.46, P<0.0001)].

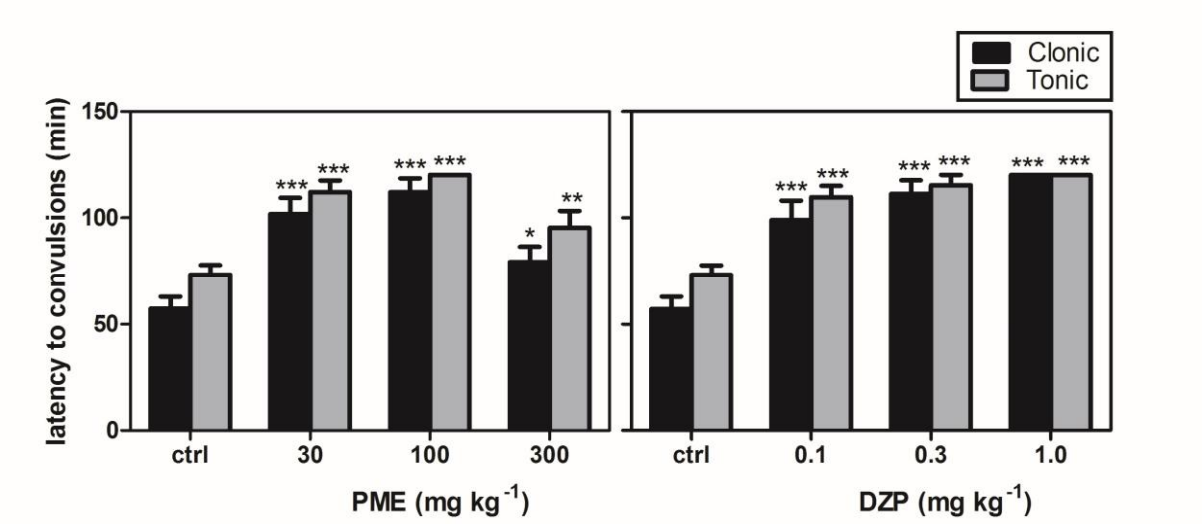


Figure 4.5 Effect of PME (30-300 mg kg^{-1}) and diazepam (0.1-1.0 mg kg^{-1}) on latency to isoniazid-induced clonic seizures in mice. Data are expressed as mean±SEM (n=10). Each group consisted of at least 10 mice. **P*<0.05; ***P*<0.01; ****P*<0.001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

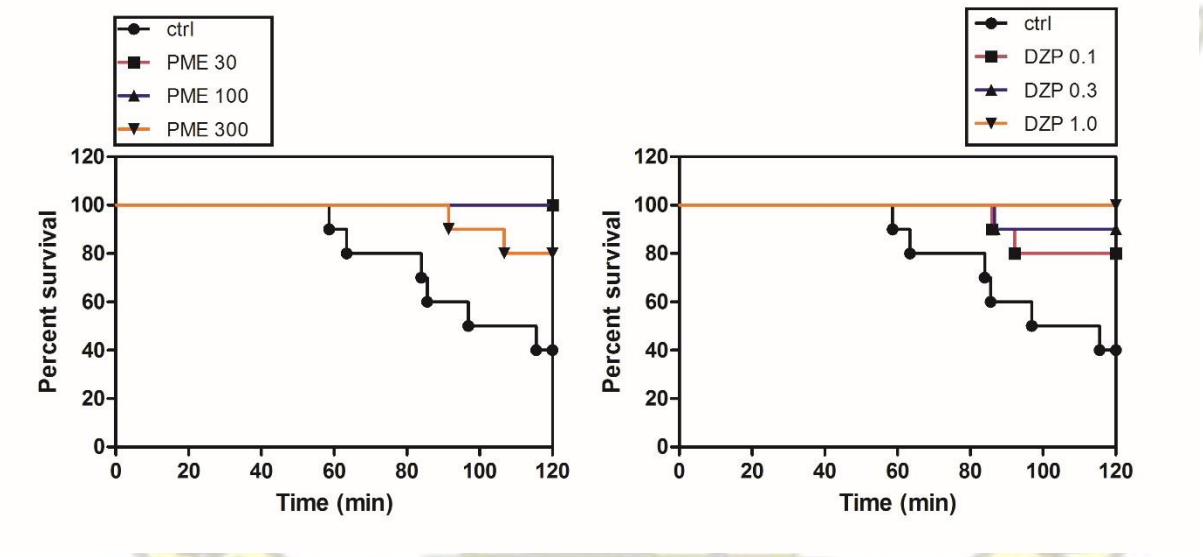


Figure 4.6 Kaplan–Meier estimates of overall survival of animals treated with PME (30, 100 and 300 mg kg^{-1}) and diazepam (0.1, 0.3 and 1 mg kg^{-1}) in the isoniazid-induced seizure test over a 2 hour observation period (n=10).

4.3.4 **Strychnine-Induced Seizures**

Figure 4.7 indicates the effects of PME (30-300 mg kg^{-1}, *p.o.*) and diazepam (0.1-1 mg kg^{-1}, i.p.) on latency, frequency and duration of clonic convulsions induced by strychnine in mice. ANOVA revealed that the extract exhibited a dose-dependent anticonvulsant effect

against strychnine-induced clonic seizures by significantly increasing latency to convulsions ($F_{3,24}$=4.208, P=0.0159) and significantly reducing the frequency ($F_{3,24}$=7.569, P=0.0010). PME provided 29 % (at 30 mg kg^{-1}), 43 % (at 100 mg kg^{-1}) and 57 % (at 300 mg kg^{-1}) protection against the strychnine-induced seizures. Diazepam significantly delayed the onset of convulsions ($F_{3,24}$=13.32, P<0.0001) and reduced the frequency ($F_{3,24}$=7.768, P=0.0009) and duration ($F_{3,24}$=6.721, P=0.0019) of convulsions. Diazepam provided 29 % (at 0.1 mg kg^{-1}), 71 % (at 0.3 mg kg^{-1}) and 100 % (at 1 mg kg^{-1}) protection against the strychnine-induced clonic seizures. Administration of diazepam was more efficacious and potent than the extract in delaying the onset of convulsions as well as reducing frequency and duration of convulsions.

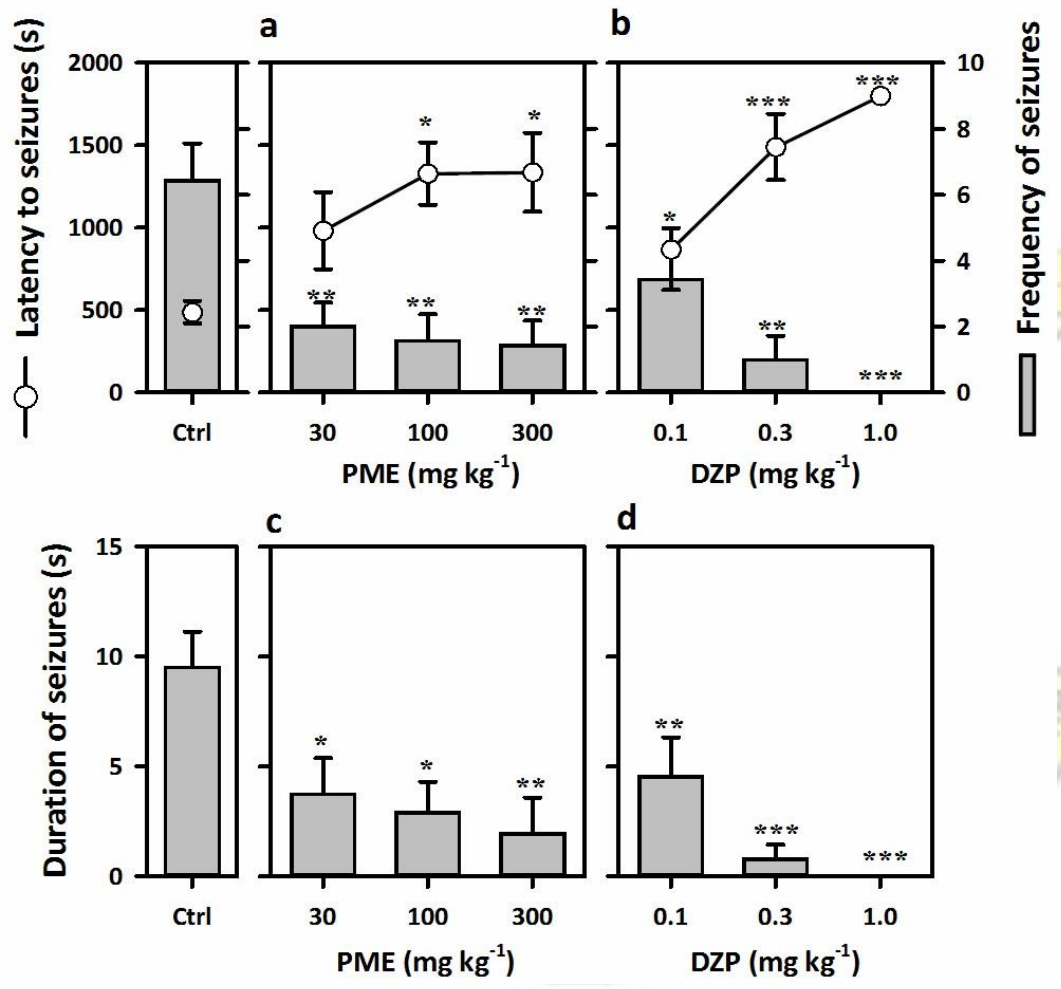


Figure 4.7 Effect of PME (30-300 mg kg^{-1}) and diazepam (0.1-1.0 mg kg^{-1}) on frequency (a and b), latency (a and b) and duration (c and d) of strychnine-induced clonic seizures in mice. Data are expressed as mean±SEM. Each group consisted of at least 7 mice. *P<0.05; **P<0.01; ***P<0.001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

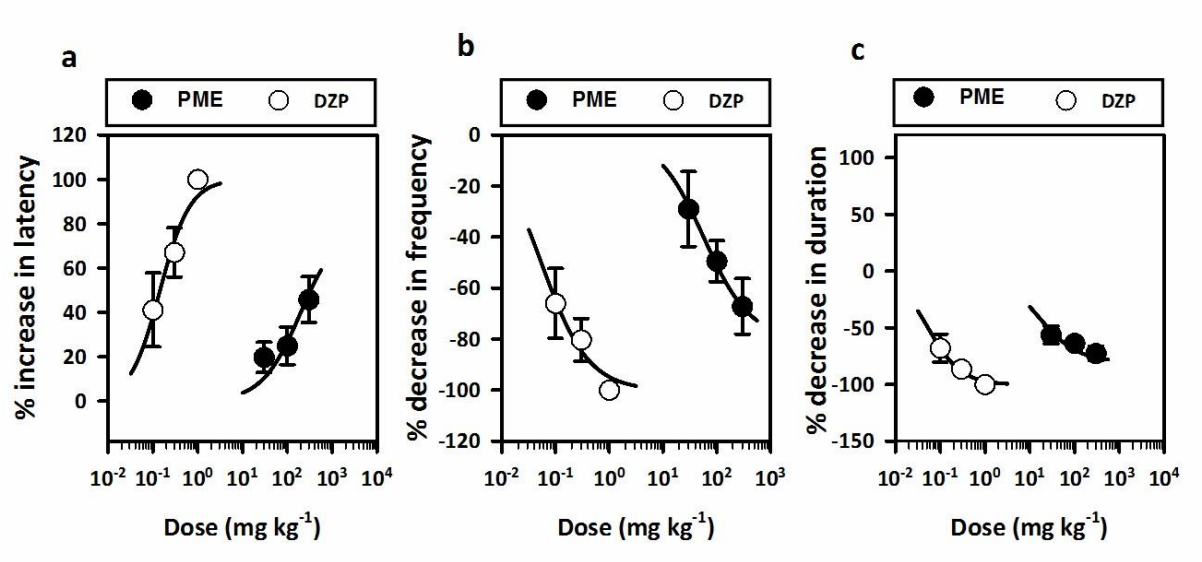


Figure 4.8 Dose-response curves of PME and diazepam on the % increase in latency to seizures (a), % decrease in frequency (b) and % decrease in duration of seizures (c) in strychnine-induced seizures. Each point represents mean±SEM (n=7).

4.3.5 **4-Aminopyridine-Induced Seizures**

A single administration of 4-AP (12 mg kg^{-1}, i.p.) caused seizures (clonic and tonic) and 100 % mortality in all saline-control mice,. In contrast, pretreatment of animals with PME (30-300 mg kg^{-1}, *p.o.*) as shown in figure 4.9 caused a significant delay in the latency of clonic ($F_{3,36}$=10.81, P<0.0001) and tonic seizures ($F_{3,36}$=9.513, P<0.0001). The anticonvulsant effects of the extract on 4-AP-induced seizures decreased with increasing dose. PME provided 40 % (at 30 mg kg^{-1}), 30 % (at 100 mg kg^{-1}) and 0 % (at 300 mg kg^{-1}) protection against 4-AP-induced clonic seizures. Furthermore, the extract provided 50 % (at 30 mg kg^{-1}), 50 % (at 100 mg kg^{-1}) and 20 % (at 300 mg kg^{-1}) protection against 4-APinduced tonic seizures in mice. Carbamazipine (CBZ) produced effects analogous to the extract in the 4AP-induced seizure test but the effects increased with increasing dose. It caused a significant delay (figure 4.9) in the latency of clonic ($F_{3,36}$=9.040, P<0.0001) and tonic seizures ($F_{3,36}$=12.06, P<0.0001). Carbamazepine provided 10 % (at 30 mg kg^{-1}), 50 % (at 100 mg kg^{-1}) and 60 % (at 300 mg kg^{-1}) protection against 4-AP-induced clonic seizures. Furthermore, it provided 10 % (at 30 mg kg^{-1}), 60 % (at 100 mg kg^{-1}) and 80 % (at 300 mg kg^{-1}) protection against 4-AP-induced tonic seizures in mice. From the dose response curves, oral administration of carbamazepine was more efficacious and potent

than the extract in delaying the occurrence of convulsions. The extract significantly (P<0.0001, χ^2 (df =3) =30.27; figure 4.10) improved survival of the animals after induction of convulsions. Carbamazepine also produced similar effects on survival (P<0.0001, χ^2 (df =3) =36.61; figure 4.10).

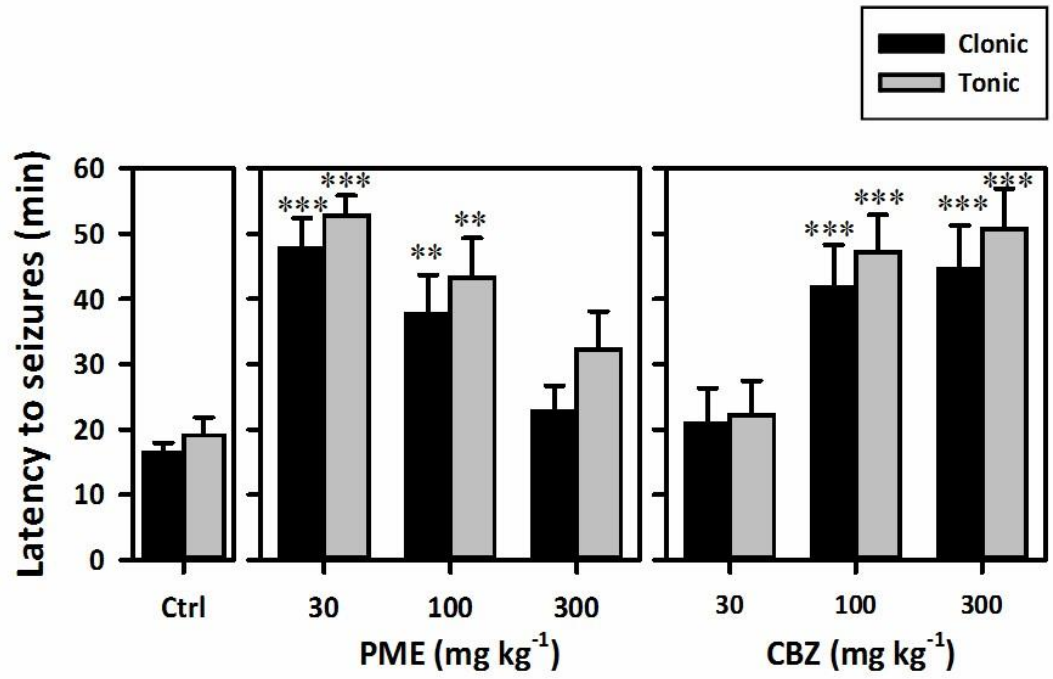


Figure 4.9 Effect of PME (30-300 mg kg^{-1}) and carbamazepine (30-300 mg kg^{-1}) on latency of 4-APinduced seizures in mice. PME and carbamazepine were administered *p.o.* 60 min before behavioural assessments for 1 h. Data are expressed as mean±SEM (n=10). Each group consisted of 10 mice. ***P*<0.01; ****P* <0.001 compared to vehicle-treated group (One-way ANOVA followed by NewmanKeuls' *post hoc* test)

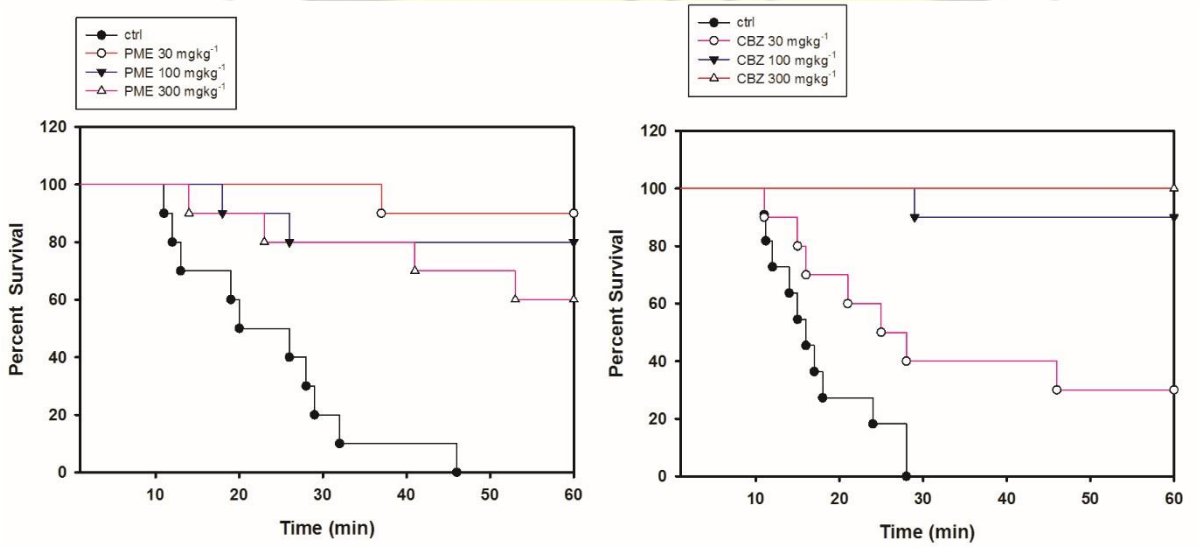


Figure 4.10 Kaplan–Meier estimates of overall survival of animals treated with PME (30, 100 and 300 mg kg^{-1}) and carbamazepine (30, 100 and 300 mg kg^{-1}) in the 4-aminopyridine seizure test over a one hour observation period (n=10).

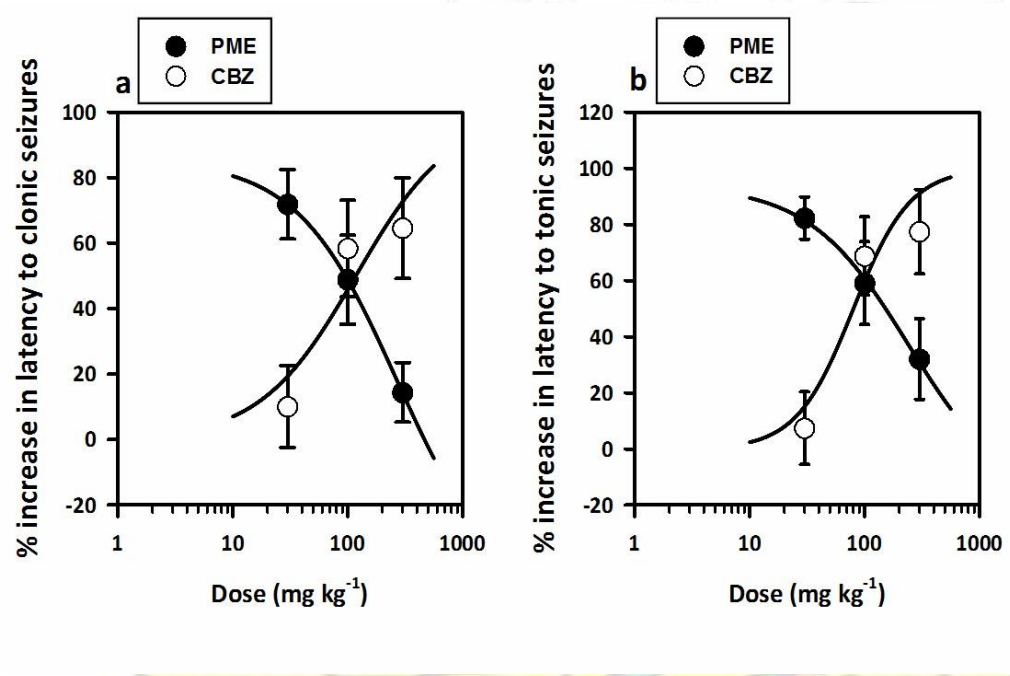


Figure 4.11 Dose-response curves of PME and carbamazepine on the % increase in latencies to clonic (a) and tonic (b) seizures in 4-AP-induced seizures. Each point represents mean±SEM (n=10).

4.3.6 **Effect on Maximal Electroshock Seizures**

Electrical stimulation produced tonic hind limb extensions (HLEs) in all saline-control mice. The extract did not protect against tonic hind limb extensions. In contrast to PME, carbamazepine at the dose of 30 mg kg^{-1}, completely protected mice against tonic hind limb extensions. In addition, no deaths were recorded at 10 and 30 mg kg^{-1} (table 4.1).

Table 4.1 Effects of PME and carbamazepine on maximal electroshock (MES)-induced seizure in mice

Group	**Dose (mg kg^{-1})**	**Incidence of tonic extensions (%)**	**Death (%)**
Control		100	33.3
PME	30	100	33.3

	100	100	50.0
	300	100	33.3
	1000	100	33.3
CBZ	3	100	16.7
	10	83.3	0
	30	0	0

Data shows the percentage of mice (n=10) that produced tonic convulsions and deaths.

4.3.7 **Effect on Psychomotor seizures**

All saline control mice exhibited psychomotor seizures (0 % protection) after electrical stimulation that lasted 60-120 s (table 4.2). Acute treatment with PME protected against 6 Hz-induced seizures. The extract provided 20 % (at 30 mg kg^{-1}), 40 % (at 100 mg kg^{-1}), 60 % (at 300 mg kg^{-1}) and 90 % (at 1000 mg kg^{-1}) protection against 6 Hz-induced seizures. Sodium valproate (VPA), the reference anticonvulsant, produced effects similar to the extract. It provided 10 % (at 100 mg kg^{-1}), 60 % (at 200 mg kg^{-1}) and 90 % (at 400 mg kg^{-1}) protection against 6 Hz-induced seizures. The ED_{50} value of PME was found to be 141.6 mg kg^{-1} (95 % confidence interval: 67.34-297.7 mg kg^{-1}) and that of sodium valproate, 155.6 mg kg^{-1} (95 % confidence interval: 20.41-1186 mg kg^{-1}).

Table 4.2 Effects of PME and sodium valproate in the mouse 6 Hz-induced limbic seizure model

Group	**Dose (mg kg^{-1})**	**% protection**
control		0
PME	30	30
	100	40
	300	60
	1000	90

VPA	100	20
	200	60
	400	90

Data indicates the percentage of mice (n=10) that were protected.

4.3.8 **Effect on $GABA_A$**

PME (100 mg kg^{-1}, *p.o*) exerted anticonvulsant activity by significantly increasing latency and decreasing both frequency and duration of clonic convulsions. Administration of flumazenil (FMZ; 2 mg kg^{-1}, i.p.) had no effects on latency, duration and frequency of convulsions as compared with saline (vehicle)-treated animals. However, pre-treatment with flumazenil significantly reversed the anticonvulsant effect of PME (100 mg kg^{-1}, *p.o*) by decreasing latency ($F_{1,36}$=36.48, P<0.0001) as well as increasing duration ($F_{1,36}$=24.04, P<0.0001) and frequency ($F_{1,36}$=23.10, P<0.0001) of clonic seizures induced by PTZ. Similar results were obtained for diazepam (figure 4.12).

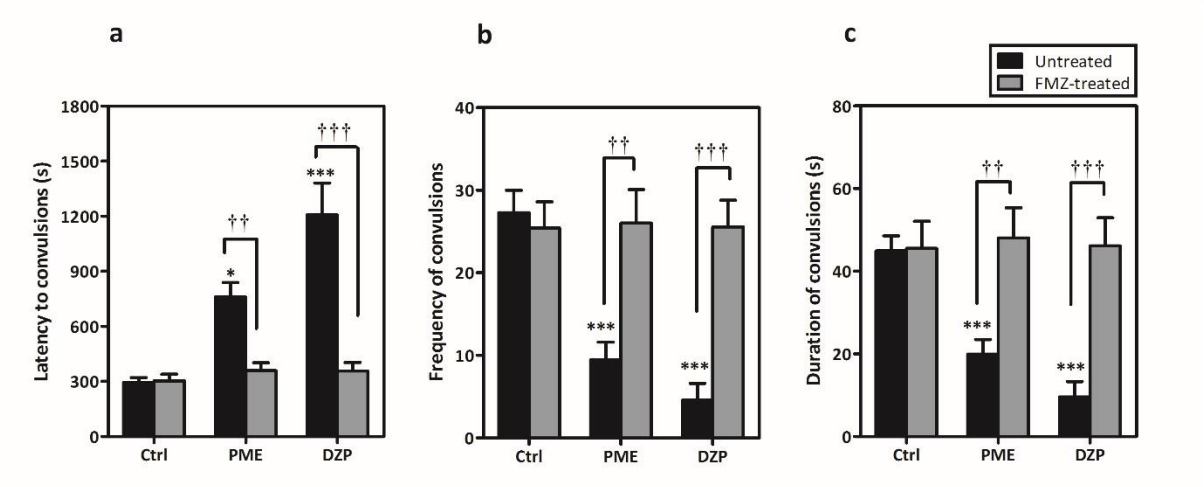


Figure 4.12 Effect of flumazenil (2 mg kg^{-1}, i.p.) on the latency (a), frequency (b), and duration of seizures (c) of PME (100 mg kg^{-1}, *p.o.*) and diazepam (0.3 mg kg^{-1}, i.p.) in PTZ-induced seizures. Data are presented as mean±SEM (n=7). $^{*}P$<0.05 and $_{***}$ <0.001 compared to vehicle-treated group (One-way analysis of variance followed by Newman-Keuls' *post hoc* test). ††† <0.001 (Two-way ANOVA followed by Bonferroni's *post hoc* test).

4.3.9 **Effect of PME on L-arginine-NO-cGMP Pathway**

Acute PME (100 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 (vehicle)-treated animals (figure 4.14). However, pre-treatment with L-arginine significantly inhibited the anticonvulsant effect of PME (100 mg kg^{-1}, *p.o*) by decreasing latency ($P<0.01$) and increasing duration ($P<0.05$) of clonic seizures as revealed by post hoc analysis. Pre-treatment with a sub-effective dose of either L-NAME (30 mg kg^{-1}, i.p., a non-selective nitric oxide synthase inhibitor) or methylene blue (1 mg kg^{-1}, i.p., an inhibitor of both nitric oxide synthase and soluble guanylate cyclase) produced significant anticonvulsant action with an effective dose of PME (100 mg kg^{-1}, *p.o*) as evident from the delayed expression of clonic convulsions [L-NAME ($F_{1,24}=4.474$, $P<0.05$); MB ($F_{1,24}=6.005$, $P<0.05$)] induced by PTZ (figure 4.13).

Figure 4.14 shows the effect of pretreatment of sildenafil, a phosphodiesterase 5 inhibitor on PTZ-induced clonic seizures. Concomitant administration with sildenafil (5 mg kg^{-1}, i.p., a PDE5 inhibitor) significantly reversed the anticonvulsant action of PME (100 mg kg^{-1}, *p.o*). This is observed as a decrease in the onset ($P<0.05$) and increase in the duration of clonic ($P<0.05$) convulsions induced by PTZ.

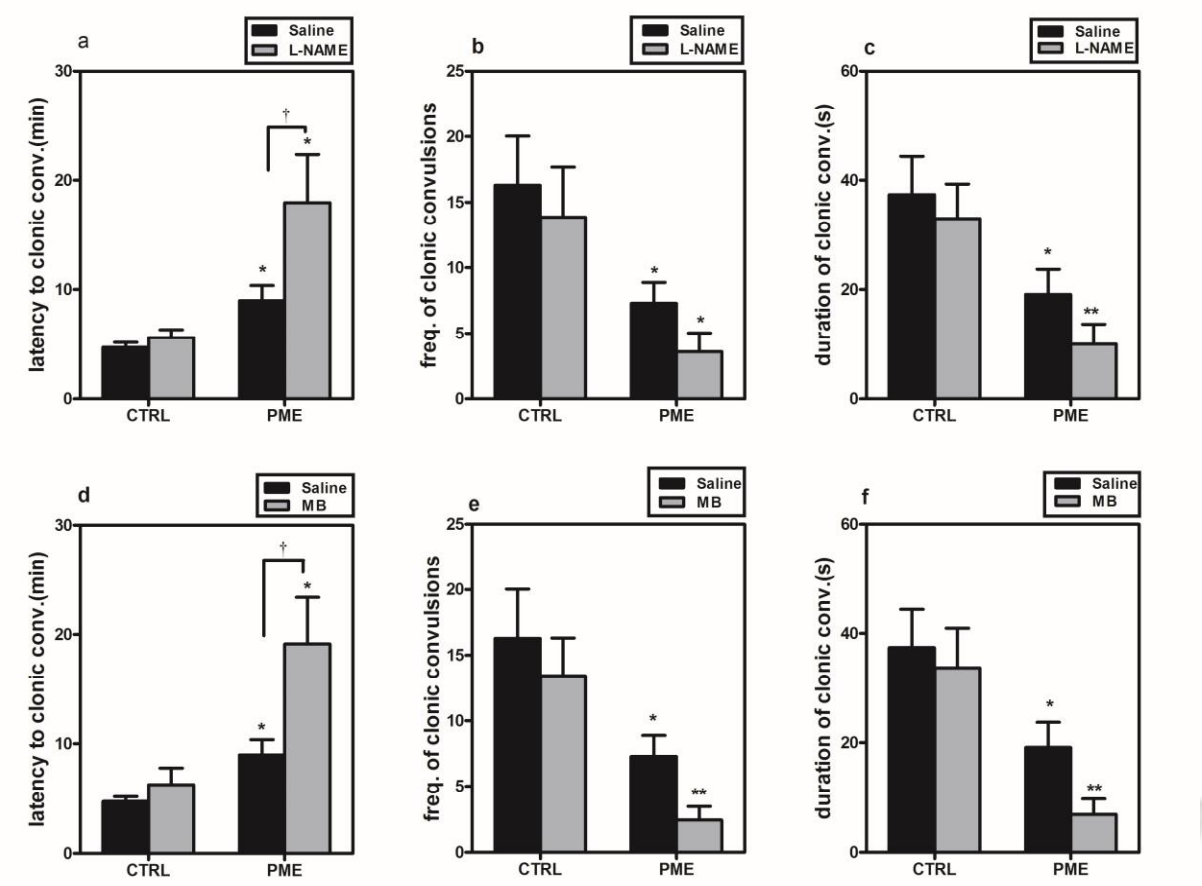


Figure 4.13 Effects of pretreatment of L-NAME [30 mg kg^{-1}, i.p., a non-selective nitric oxide synthase (NOS) inhibitor] and methylene blue [1 mg kg^{-1}, i.p., an inhibitor of NO synthase and an inhibitor of soluble guanylate cyclase (sGC)] on the anticonvulsant effect of PME (100 mg kg^{-1}, *p.o.*) in the PTZinduced seizure test. L-NAME, MB or saline was administered 15 min before administration of PME and 45 min before determination of PTZ-induced seizures. Data are presented as group mean±SEM (n=7). **P*<0.05, ***P*<0.01 versus vehicle-treated animals (One-way ANOVA followed by Newman-Keuls' test). Significant difference between treatments: †*P*<0.05 (Two-way ANOVA followed by Bonferroni's test).

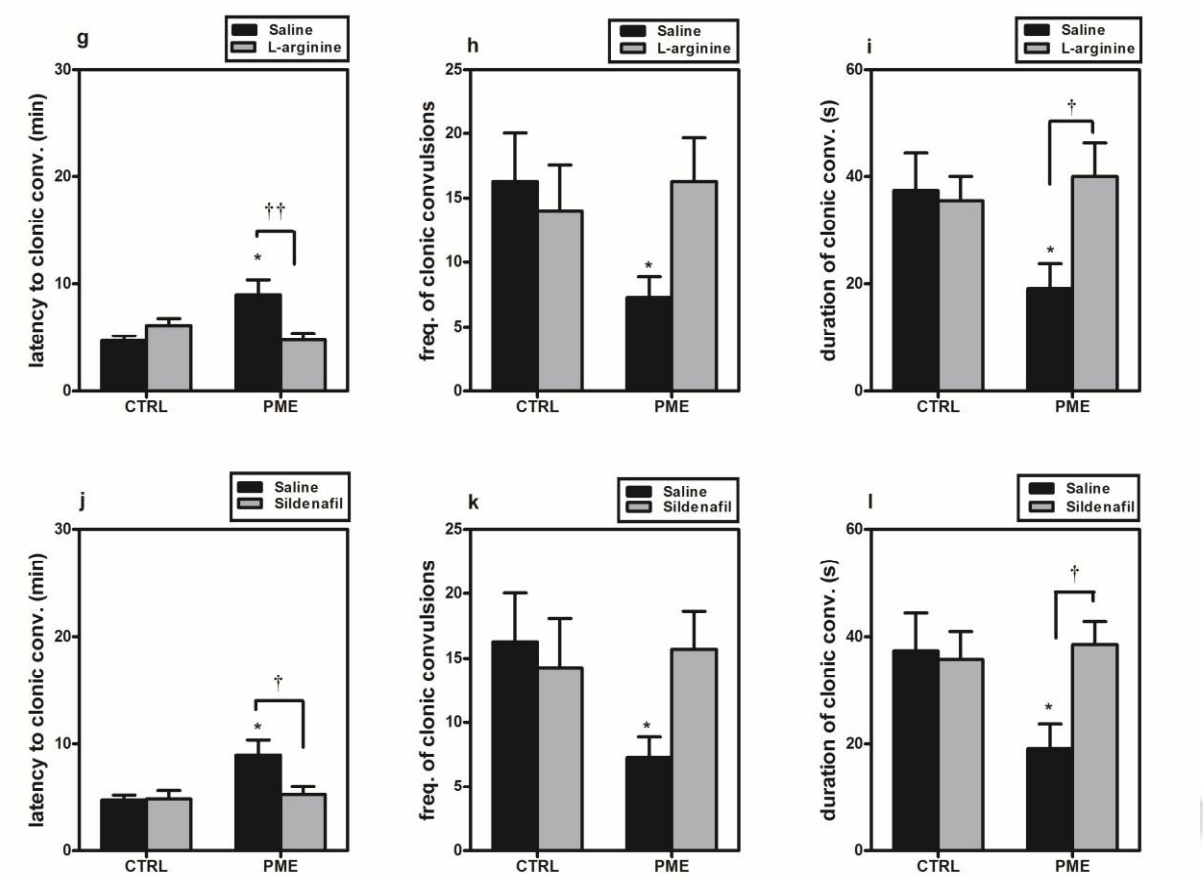


Figure 4.14 Effects of pretreatment of L-arginine [150 mg kg^{-1}, i.p., a precursor of nitric oxide (NO)] and sildenafil [5 mg kg^{-1}, i.p., a phosphodiesterase 5 (PDE5) inhibitor] on the anticonvulsant effect of PME (100 mg kg^{-1}, *p.o.*) in the PTZ-induced seizure test. L-arginine, sildenafil or saline was administered 15 min before administration of PME and 45 min before determination of PTZ-induced seizures. Data are presented as group mean±SEM (n=7). **P*<0.05 versus vehicle-treated animals (One-way ANOVA followed by Newman-Keuls' test). Significant difference between treatments: $^{\dagger}P$<0.05 (Two-way ANOVA followed by Bonferroni's test).

4.3.10 **Grip-Strength Test**

Figure 4.15 shows the results of the effect of PME and diazepam on skeletal muscle strength in the grip-strength test. ANOVA revealed that pre-treatment of mice with PME (30-300

mg kg^{-1}, *p.o.*) did not significantly affect the grip-strength in mice (P>0.05). In contrast to PME, diazepam (0.1-3 mg kg^{-1}, i.p.) significantly and dose-dependently decreased ($F_{3,16}$=4.308, P=0.0113) the grip-strength in mice with Newman-Keuls' *post hoc* analysis revealing a significant effect at the dose of 3 mg kg^{-1} (P<0.05).

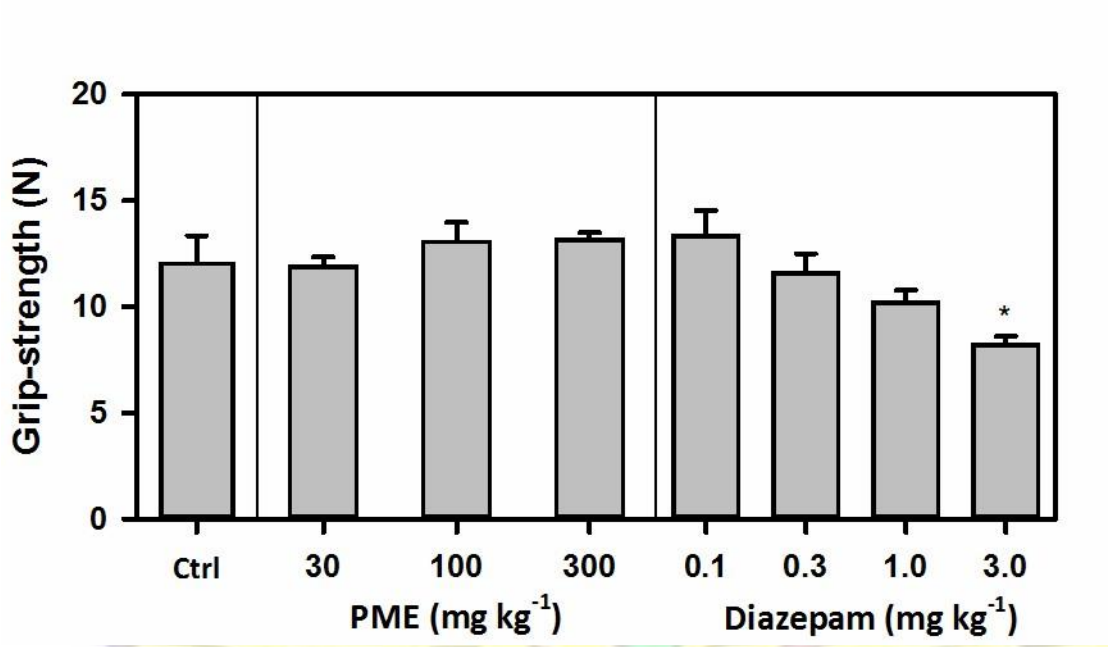


Figure 4.15 Behavioural effects of PME and DZP on muscle relaxant activity in the grip-strength test in mice. Data are expressed as group mean±SEM (n=6). *P<0.05 compared to control group (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

4.4 DISCUSSION

Results of the present study provide evidence that a hydroethanolic leaf extract of the plant possesses anticonvulsant activity in the pharmacologically validated experimental animal models used.

Pentylenetetrazole (PTZ) test is the most frequently used acute chemical experimental model employed in the search for new antiepileptic drugs (AEDs) (Löscher, 2011). PTZ blocks GABA-mediated Cl^- influx through an allosteric interaction in the Cl^- channel, thus leading to induction of convulsions in animals (Kubova, 2009; Velíšek, 2006). The GABAergic system is implicated in epilepsy since enhancement and inhibition of the neurotransmission of GABA will attenuate and enhance convulsion respectively (Meldrum, 1981; Gale, 1992; Quintans-Júnior *et al.*, 2008). Defects in GABA neurotransmission are linked to epilepsy in both experimental animal models and human syndromes (Ambavade

et al., 2009; Velíšek, 2006). Drugs such as benzodiazepines, phenobarbitone, valproate and felbamate that enhance $GABA_A$ receptor-mediated inhibitory neurotransmission can also block PTZ-induced clonic seizures (Macdonald and Kelly, 1995). Ability of an agent to prevent or delay the onset of clonic and tonic-clonic convulsions induced by PTZ in animals is an indication of anticonvulsant activity (Amabeoku and Chikuni, 1993; Vellucci and Webster, 1984). In this study, acute administration of PME and the benzodiazepine diazepam, exhibited anticonvulsant activity against PTZ-induced seizures by significantly and dose-dependently delaying the occurrence of clonic seizures. In addition, they decreased the frequency and duration of the clonic seizures in mice. This potent effect of diazepam as evident in the PTZ-induced convulsions agrees with its enhancing effects in GABAergic neurotransmission (Patil *et al.*, 2011). Therefore, the blocking effect of *P. microcarpa* extract in the PTZ-seizure model suggests its anticonvulsant action may probably be due to its interference with GABAergic mechanism(s). It has also been suggested that PTZ-induced clonic seizures model myoclonic seizures (Kubova, 2009). Thus, the extract could protect against myoclonic seizures.

The convulsant action of picrotoxin is via blockade of the $GABA_A$ receptor-linked chloride ion channel, which normally opens to allow increased chloride ion conductance following the activation of $GABA_A$ receptors by GABA (De Deyn *et al.*, 1992; Nicoll, 2001; Velíšek, 2006). Data from this study shows that PME and diazepam exhibited anticonvulsant activity against picrotoxin-induced seizures by significantly and dose-dependently delaying the occurrence as well as decreasing the frequency and duration of clonic seizures. It is probable that PME attenuated picrotoxin-induced convulsions by enhancing GABA neurotransmission. This further supports the hypothesis that *P. microcarpa* may be affecting GABAergic mechanism(s) to exert its anticonvulsant activity.

Isoniazid induces convulsions by inhibiting GABA synthesis (Costa *et al.*, 1975). It inhibits glutamic acid decarboxylase (GAD) activity (enzyme involved in GABA synthesis), resulting in decreased levels of GABA (Raygude *et al.*, 2012; Vergnes *et al.*, 2000). Similar to diazepam, PME significantly delayed convulsions (clonic and tonic) and reduced mortality indicating anticonvulsant effect against INH-induced convulsions. Action against INH-induced convulsions could therefore indicate increased GABA synthesis by PME. This further confirms the possible contribution of the GABAergic system in the

anticonvulsant activity of the plant extract.

To further confirm the possible involvement of GABAergic system in the anticonvulsant activity of PME, flumazenil, a specific antagonist of the benzodiazepine site in the $GABA_ABZD$ receptor complex (File and Pellow, 1986; Brogden and Goa, 1991), was used. Pretreatment with flumazenil antagonised the anticonvulsant effect of PME suggesting involvement of the $GABA_A$ receptor complex.

Activation of glycine receptors results in an influx of chloride ions into the neuron, which is then hyperpolarized and inhibited. Strychnine acts as a selective competitive antagonist that blocks the inhibitory effect of chloride channel associated with glycine at all glycine receptors (Curtis *et al.*, 1971; Patil *et al.*, 2011). Thus, the observed protection of diazepam or the extract in the strychnine-induced seizure test is presumably mediated through the glycinergic pathway.

Pretreatment of PME and carbamazepine before administration of 4-AP significantly increased the latency to seizures and reduced the incidence of mortality. Action of 4-AP occurs through a K^+-channel blockade at the presynaptic neuronal level (Thesleff, 1980; Rogawski and Barker, 1983; Brito *et al.*, 2009). As a result, efflux of intracellular K^+ is suppressed and calcium influx is enhanced, leading to an increase in neurotransmitter release and therefore, to an increase in nervous signalling (Molgo *et al.*, 1985). At the neuronal level, K^+ channels are involved in excitability (Pongs, 1999). Thus, direct activation of voltage dependent K^+ channels hyperpolarizes the neuronal membrane and limits the firing of an action potential (Porter and Rogawski, 1992). Accordingly, K^+ channel activators possess anticonvulsant effects in some seizure models (Gandolfo *et al.*, 1989; Rostock *et al.*, 1996). The extract exhibited anticonvulsant activity against 4-APinduced seizures probably by activating K^+ channels and may therefore contribute to membrane hyperpolarization (Herrero *et al.*, 2002).

Furthermore, the convulsant effect of 4-AP is due to the release of excitatory neurotransmitters such as glutamate (Morales-Villagrán *et al.*, 1996). The release of glutamate results in over activation of excitatory amino-acid receptors, mainly the NMDAtype. Indeed, an enhancement in the glutamatergic neurotransmission has been linked to the 4-AP convulsant action (Tapia *et al.*, 1999), since the administration of NMDA receptor antagonists protects against 4-AP induced seizures (Fragoso-Veloz and Tapia,

1992). Anticonvulsant activity of PME against seizures induced by 4-AP might also be due to its inhibition of the glutamate signal pathway, probably NMDA receptors.

As a well-validated preclinical model, the maximal electroshock seizure test predicts anticonvulsant drug efficacy against generalized tonic-clonic (grand mal) seizures (Löscher, 1998; Holmes, 2007; White, 1997). In addition, it permits the evaluation of the ability of a substance to prevent seizure spread through neural tissue (Piredda *et al.*, 1985; Swinyard and Kupferberg, 1985; Castel-Branco *et al.*, 2009).The extract produced no anticonvulsant effect against MES-induced tonic seizures in mice. This therefore indicates its inability to protect against generalized tonic-clonic seizures as well as prevent seizure spread.

The 6-Hz seizure test uses a low-frequency, long-duration stimulation paradigm to induce psychomotor seizures that involve forelimb clonic convulsions and stereotyped behaviours similar to those seen in complex partial epilepsy (Brown *et al.*, 1953; Giardina and Gasior, 2001). In addition, it has been suggested that the 6-Hz seizure test might identify anticonvulsant compounds with novel mechanisms of action and serve as a test of human drug-resistant epilepsy (Barton *et al.*, 2001; Duncan and Kohn, 2005). In this study, PME protected against 6 Hz-induced psychomotor seizures suggesting anticonvulsant activity against complex partial seizures. Moreover, like levetiracetam (Surges *et al.*, 2008), PME could as well possess a distinct profile of activity from the commonly used antiepileptic drugs, suggesting possible efficacy in pharmacoresistant epilepsies.

Considerable lines of evidence suggest nitric oxide (NO) as a modulator of seizure activity with divergent anticonvulsant (Tsuda *et al.*, 1997; Noh *et al.*, 2006) and proconvulsant (Van Leeuwen *et al.*, 1995; Nidhi *et al.*, 1999; Royes *et al.*, 2007) effects. These effects are based on the type of seizure, the route of administration, source of NO and other neurotransmitter systems involved in the experiments (Riazi *et al.*, 2006). Osonoe *et al.* (1994), demonstrated that decrease NO levels result in suppression of convulsions, and inhibition of NOS activity shows anticonvulsant property against pentylenetetrazole (PTZ)-induced seizures in rats. Studies have shown that L-NAME inhibits the activity of both endothelial and neuronal NOS (Rees *et al.*, 1990; Talarek and Fidecka, 2003). It has also been shown that L-NAME inhibits pentylenetetrazole and strychnine-induced seizures in mice (Kaputlu and Uzbay, 1997). In the present study, L-NAME given alone did not influence

pentylenetetrazoleinduced seizures. However, administration of L-NAME potentiated the anticonvulsant effect of PME by delaying latency as well as decreasing frequency and duration of PTZinduced clonic seizures. This potentiating effect has been observed for anticonvulsants that act via the GABAergic pathway such as the benzodiazepines (Talarek and Fidecka, 2003). This shows that PME also elicits its anticonvulsant effect in the pentylenetetrazole seizure model probably by interacting with the nitric oxide pathway.

Administration of large doses of L-arginine enhances seizure susceptibility through excessive release of NO in chemical seizure models induced by GABA antagonists. This effect is likely a consequence of hyperexcitability caused by NO-induced cGMP synthesis (Garthwaite, 1991; Riazi *et al.*, 2006). L-arginine [nitric oxide synthase (NOS) substrate] at the dose used attenuated the anticonvulsant activity of PME. This is consistent with various reports where L-arginine (NO donor) decreased the antiepileptic effect of compounds in the pentylenetetrazole model of epilepsy (Akula *et al.*, 2008; Gholipour *et al.*, 2008; Bahremand *et al.*, 2010). This further confirms the possible involvement of the nitric oxide pathway in the anticonvulsant effect of PME.

Nitric Oxide (NO) is also a major stimulator of cGMP generation via soluble guanylate cyclase, which is assumed to play a major role in seizure (Snyder and Bredt, 1991). Methylene blue inhibits soluble guanylate cyclase (Meller and Gebhart, 1993). It has been widely applied in experiments to determine the contribution of the cGMP pathway in the effects of nitricoxidergic system (Talarek and Fidecka, 2003). Methylene blue given alone did not affect the convulsions induced by pentylenetetrazole. However, it potentiated the anticonvulsant effect of PME in the PTZ-induced clonic seizures in mice. This further confirms the role of the nitric oxide pathway in the anticonvulsant effect of PME. The potentiating effect of methylene blue has also been demonstrated in the anticonvulsant effect of diazepam and clonazepam in PTZ-induced seizures in mice (Talarek and Fidecka, 2003).

The intra-cellular cGMP concentrations are also regulated not only by soluble guanylate cyclase (sGC), but also by PDE5, which catalyses the hydrolysis of the second messengers cAMP and cGMP to yield AMP and GMP, respectively (Denninger and Marletta, 1999; Akula *et al.*, 2008). Sildenafil is a PDE5 inhibitor and enhance the NO-mediated effects by inhibiting cGMP degradation in target tissues (Boolell *et al.*, 1996; Jackson *et al.*, 1999;

Gholipour *et al.*, 2009). Sildenafil inhibited the anticonvulsant effect of PME. This is in agreement with reports establishing the proconvulsant effect of sildenafil (Riazi *et al.*, 2006; Akula *et al.*, 2008). Results of this study therefore suggest the possible contribution of the cGMP pathway in the anticonvulsant effect of the extract.

Several studies have demonstrated potential levels of interaction between GABA and the NO system in the regulation of seizure susceptibility (Paul, 2003; Paul and Subramanian, 2002). For instance, increased synthesis of NO can decrease GABA-stimulated chloride ion influx by inhibiting $GABA_A$ receptor function (Zarri *et al.*, 1994; Gholipour *et al.*, 2008). In addition, release of endogenous NO participates in the excitatory transmission through NMDA receptors (Manzoni *et al.*, 1992; Riazi *et al.*, 2006), where activation of NMDAtype glutamate receptors causes a reduction in the effect of GABA (Robello *et al.*, 1997; Talarek and Fidecka, 2003). Moreover, NMDA receptor blockade or suppression has been shown to decrease susceptibility to seizure development (Borris *et al.*, 2000; Ahmed *et al.*, 2005; Ghasemi *et al.*, 2010b). Just like the benzodiazepines, PME has shown to possess anticonvulsant activity against convulsions induced by GABA antagonists probably by interacting with $GABA_A$ receptors. PME has also been shown to elicit its anticonvulsant effect probably via an interaction with the NMDA receptor complex. Therefore, the influence of NO modulators in the anticonvulsant activity of PME could possibly be through its interaction with GABA and/or NMDA receptors.

In this study, the neuromuscular tone of animals was evaluated by use of the grip-strength test. This test is a widely-used non-invasive method designed to evaluate mouse limb strength and has been used to investigate the effects of neuromuscular disorders and drugs. It is based on the natural tendency of the mouse to grasp a grid when it is suspended by the tail. PME had no effect on skeletal muscular strength. In contrast, diazepam at the highest dose (3 mg kg^{-1}) impaired neuromuscular strength by decreasing the force (N). This effect is in agreement with various reports in which some classical and second generation antiepileptic drugs—diazepam, carbamazepine, valproate, clonazepam, phenytoin, phenobarbital, lamotrigine, oxcarbazepine and topiramate—produced impairment of skeletal muscular strength in mice in a dose-dependent manner (Łuszczki *et al.*, 2008; Łuszczki, 2009; Zadrozniak *et al.*, 2009).

4.5 CONCLUSION

Findings of the present study suggest that administration of *P. microcarpa* hydroethanolic leaf extract has anticonvulsant activity and may probably be affecting GABAergic, NMDA, K^+ channels and nitric oxide-cyclic GMP mechanisms to exert its effect.

Chapter 5 ACUTE ANTIDEPRESSANT ACTIVITY

5.1 INTRODUCTION

Major depression is one of the most prevalent psychiatric disorders and is characterized by change in mood, lack of interest in the surroundings as well as psychosocial and physical impairment (Girish *et al.*, 2012; Nakajima *et al.*, 2010). Depression represents one of the most frequent comorbid disorders in epilepsy patients (Kanner and Balabanov, 2002; Harden, 2002). Both clinical and experimental evidence suggest that imbalances in various neurotransmitters as GABA, glutamate, norepinephrine and serotonin, which are frequently observed in epilepsy patients, may concurrently contribute to the development of depression (Kanner and Balabanov, 2002; Jobe, 2003; Kondziella *et al.*, 2007; Kanner, 2005).

There are several antidepressant drugs available, most of them affecting directly or indirectly the monoaminergic system (Elhwuegi, 2004; Brocardo *et al.*, 2008; Freitas *et al.*, 2010; Lee *et al.*, 2010). These classical antidepressant agents are designed to increase monoamine transmission, either by inhibiting neuronal reuptake (imipramine, fluoxetine and desipramine), or by inhibiting degradation (for example, monoamine oxidase inhibitors such as iproniazide) (Freitas *et al.*, 2010; Zhou *et al.*, 2010; Lee *et al.*, 2010; Dwyer *et al.*, 2010). However, a major limitation of these antidepressants are side effects such as sedation, blurred vision, constipation, seizures, sexual dysfunction, anxiety and weight gain (Dhingra *et al.*, 2012; Hyman and Nestler, 1996; Andreasen *et al.*, 2009). Furthermore, although these are effective in treating most depressive episodes, a significant proportion of depressed patients do not display signs of mood improvement until 2–3 weeks after the start of the treatment (Poleszak *et al.*, 2011).

Accordingly, medicinal plants may be important sources of new antidepressant drugs and the safety of such plant extracts may be better than that of synthetic antidepressants (Schulz, 2006; Wang *et al.*, 2010). It is therefore desirable to research and develop more effective antidepressants with fewer adverse effects. Plant extracts are some of the most attractive sources of new drugs, and have been shown to produce promising results for the treatment of depression. For instance, the excellent patient acceptance of St. John's Wort (*Hypericum perforatum* L., Hypericaceae) and its extensive use in Europe and USA for the treatment of mood disorders, especially conditions of mild to moderate depression has drawn attention to plant extracts as potential sources of highly desirable, new and

innovative antidepressant agents (Isacchi *et al.*, 2009; Piato *et al.*, 2009; Schulz, 2006). *Pseudospondias microcarpa* is one of such plants used for managing various diseases including CNS disorders (Burkill, 1985). However, despite the wide use of the plant, there is no data in literature on its probable antidepressant activity.

Therefore, the present study evaluated the antidepressant-like effect of the ethanolic extract obtained from the leaves of *Pseudospondias microcarpa* (PME) in two predictive models of depression: forced swimming test (FST) and tail suspension test (TST) in mice. Moreover, the mechanisms through which PME elicits its antidepressant-like action were investigated in the TST.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Male ICR mice were used in this experiment and experimental conditions were as described in section 3.2.1.

5.2.2 Drugs and Chemicals

Desipramine hydrochloride, α-methyl-para-tyrosine methyl ester (AMPT), parachlorophenylalanine (*p*CPA), D-cycloserine (DCS, D-4-amino-3-isoxazolidone), D-serine (DS), 5-hydroxy-L-tryptophan (5-HTP), reserpine, N-nitro-L-arginine methyl ester (LNAME), L-arginine, norepinephrine, yohimbine and methylene blue were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA. Cyproheptadine (LETAP Pharmaceuticals Ltd., Accra, Ghana). Sildefanil, prazosin hydrochloride from Pfizer, U.S.A. Propranolol hydrochloride from Watson Pharma Private Ltd., India and fluoxetine hydrochloride (Prozac®), Eli Lilly and Company Ltd., Basingstoke, England.

All drugs were dissolved in normal saline with the exception of reserpine which was first dissolved with few drops of 5 % glacial acetic acid and then subsequently diluted with normal saline to obtain the appropriate dose. All drugs were administered intraperitoneally (i.p.) except fluoxetine and reserpine/norepinephrine which were administered orally (*p.o.*) and subcutaneously (s.c.) respectively.

5.2.3 Forced Swimming Test

This experiment was performed according to the procedure of Porsolt *et al.* (1977) with modifications. Briefly, mice were divided into 10 groups (n=5) and pretreated with vehicle

(10 ml kg^{-1} of 0.9 % NaCl, i.p), PME (30, 100 and 300 mg kg^{-1}, *p.o.*), fluoxetine (3, 10 and 30 mg kg^{-1}, *p.o.*) or desipramine (3, 10 and 30 mg kg^{-1}, i.p.) 60 min (*p.o.*) or 30 min (i.p.) before placed individually in polypropylene cylinders (height 25 cm, diameter 10 cm) containing 10 cm of water, maintained at 25 °C. With a public domain software JWatcher™, version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia), behavioural assessment was measured during the last 4 min of the 6 min test period according to Detke *et al.* (1995). Three different behaviours were rated: 1) immobility, defined as when mice remained floating passively in the water; 2) swimming, when mice made active swimming motions, more than necessary to solely maintain their head above water; and 3) climbing, mice were judged to be climbing when they were making active movements in and out of the water with their forepaws, usually directed against the walls. Duration of immobility, swimming, and climbing was measured.

5.2.4 **Tail Suspension Test**

The tail suspension test (TST) was conducted as initially described by Steru *et al.* (1985) with modifications (Berrocoso *et al.*, 2006; 2012). Animals were similarly grouped as in the FST. One hour after oral administration and 30 min after intraperitoneal injection of test compounds, mice were individually suspended by the tail from a horizontal ring-stand bar raised 30 cm above the floor using adhesive tape placed 1 cm from the tip of tail and positioned such that the base of their tail was aligned with the horizontal plane. Test sessions lasted for 6 min and were videotaped. Behaviours for the last 4 of the 6-minute period were then analyzed. Behaviours rated were: (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 while 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) Pedalling – defined as when the animal moved its paws continuously without moving its body.

5.2.5 **Mechanism(s) of Action**

The TST presents some advantages over the FST in allowing an objective measure of immobility and does not induce hypothermia by immersion in water (Ripoll *et al.*, 2003; Cryan *et al.*, 2005a). Thus, this model was therefore used to assess the possible mechanism of action.

5.2.5.1 *Serotonergic Depletion*

In order to investigate the possible contribution of the serotoninergic system to the effect of PME in the TST, mice were pretreated with para-chlorophenylalanine (*p*CPA). *p*CPA is known to reduce the concentration of brain serotonin by inhibiting its biosynthesis (O'Leary *et al.*, 2007; Poleszak *et al.*, 2007). In the present experiment mice were injected i.p. either with saline (control group) or with *p*CPA. *p*CPA was administered at the dose of 300 mg kg^{-1} once daily for 3 consecutive days. On the fourth day (24 h after the last pCPA administration), mice received PME (100 mg kg^{-1}, *p.o.*), FLX (10 mg kg^{-1}, *p.o.*) , DES (10 mg kg^{-1}, i.p.) or saline 60 min (*p.o.*) or 30 min (i.p.) before the test.

5.2.5.2 *Inhibition of Biosynthesis and/or Depletion of Catecholamines*

To assess the possible involvement of catecholamines, dopamine (DA) and noradrenaline, (NA), in the antidepressant-like effect of PME, mice were pretreated with AMPT (400 mg kg^{-1}, i.p., an inhibitor of the enzyme tyrosine hydroxylase) (Kwon *et al.*, 2010) before behavioural assessment in the tail suspension test. This was done to deplete newly synthesized pools of catecholamines (NA and DA). Moreover, to deplete vesicular pools of NA and DA, mice were treated with a single dose of reserpine (1 mg kg^{-1}, s.c.) 24 h before behavioural testing. In an effort to deplete both the vesicular and cytoplasmic pools of NA and DA, mice were pretreated with a combination of reserpine (1 mg kg^{-1}, s.c., 24 h before behavioural testing) and AMPT (200 mg kg^{-1}, i.p., 3.5 h before behavioural testing) respectively (O'Leary *et al.*, 2007).

5.2.5.3 *Effects of Some Antagonists on PME Actions in the TST*

Appropriate doses for antagonists were selected from literature (Luscombe *et al.*, 1993) as well as pilot experiments. Groups of mice received saline or antagonists (cyproheptadine – 8 mg kg^{-1}, *p.o.*, a non-selective 5-HT receptor antagonist; prazosin – 3 mg kg^{-1}, *p.o.*, a selective α_1- receptor antagonist; propranolol – 3 mg kg^{-1} *p.o.*, β- receptor antagonist or yohimbine 3 mg kg^{-1} *p.o.* – α_2-receptor antagonist) 30 min before vehicle or PME (100 mg kg^{-1}, *p.o*) and were assessed 45 min later for immobility time in the TST.

5.2.5.4 *5-HTP Induced Head-Twitch Response*

PME (100 mg kg^{-1} *p.o.*), FLX (10 mg kg^{-1} *p.o.*), DES (10 mg kg^{-1} i.p.) or saline were administered 60 min (*p.o.*) or 30 min (i.p.) before intraperitoneal administration of 5-HTP (200 mg kg^{-1}). Mice were then placed into plastic cages and the number of head twitches

(rapid movements of the head with little or no involvement of the trunk) was counted for 8 min (from 15 to 23 min) after the injection of 5-HTP (Shelkunov, 1978; Mahesh *et al.*, 2011).

5.2.5.5 *Potentiation of Norepinephrine Toxicity*

Mice were randomly assigned to test groups of 10 subjects. Mice were pre-treated with vehicle (10 ml kg^{-1} of 0.9 % NaCl, i.p), PME (30, 100 and 300 mg kg^{-1}, *p.o.*), fluoxetine (30 mg kg^{-1}, *p.o.*) or desipramine (30 mg kg^{-1}, i.p.) 60 min (*p.o.*) or 30 min (i.p.) prior to the s.c. injection of the sub lethal dose of noradrenaline (3 mg kg^{-1}). Mice were then placed in plastic cages with free access to food and water, and mortality rate was assessed 48 hours post-dosing.

5.2.5.6 *N-Methyl-D-Aspartate (NMDA) Interaction*

To evaluate the effects of D-cycloserine (DCS) and D-serine (DS) in the mouse TST, the drugs were administered 30 min before the test to different experimental groups of animals. Immobility time was compared with a control group in which saline (as a vehicle) was injected 30 min before the test. For the present report, the doses of these drugs were chosen based on a pilot study and in accordance with previous studies (Poleszak *et al.*, 2011; Wlaz *et al.*, 2011).

To evaluate the possible involvement of the NMDA receptor system in the effect of PME in the TST, subeffective doses of D-cycloserine (2.5 mg kg^{-1}, i.p.) and D-serine (600 mg kg^{-1}, i.p.) were separately administered 15 min before administration of PME (100 mg kg *p.o.*), FLX (10 mg kg^{-1} *p.o.*), DES (10 mg kg^{-1} i.p.) or saline. Forty five minutes after administration, the mice were assessed in the TST for duration of immobility.

5.2.5.7 *Involvement of L-arginine-NO-cGMP Pathway*

An appreciable number of studies have attributed a significant role to the L-arginine–NOcGMP pathway in the pathophysiology of depression. Therefore, the possible participation of this pathway in the antidepressant effect of PME was investigated. Doses of the drugs were chosen based on a pilot study and in accordance with previous studies (Dhir and Kulkarni, 2007; Ghasemi *et al.*, 2009).

To investigate the possible involvement of the L-arginine-nitric oxide pathway in the antiimmobility effects of PME in the TST, mice were pre-treated with a sub-effective dose of L-arginine [750 mg kg^{-1}, i.p., a precursor of nitric oxide (NO)] or vehicle 20 min before PME (100 mg kg^{-1}, *p.o*) administration and assessed 45 min later for immobility time. In separate experiments, the enhanced anti-immobility effect of PME with a sub-effective dose L-NAME [30 mg kg^{-1}, i.p., a non-selective nitric oxide synthase (NOS) inhibitor] or methylene blue [10 mg kg^{-1}, i.p., an inhibitor of nitric oxide synthase and an inhibitor of soluble guanylate cyclase (sGC)] was investigated. Mice were administered with these inhibitors 20 min before PME or vehicle and assessed 45 min later for immobility time in the TST.

To observe the role of cyclic guanosine monophosphate (cGMP) in the antidepressant action of PME, mice received an injection of sildenafil [5 mg kg^{-1}, i.p., a phosphodiesterase 5 inhibitor (PDE5)] or vehicle 20 min before PME (100 mg kg^{-1}, *p.o*). Forty five minutes after PME administration, the mice were subjected to TST to evaluate immobility duration.

5.2.6 **Rotarod Test**

The effect of PME on motor co-ordination was assessed using a rotarod apparatus (Ugo Basile, model 7600, Cormerio, Milan, Italy). The rotarod consisted of a rotating rod (diameter: 3 cm) and individual compartments for each mouse. Mice were trained for 3 days before the test to stay on the rotating rod (speed 20 rpm) for at least 5 min. On the test day, mice were randomly divided into seven groups (n=5): saline-treated control group; diazepam group (0.1, 0.3 and 1 mg kg^{-1}, i.p.) and PME group (30, 100 and 300 mg kg^{-1}, *p.o*.). One hour (*p.o*.) or 30 (i.p.) min after administration of test compounds, mice were put on the rotating rod and latency until fall during the 5-min session was recorded. Animals that stayed on the bar for more than 5 min were given the maximum score of 5 min.

5.2.7 **Statistical Analysis**

In all experiments, a sample size of 5-10 animals was utilized. All data are presented as mean±SEM. To compare differences between groups, one-way ANOVA was performed with Newman-Keuls' test as *post hoc.* Two-way ANOVA followed by Bonferroni's test as *post hoc* was used in the forced smimming and tail suspension tests. GraphPad® Prism Version 5.0 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis.

P<0.05 (Newman-Keuls test or Bonferroni's test) was considered statistically significant. Doses for 50 % of the maximal effect (ED_{50}) for each drug were determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation:

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

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.

5.3 RESULTS

5.3.1 Forced Swimming Test

Figure 5.1 depicts the effect of acute administration of PME (30-300 mg kg^{-1}, *p.o.*) and the classical antidepressant drugs fluoxetine (3-30 mg kg^{-1}, *p.o.*) and desipramine (3-30 mg kg^{-1}, i.p.) on mice behaviours in the FST.

ANOVA analysis revealed that all the doses of PME significantly decreased the immobility time ($F_{3,16}$=7.995, P=0.0018) and increased swimming time ($F_{3,16}$=8.462, P=0.0013) of mice in the FST by a maximum of 62.83±9.98 % and 94.53±17.31 % respectively, indicating significant antidepressant-like activity (figure 5.1a). *Post hoc* analysis revealed statistical significance for the effect of PME on swimming at all doses used (figure 5.1a). One-way ANOVA revealed that PME did not significantly affect latency to immobility ($F_{3,16}$=3.062, P=0.0583). However, *post hoc* analysis showed statistical significance at 300 mg kg^{-1} (P<0.05; figure 5.1b). Climbing time was not affected ($F_{3,16}$=1.620, P=0.966; figure 5.1c). One-way ANOVA analysis revealed that fluoxetine significantly decreased the immobility time ($F_{3,16}$=7.995, P=0.0043; figure 5.1d) and increased swimming time ($F_{3,16}$=8.462, P=0.0060; figure 5.1d) of mice in FST reaching statistical significance at 10 and 30 mg kg^{-1} (both P<0.01). Latency to immobility ($F_{3,16}$=4.490, P=0.0181; figure 5.1e) was significantly affected but not climbing behaviour (figure 5.1f). In figure 5g, swimming behaviour of desipramine was not significantly affected ($F_{3,16}$=3.350, P=0.669). However,

one-way ANOVA revealed a significant reduction of immobility time ($F_{3,16}$=11.95, P=0.0002; figure 5.1g) and significant increase in immobility latency ($F_{3,16}$=6.785, P=0.0037) (figure 5.1h). Climbing time was also significantly increased ($F_{3,16}$=6.850, P=0.0035; figure 5.1i). From the dose-response curves (% decrease in immobility; figure 5.2), ED_{50} values show the following order of potency for the test compounds: desipramine>fluoxetine>PME. However, E_{max} values show that fluoxetine was efficacious (72.77 %) than desipramine (69.14 %) and PME (62.83 %). For % increase in swimming, order of potency was as follows: desipramine>fluoxetine>PME. E_{max} values however show desipramine to be the least in efficacy. Slopes of the PME swimming curves were similar to that of fluoxetine indicating possible action at similar receptor sites.

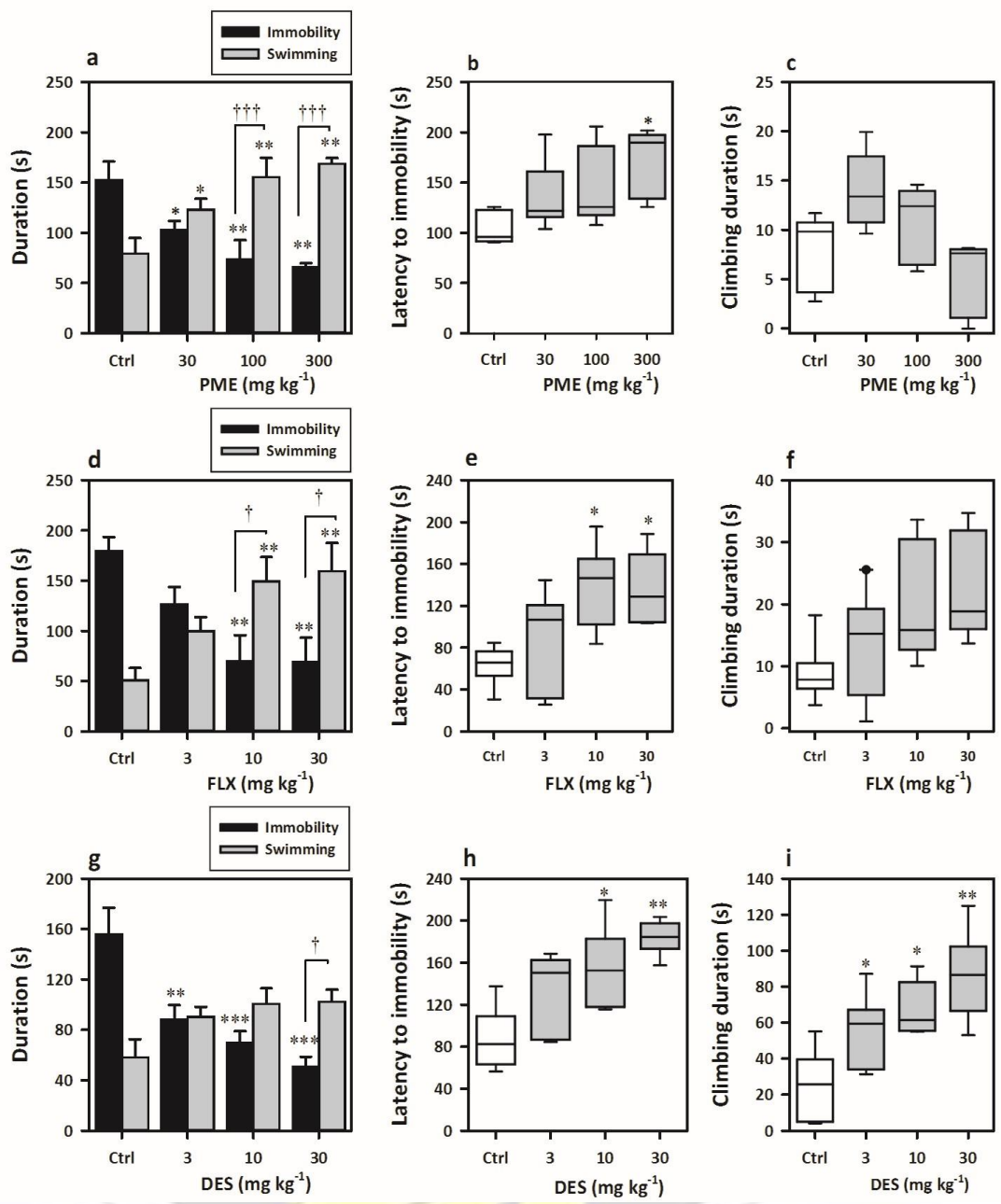


Figure 5.1 Performance of mice in the FST: behavioural assessment including immobility and swimming duration (a, d and g), immobility latency (b, e and h) and climbing duration (c, f and i) after acute treatment of mice with PME, fluoxetine and desipramine. PME (30-300 mg kg^{-1}) and FLX (3-30 mg kg^{-1}) were *p.o.* administered 60 min before behavioural assessment. DES (3-30 mg kg^{-1}) was i.p. injected 30 min before the test. Data are expressed as group mean±SEM (n=5). The lower and upper margins of the boxes represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box and symbols represent outliers. Significantly different from control: **P*<0.05; ***P*<0.01; ****P* <0.001 (One-way ANOVA followed by Newman-Keuls' *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).

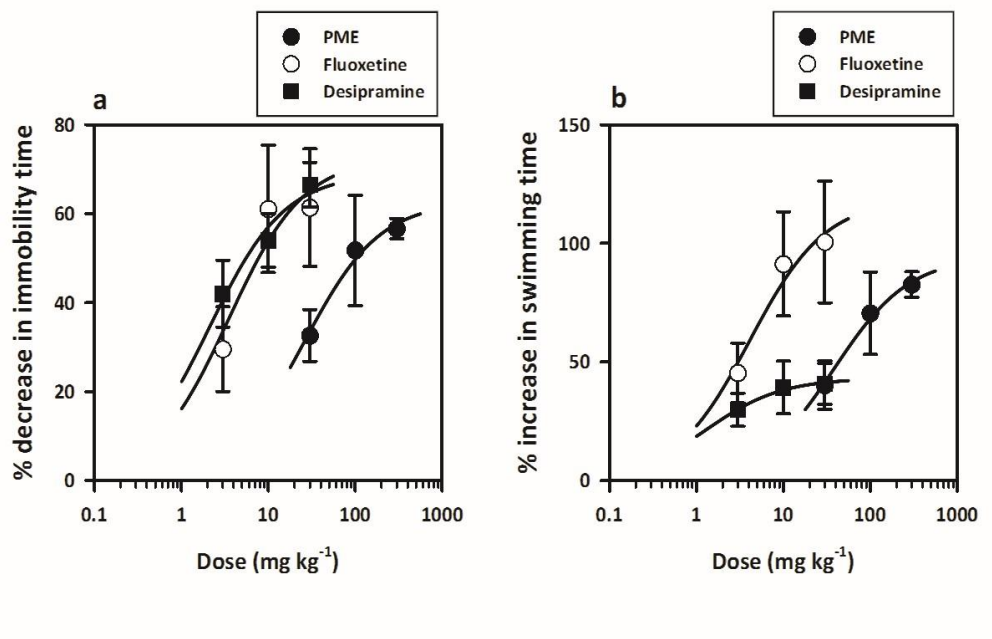


Figure 5.2 Dose response curves for PME (10-300 mg kg^{-1}, *p.o.*), fluoxetine (3-30 mg kg^{-1}, *p.o.*) and desipramine (3-30 mg kg^{-1}, i.p.) with respect to immobility time (a) and swimming time (b) in the forced swim test in mice. Each point represents the mean±SEM (n=5).

5.3.2 **Tail Suspension Test**

Figures 5.3 and 5.5 represent the effect of acute administration of PME (30-300 mg kg^{-1}, *p.o.*) and the classical antidepressant drugs fluoxetine (3-30 mg kg^{-1}, *p.o.*) and desipramine (3-30 mg kg^{-1}, i.p.) on mice behaviours in the TST. Administration of PME (30-300 mg kg^{-1}, *p.o.*) 1 h before the test period significantly decreased the immobility periods of mice by maximum of 80.98±18.75 % when compared to control group, indicating significant antidepressant-like activity. Newman-Keuls post-hoc test indicated statistically significant anti-immobility effects of PME at doses of 100-300 mg kg^{-1} (P<0.01 at 100 and 300 mg kg^{-1}). PME did not significantly affect pedaling but caused an increase in time spent swinging ($F_{3,16}$=6.951, P=0.0033) and curling ($F_{3,16}$=7.580, P=0.0022). The SSRI fluoxetine significantly increased anti-immobility effects by a maximum of 62.21±25.07 %. Swinging time was also significantly increased ($F_{3,16}$=11.59, P=0.0003) reaching statistical significance at 10 mg kg^{-1} (P<0.05) and 30 mg kg^{-1} (P<0.01). However, ANOVA did not indicate any significant effect of fluoxetine on pedaling ($F_{3,16}$=2.039, P>0.05) or curling ($F_{3,16}$=1.246, P=0.326) times. Administration of desipramine significantly reduced immobility time in a dose dependent manner by a maximum of 80.10±17.38 %. Swinging

time was also significantly increased ($F_{3,16}$=6.248, P=0.0052). Just like fluoxetine, one-way ANOVA did not reveal any significant effect of desipramine on pedaling and curling times.

From the dose response curves (% decrease in immobility; figure 5.4), ED_{50} values show the following order of potency for the test compounds: fluoxetine>desipramine>PME. However, E_{max} values indicates that PME (80.98 %) was efficacious than desipramine (80.10 %) and fluoxetine (62.21 %).

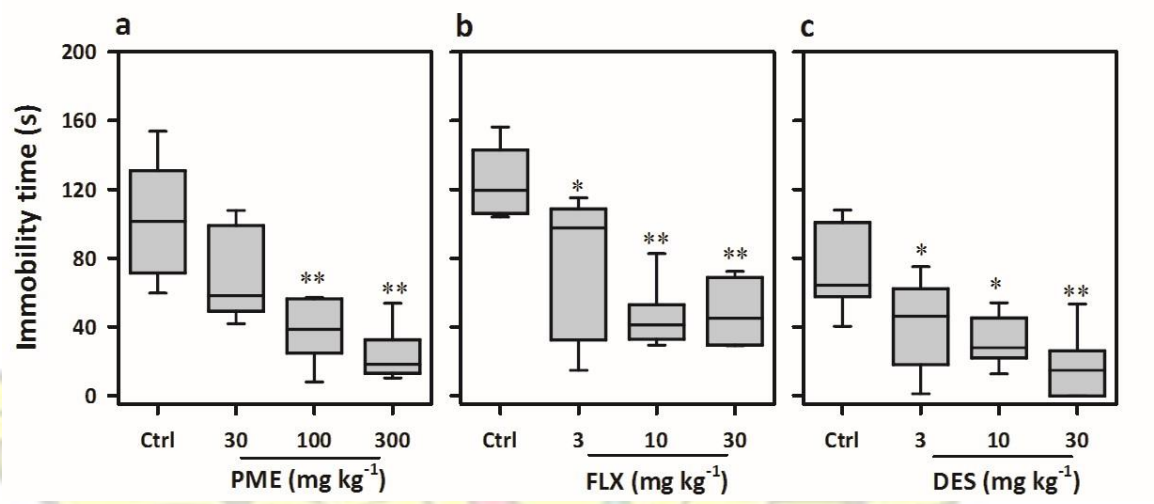


Figure 5.3 Effects of PME (30-300 mg kg^{-1}), fluoxetine (3-30 mg kg^{-1}) and desipramine (3-30 mg kg^{-1}) on the total duration of immobility in the TST. The values represent mean±SEM (n=5). The lower and upper margins of the boxes represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Significantly different from control: *P<0.05; **P<0.01 (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

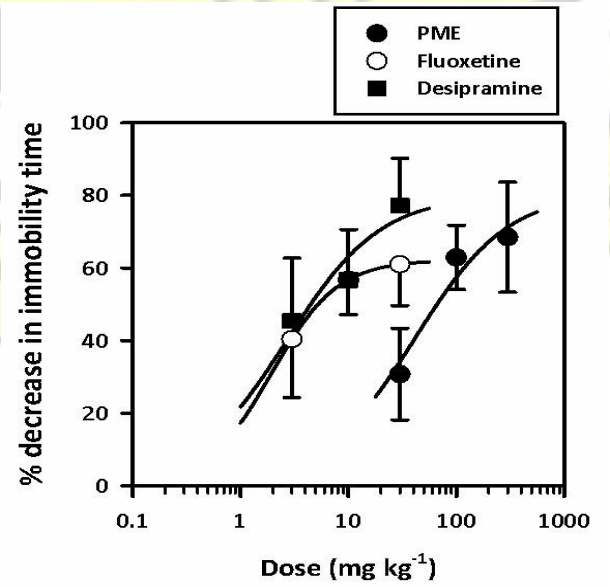


Figure 5.4 Dose response curves for PME (10-300 mg kg^{-1}, *p.o.*), fluoxetine (3-30 mg kg^{-1}, *p.o.*) and desipramine (3-30 mg kg^{-1}, i.p.) with respect to % decrease in immobility in the tail suspension test in mice. Each point represents the mean±SEM (n=5).

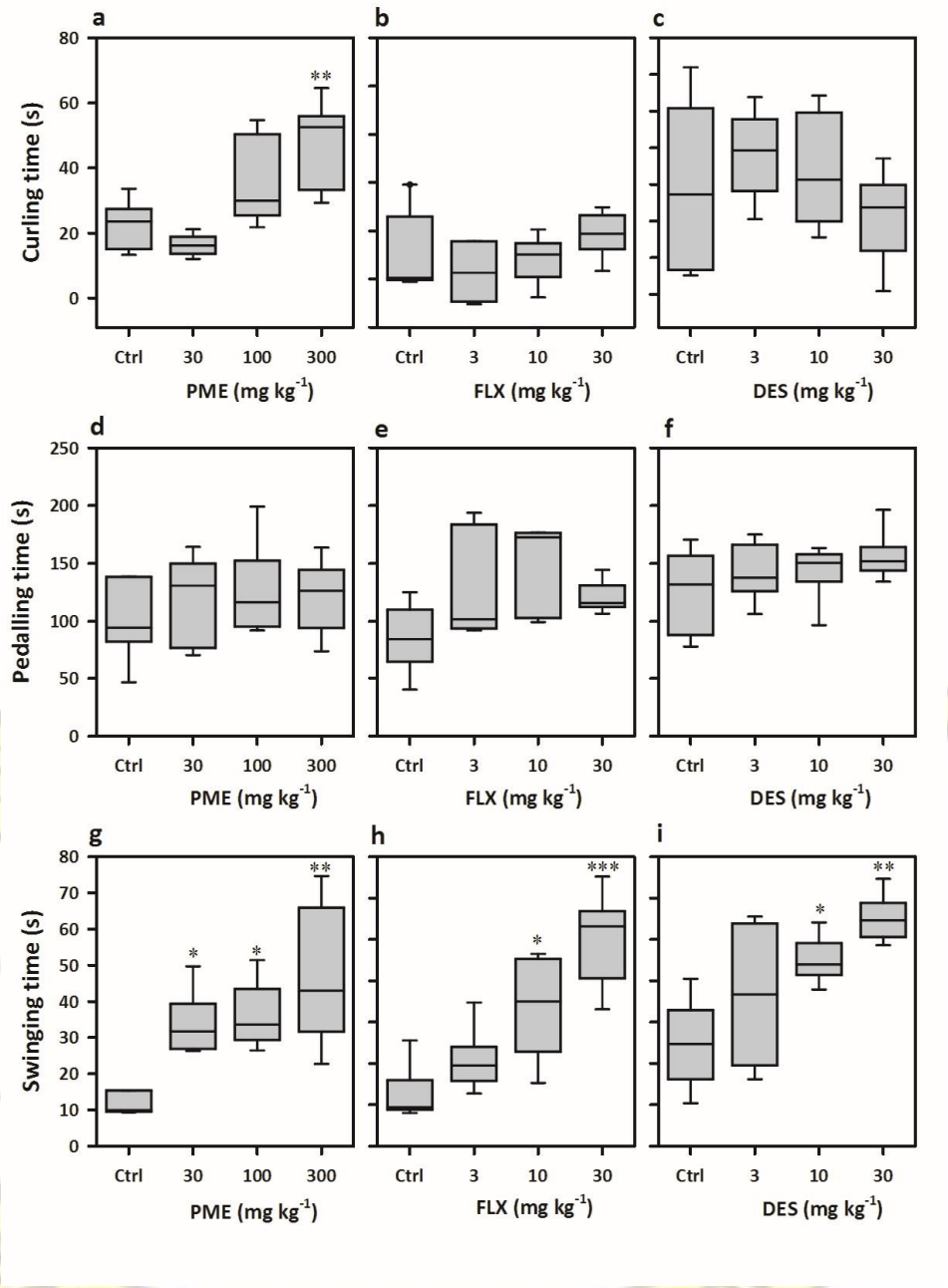


Figure 5.5 Performance of mice in the TST: behavioural assessment including curling (a, b and c), pedaling (e, f and g) and swinging (g, h and i) after acute treatment of mice with PME, fluoxetine and desipramine. PME (30-300 mg kg^{-1}) and FLX (3-30 mg kg^{-1}) were *p.o.* administered 60 min before behavioural assessment. DES (3-30 mg kg^{-1}) was i.p. injected before the test Data are expressed as group mean±SEM (n=5). The lower and upper margins of the boxes represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Significantly different from control: **P*<0.05; ***P*<0.01; ****P*<0.001 (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

5.3.3 **Mechanism(s) of Antidepressant Action of PME**

5.3.3.1 ***Pretreatment with pCPA***

The results in figure 5.6 show that *p*CPA alone (300 mg kg^{-1} for 3 consecutive days) did not modify the immobility time, while pretreatment of mice with *p*CPA significantly blocked the reduction in the immobility time elicited by PME (100 mg kg^{-1}, *p.o.*) in the TST ($F_{1,24}$=40.45, P=0.0002). Fluoxetine, the SSRI reduced immobility in saline-pretreated mice. However, it significantly increased immobility in *p*CPA -pretreated animals when compared with the corresponding group given saline. Prior administration of *p*CPA did not alter the response to desipramine.

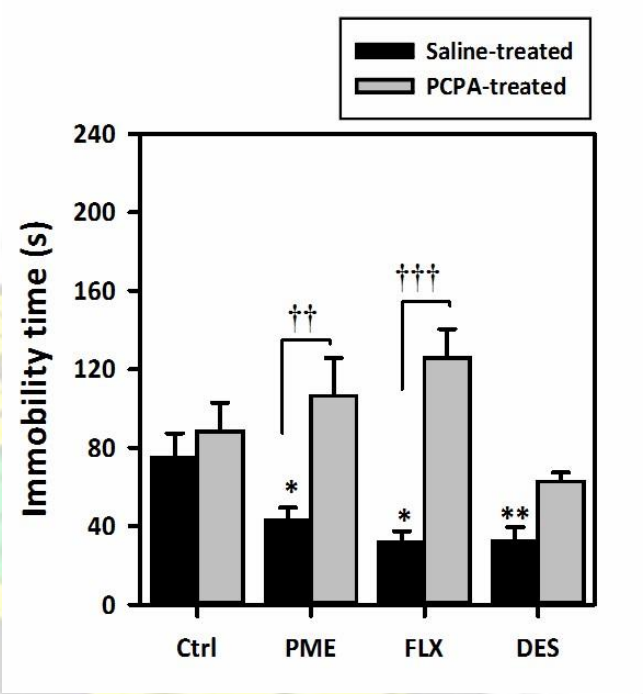


Figure 5.6 Effects of *p*CPA (300 mg kg^{-1}, i.p. for 3 consecutive days) pretreatment on the behavioural response of PME (100 mg kg^{-1}, *p.o.*), fluoxetine (10 mg kg^{-1}, *p.o.*) and desipramine (10 mg kg^{-1}, i.p.) in the tail suspension test. Data are presented as group mean±SEM (n=5). **P*<0.05, ***P*<0.01 versus vehicletreated animals (One-way ANOVA followed by Newman Keuls' test). Significant difference between treatments: ††*P*<0.01, †††*P*<0.001 (Two-way ANOVA followed by Bonferroni's test).

5.3.3.2 ***Inhibition of Biosynthesis and/or Depletion of Catecholamines***

Figure 5.7 shows the influence of AMPT on the effects of PME, fluoxetine and desipramine in the TST. Pretreatment of mice with AMPT (400 mg kg^{-1}, i.p.) for 3.5 h, increased baseline immobility values by 47 %. PME, fluoxetine and desipramine significantly reduced immobility when compared to control ($F_{3,16}$=5.782, P=0.0071) but this effect as

revealed by two-way ANOVA was blocked by pretreatment with AMPT ($F_{1,24}$=74.49, P<0.0001). Bonferroni's *post hoc* analysis showed statistical significance for PME (P<0.01), FLX (P<0.001) and DES (P<0.001).

The effect of reserpine pretreatment on the behavioural effects of antidepressants in the TST is also shown in figure 5.7b. Pretreatment of mice with reserpine increased baseline immobility values by 53 %. PME and the classical antidepressants significantly reduced immobility when compared to corresponding group given saline ($F_{3,16}$=6.527, P=0.0043). This effect was however reversed by pretreatment with resrpine ($F_{1,24}$=70.44, P<0.0001).

Figure 5.7c also shows the effects of reserpine+AMPT pretreatment on the behavioural effects in the TST. Vesicular stores of catecholamines were depleted by pretreating mice with reserpine (1 mg kg^{-1}, s.c.) 24 h before the TST, whereas newly formed stores of catecholamines were depleted by pretreatment with AMPT (200 mg kg^{-1}, i.p.) 3.5 h before the TST. This was done since the behavioural effects of antidepressants could involve catecholamines located in different cellular pools or compartments. Prior administration of the reserpine+AMPT combination significantly increased baseline immobility in mice treated with saline by 75 % (P<0.05). The anti-immobility effects of PME, fluoxetine and desipramine were blocked after pretreatment with reserpine+AMPT as shown by two-way ANOVA ($F_{1,24}$=60.17, P<0.0001; figure 5.7c).

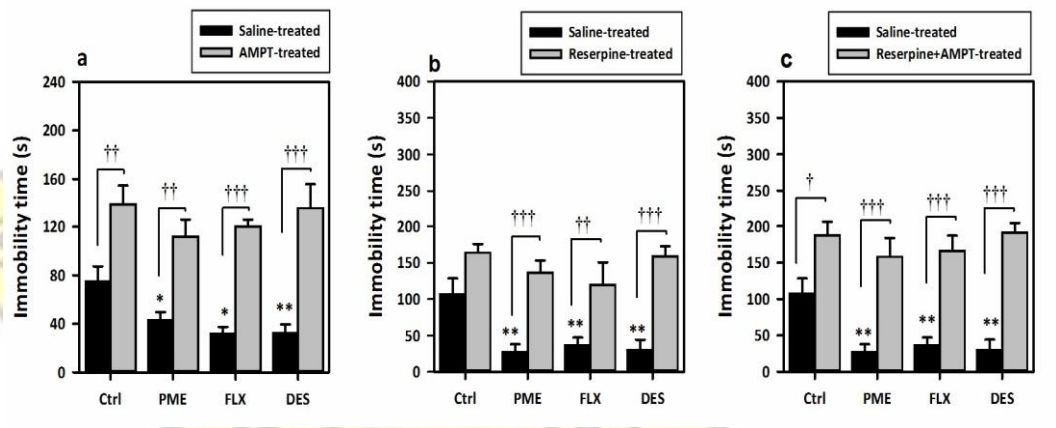


Figure 5.7 Effects of AMPT (200 mg kg^{-1}, i.p.) (a), Reserpine (1 mg kg^{-1}, s.c.) (b) and Reserpine+AMPT (c) pretreatment on the behavioural response of PME (100 mg kg^{-1}, *p.o.*), fluoxetine (10 mg kg^{-1}, *p.o.*) and desipramine (10 mg kg^{-1}, i.p.) in the tail suspension test. Data are presented as group mean±SEM (n=5). *P<0.05, **P<0.01 versus vehicle-treated animals (One-way ANOVA followed by Newman-Keuls' test). Significant difference between treatments: †P<0.05, ††P<0.01, †††P<0.001 (Two-way ANOVA followed by Bonferroni's test).

5.3.3.3 *Effects of Antagonists on PME Actions in the TST*

Cyproheptadine (8 mg kg^{-1}, *p.o.*), prazosin (3 mg kg^{-1}, *p.o.*), propranolol (3 mg kg^{-1}, *p.o.*) and yohimbine (3 mg kg^{-1}, *p.o.*) were administered 30 min before PME and the tail suspension test was performed 45 min after PME administration. The anti-immobility effect caused by PME (100 mg kg^{-1}, *p.o.*) was significantly prevented by pretreatment of mice with cyproheptadine (figure 5.8a). Prazosin (figure 5.8b), propranolol (figure 5.8c) and yohimbine (figure 5.8d) had no effect on the anti-immobility effect of the extract.

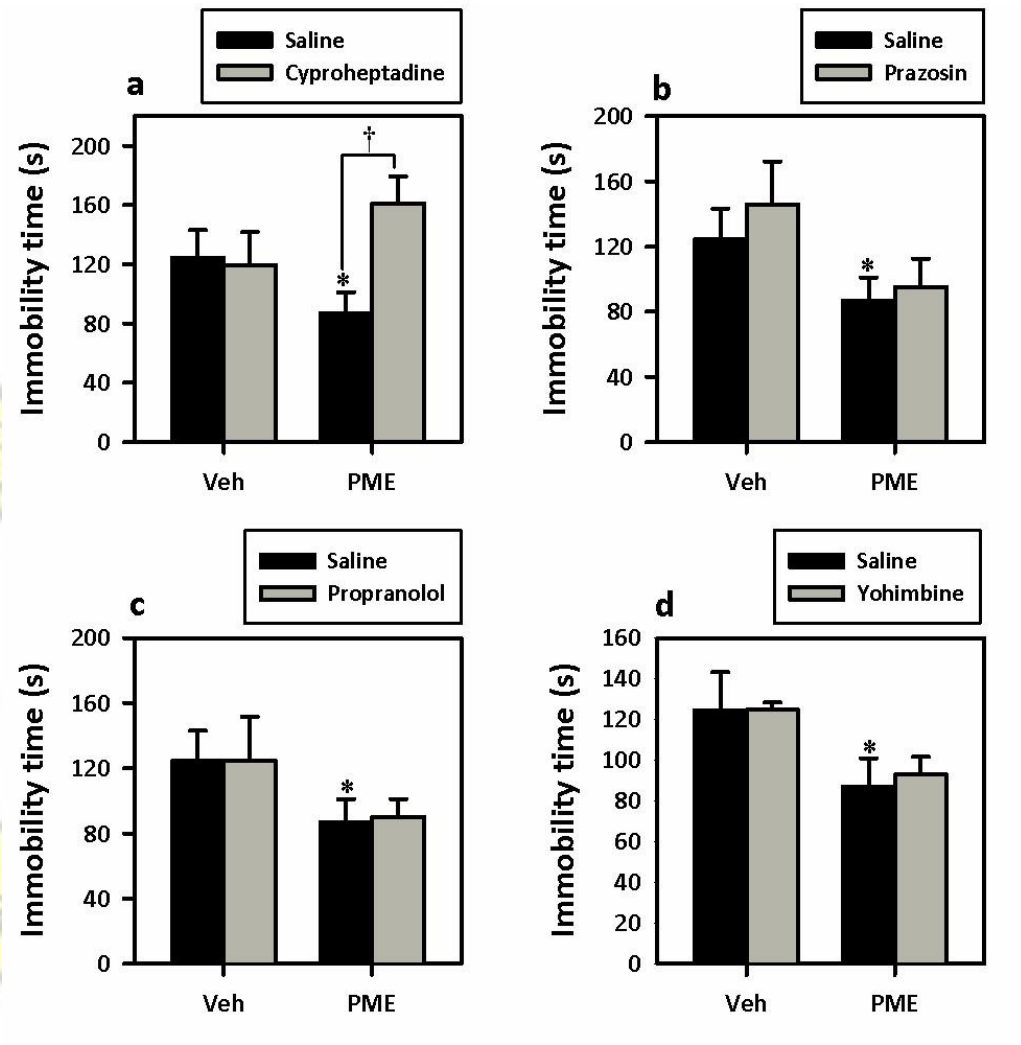


Figure 5.8 Effect of pre-treatment of mice with cyproheptadine (8 mg kg^{-1}, *p.o.*, a 5-HT receptor antagonist, panel a), prazosin (3 mg kg^{-1}, *p.o.*, a selective α_1-receptor antagonist, panel b), propranolol (3 mg kg^{-1}, *p.o.*, β-receptor antagonist, panel c), yohimbine (3 mg kg^{-1}, *p.o.*, α_2-receptor antagonist, panel d) on PME (100 mgkg^{-1}, *p.o.*)-induced reduction in immobility time in the TST. Each column represents the mean±SEM (n=6). *P<0.05 as compared with the vehicle-treated control. †P<0.05 as compared with the group pretreated with vehicle and PME.

5.3.3.4 *5-HTP Induced Head-Twitch Response in Mice*

PME and fluoxetine potentiated the number of head-twitch responses by maximum of 54.08 % and 80.88 % as compared to the control respectively (figure 5.9). Unlike PME and fluoxetine, desipramine significantly decreased the number of head-twitch response in comparison to the control group ($P<0.05$).

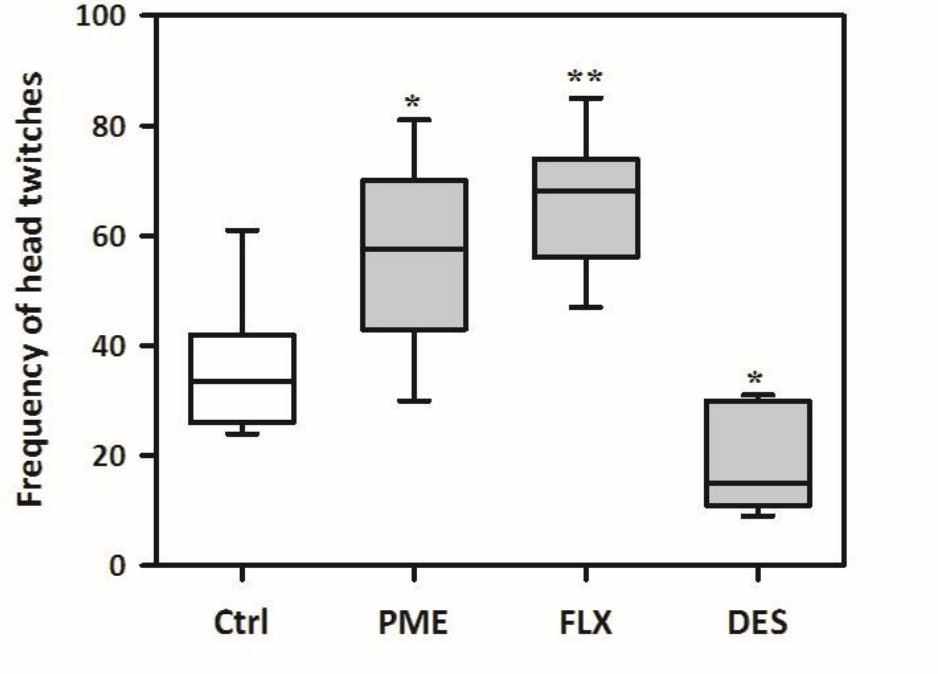


Figure 5.9 Effects of PME (100 mg kg^{-1}), FLX (10 mg kg^{-1}) and DES (10 mg kg^{-1}) on the number of 5HTP-induced head twitches in mice. Data are expressed as mean±SEM (n=6). The lower and upper margins of the boxes represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. *$P<0.05$, **$P<0.01$ compared with the saline-treated control (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

5.3.3.5 *Potentiation of Norepinephrine Toxicity*

As shown in table 5.1, injection of the sub-lethal dose of noradrenaline (3 mg kg^{-1}, s.c.), caused no mortality in PME- and FLX-treated mice. However, desipramine pretreatment potentiated markedly and significantly NE toxicity in mice.

Table 5.1 Effect of PME, fluoxetine and desipramine on norepinephrine-induced toxicity in mice

Group	Dose (mg kg^{-1})	Number of deaths	% mortality
control		0	0
PME	30	0	0
	100	0	0
	300	0	0
FLX	30	0	0
DES	30	5	50

Data indicates the number and percentage of mice (n=10) that died

5.3.3.6 *Effect of Joint Administration of D-Serine or DCS and PME, FLX or DES in the TST*

The effects of a combined administration of DCS and PME, FLX or DES on total duration of immobility in mice are shown in figure 5.10b. Administration of DCS at a dose of 2.5 mg kg^{-1} had no effect on the immobility time in mice. Concomitant administration of DCS (2.5 mg kg^{-1}) with PME (100 mg kg^{-1}) significantly reduced the immobility time in mice ($F_{1,24}$=16.42, P=0.0037) with Bonferroni's *post hoc* analysis showing significance of P<0.01. Similar effects were observed for fluoxetine (P<0.01) but not desipramine.

The effects of a combined administration of PME, FLX or DES and D-serine on total duration of immobility in mice are shown in figure 5.10a. PME (100 mg kg^{-1}), FLX (10 mg kg^{-1}) and DES (10 mg kg^{-1}) significantly reduced the immobility time in mice as revealed by ANOVA ($F_{3,16}$=4.483, P=0.0182). D-serine given alone at a dose of 600 mg kg^{-1} had no effect on immobility time, but when combined with PME or FLX, abolished their

antidepressant-like effects ($F_{1,24}$=8.849, P=0.0177). D-serine however did not completely abolish the anti-immobility of DES (P>0.05).

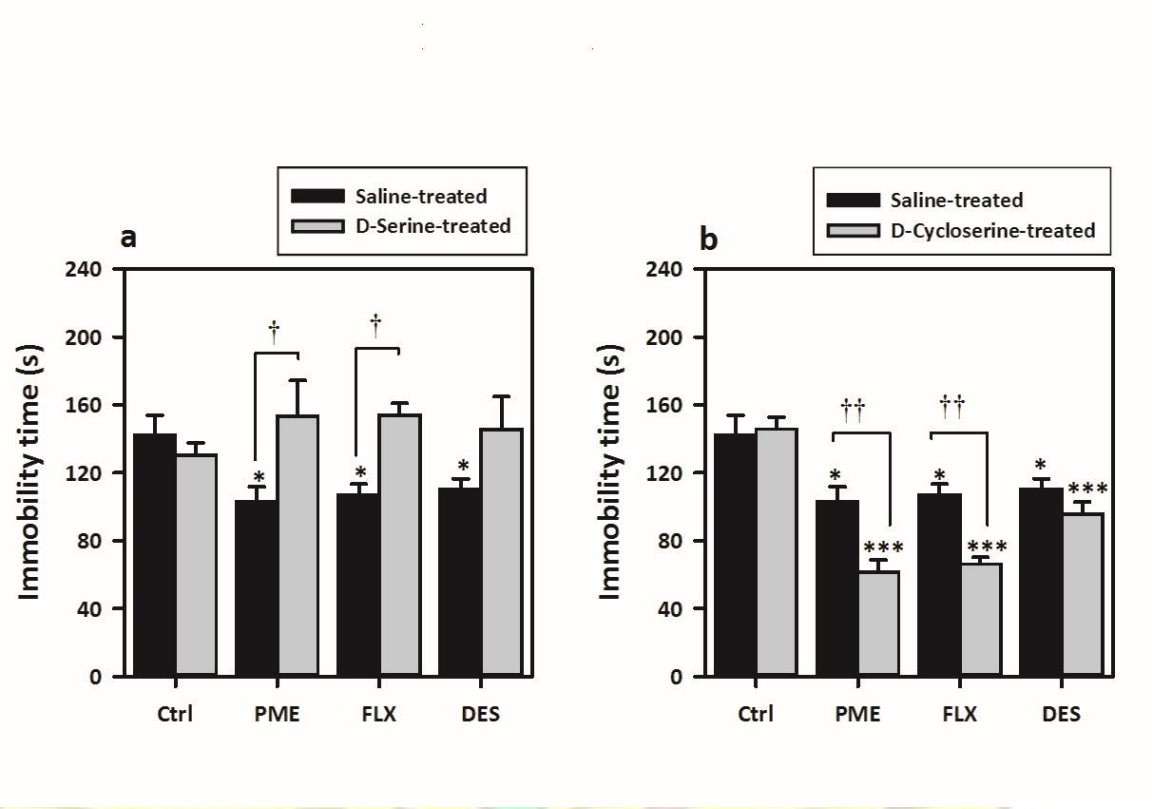


Figure 5.10 Effect of joint administration of (a) D-serine (DS) or (b) D-cycloserine (DCS) and PME, fluoxetine (FLX) or desipramine (DES) on the total duration of immobility in the TST in mice. The values represent means±SEM of 5 mice. *P<0.05, ***P<0.001 versus vehicle-treated animals (One-way ANOVA followed by Newman-Keuls' test). Significant difference between treatments: †P<0.05, ††P<0.01 (Twoway ANOVA followed by Bonferroni's test).

5.3.3.7 *Involvement of L-arginine-NO-cGMP Pathway*

PME (100 mg kg^{-1}, *p.o*) significantly decreased the immobility time of mice in the TST (P<0.05). Administration of L-arginine (750 mg kg^{-1}, i.p., a precursor of nitric oxide) had no anti-immobility effects on mice in the TST compared with saline (vehicle)-treated animals. However, pre-treatment with L-arginine prevented the antidepressant-like effect of PME (100 mg kg^{-1}, *p.o*) with a Bonferroni's *post hoc* analysis showing statistical significance of P<0.01 (figure 5.11a). L-NAME (30 mg kg^{-1}, i.p., a non-selective nitric oxide synthase inhibitor) enhanced the antidepressant effect of an effective dose of PME (100 mg kg^{-1}, *p.o*) ($F_{1,20}$=7.786, P=0.0113; figure 5.11b). Methylene blue (10 mg kg^{-1}, i.p., an inhibitor of NO synthase and an inhibitor of sGC) given alone did not affect immobility time when compared to the control group. However, methylene blue significantly enhanced the antidepressant effect of PME (100 mg kg^{-1}, *p.o*) ($F_{1,20}$=9.357, P=0.0062; figure 5.11c).

Figure 5.11d shows that the pretreatment of animals with sildenafil (5 mg kg^{-1}, i.p., a phosphodiesterase 5 inhibitor) significantly inhibited the reduction in immobility time elicited by PME in TST (P<0.05).

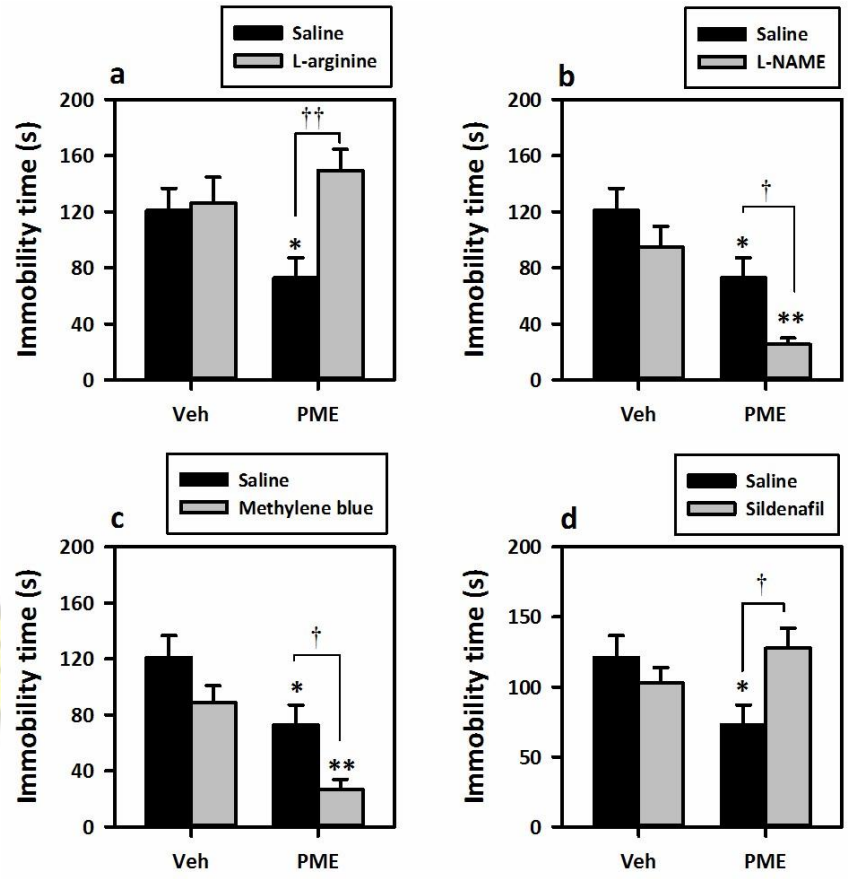


Figure 5.11 Effects of pre-treatment of mice with L-arginine (750 mg kg^{-1}, i.p., a precursor of nitric oxide, panel a), L-NAME (30 mg kg^{-1}, i.p., a non-selective nitric oxide synthase inhibitor, panel b), methylene blue (10 mg kg^{-1}, i.p., an inhibitor of NO synthase and an inhibitor of sGC, panel c), sildenafil (5 mg kg^{-1}, i.p., a phosphodiesterase 5 inhibitor, panel d) on PME (100 $mgkg^{-1}$, *p.o.*)-induced reduction in immobility time in the TST. Each column represents the mean±SEM (n=6). *P<0.05, **P<0.01 versus vehicle-treated animals (One-way ANOVA followed by Newman-Keuls' test). Significant difference between treatments: †P<0.05, ††P<0.01 (Two-way ANOVA followed by Bonferroni's test).

5.3.4 **Effect of PME on Rotarod Performance**

The extract caused no significant effect on the time taken by mice to fall off the rotarod compared to the control at all the doses used (P>0.05 at 30-300 mg kg^{-1}). Diazepam at the dose of 1.0 mg kg^{-1} caused significant decrease in the latency to fall off the rotating rod (P<0.01) (figure 5.12).

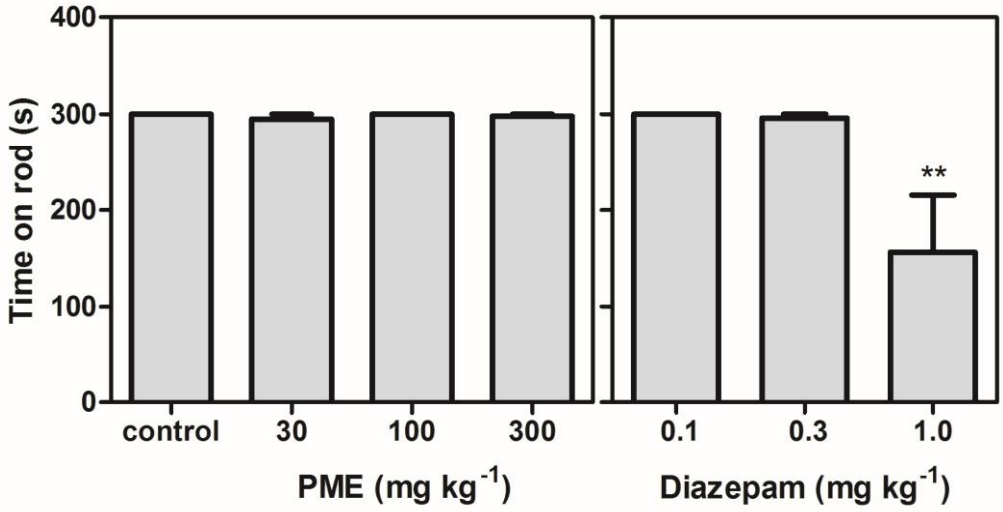


Figure 5.12 Behavioural effects of PME and DZP on muscle relaxant activity in the rotarod test in mice. Data are expressed as group mean±SEM (n=5). **P<0.01 compared to control group (one-way ANOVA followed by Newman-Keuls' test).

5.4 DISCUSSION

The present study provides convincing evidence that PME, when administered orally, produces an antidepressant-like effect in both the FST and TST and also elicits its action to a similar extent as the SSRI fluoxetine.

The FST and TST are widely used for screening potential antidepressant agents and are sensitive and relatively specific to all major classes of antidepressants including tricyclics, SSRIs, monoamine oxidase inhibitors (MAOIs) and atypical antidepressants (Detke *et al.*, 1995). These tests are based on the observation that rodents, after initial escape-oriented movements, develop an immobile posture when placed in an inescapable stressful situation. This immobility behaviour displayed in rodents has been hypothesized to reflect behavioural despair, which in turn may reflect depressive disorders in humans (Willner, 1984; Porsolt *et al.*, 1977; Steru *et al.*, 1985). If antidepressant treatments are given prior to the test, the subjects will actively persist engaging in escape-directed behaviour for longer periods of time than after vehicle treatment (Cryan *et al.*, 2005a). There is, indeed, a significant correlation between clinical potency and effectiveness of antidepressants in both models (Teixeira *et al.*, 2011; Melo *et al.*, 2011).

In this study, the modified version of the FST, originally introduced by Detke *et al.* (1995) was used, in which three specific types of behaviour, that is, immobility, climbing, and swimming, were measured. The modified FST distinguishes between passive responses (immobility) and active responses (increases in swimming or climbing) to stress (Cryan *et al.*, 2005b). Moreover, it widely measures the effects of SSRIs in mice (Detke *et al.*, 1995). Antidepressants acting through the serotoninergic system, including the SSRIs fluoxetine, sertraline, paroxetine, and citalopram, selectively increase swimming behaviour. In addition, the modified FST differentiates between antidepressants that work through serotoninergic mechanisms or noradrenergic mechanisms, as noradrenergic compounds selectively increased climbing behaviour (Detke *et al.*, 1995; Page *et al.*, 1999) and drugs with dual effects increased both swimming and climbing (Rénéric and Lucki, 1998; Carr and Lucki, 2011). In this study, PME in a similar fashion as fluoxetine induced a dose dependent reduction in the immobility time and an increase in the swimming behaviour; whereas no changes were observed in the climbing behaviour. This profile of action may suggest that the mechanism of the antidepressant-like activity of PME is related to the modulation of the serotoninergic system. In contrast, desipramine had no effect on swimming behaviour but instead increased climbing behaviour.

Modifications have been made in terms of the measurement of specific behavioural components of active behaviours in the TST to help differentiate between standard antidepressants and other compounds with antidepressant-like effects but with different mechanisms of actions, such as opiates (Berrocoso *et al.*, 2006; 2012). While traditional antidepressants that inhibit serotonin and/or noradrenaline reuptake decrease immobility and increase swinging behaviour, opioids, having decreased immobility, increase curling behaviour (Berrocoso *et al.*, 2012). In this present study, PME, fluoxetine and desipramine showed significant antidepressant-like effects by decreasing immobility. PME did not significantly affect pedaling but caused an increase in time spent swinging and curling. Thus, PME in addition to its antidepressant like activities could possibly have opioidergic properties. The TST has many advantages over the FST including the lack of hypothermic effects of cold water, the ability to test strains that may have motor deficits that make swimming difficult and increased sensitivity to a wider range of antidepressant compounds (Ripoll *et al.*, 2003; Carr and Lucki, 2011). For this reason, the TST was used to assess the mechanisms by which PME elicits its antidepressant-like activity. The anti-immobility

effect of PME in the FST and TST seems not to be associated with any motor deficits, since mice treated with PME did not show impairment in motor coordination in the rotarod test.

Several reports have suggested involvement of the serotoninergic and noradrenergic receptors in the mechanism of action of several classes of antidepressant drugs, including tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs) and monoamine oxidase inhibitors (MAOIs) as well as in the pathophysiology of depression. The monoamine hypothesis of depression proposes that there is depletion in the levels of serotonin and noradrenaline in the central nervous system (Delgado, 2000; Yi *et al.*, 2010) Therefore, the current effective treatments for depression are considered to elevate brain serotonin and/or noradrenaline neurotransmission (Delgado and Moreno, 1999; Leonard, 2001). Thus, in this study the possible involvement of these systems in the antidepressantlike effect of PME administered orally was investigated.

To confirm a possible contribution of the serotonin transmission in the antidepressant effect of PME, mice were pre-treated with *p*CPA, an inhibitor of serotonin synthesis (Cardoso *et al.*, 2009; O'Leary *et al.*, 2007). Even though the depletion of 5-HT does not always produce behavioural depression, depletion of 5-HT with *p*CPA blocks the effects of fluoxetine in the FST and TST, while the effects of desipramine, which acts primarily as a norepinephrine reuptake inhibitor, are unaffected by 5-HT depletion (Page *et al.*, 1999; O'Leary *et al.*, 2007). According to previous reports, *p*CPA at the present dose administered for three consecutive days was able to deplete the endogenous store of serotonin successfully without affecting the noradrenergic or dopaminergic levels (Eckeli *et al.*, 2000; O'Leary *et al.*, 2007; Girish *et al.*, 2012). In this study, the antidepressant-like effect of PME was abolished by *p*CPA administration, suggesting that 5-HT in the brain is essential for its action in the TST. This effect demonstrates that the serotoninergic mechanism underlie the acute behavioural effects of PME on tests of depressive behaviour.

In the present study, the anti-immobility effect elicited by PME in the TST was also blocked by the pretreatment of mice with cyproheptadine (a non-specific 5-HT receptor antagonist) whereas prazosin, propranolol and yohimbine had no effects. This further confirms the role of the serotonniergic system in the mechanism of the antidepressant-like activity of PME.

Administration of large doses of 5-HTP, a precursor of 5-HT, induces head twitches that occur spontaneously and irregularly, probably through a central action of 5-HT.

Headtwitch response induced by 5-HTP in mice, provides a simple method of determining specific activities of potentiators and antagonists for 5-HT in the central nervous system (Corne *et al.*, 1963; Nishizawa *et al.*, 2007). Administration of PME and fluoxetine potentiated 5-HTP-induced HTR in mice. This potentiation of HTR may be due to the PME or fluoxetine-mediated inhibition of the 5-HT reuptake and resulting increase of the content of 5-HT in synapses. This finding is consistent with the fact that *p*CPA pretreatment attenuated the anti-immobility activity of PME and fluoxetine in the TST. In contrast, desipramine significantly decreased the number of 5-HTP-induced head-twitch response.

In order to assess the importance of noradrenergic and/or dopaminergic neurotransmission in the antidepressant-like effect of PME in the TST, a selective inhibitor of tyrosine hydroxylase, AMPT, was used. Tyrosine hydroxylase is the rate-limiting enzyme in the synthesis of both noradrenaline (NA) and dopamine (DA), and it has been shown that AMPT reduces DA and NA levels (57 and 53 %) in mice (Widerlöv and Lewander, 1978; Jesse *et al.*, 2010). The present study found that AMPT increased immobility values in all treatment groups in the mouse tail suspension test. Reserpine irreversibly inhibits the vesicular monoamine transporter 2 (VMAT-2) which is located primarily within the CNS and is responsible for transporting monoamines from the cytoplasm into secretory vesicles (Ji *et al.*, 2007; Woode *et al.*, 2010). This disrupts vesicular storage and release of monoamine neurotransmitters (Corrodi and Hanson, 1966). Thus, reserpine pre-treatment increased baseline immobility and attenuated the effects of PME, fluoxetine and desipramine in the TST, but did not completely block their activity. However, pretreatment of mice with resepine and AMPT completely blocked the behaviours of PME, fluoxetine and desipramine. This is consistent with results obtained by O'Leary *et al* (2007). This therefore implicates the monoaminergic neurotransmission in the antidepressant-like effects of PME.

The norepinephrine potentiation toxicity in mice reveals an adrenergic component of the pharmacological activity of antidepressants. In the present study, PME did not potentiate NE toxicity indicating non-involvement of the noradrenergic system in its antidepressantlike effects. This further confirms the previous report in which the anti-immobility effect of PME was not reversed by prazosin (selective α_1- receptor antagonist) and yohimbine ($\alpha_2$receptor antagonist). Thus from the previous studies, reversal of the anti-immobility effect

of PME by AMPT could possibly be via dopaminergic and not noradrenergic neurotransmission.

During the past years, several evidences have implicated the NMDA class of glutamate receptors in the pathophysiology of major depression and the mechanism of action of antidepressant treatment (Hashimoto, 2011). Studies have also reported antidepressant-like effects of a variety of NMDA receptor antagonists in animal models of depression such as the mouse FST (Poleszak *et al.*, 2007; Wlaz *et al.*, 2011) and TST (Kos and Popik, 2005). Furthermore, it has been proven that glycine receptor antagonists and partial agonists have favourable safety profile compared with competitive and noncompetitive NMDA receptor antagonists (Hashimoto, 2011; Poleszak *et al.*, 2011). This makes them potential candidates for new antidepressant drugs. Thus, this study further assessed the involvement of the glycine site NMDA receptors in the antidepressant-like effect of PME.

D-cycloserine is a partial agonist of glycine$_B$ site of the NMDA receptor complex (Hood *et al.*, 1989; Poleszak *et al.*, 2011). At low doses, D-cycloserine exerts an agonist profile as it mimics the action of endogenous glycine at its site and at higher doses competitively antagonize the glycine site (Poleszak *et al.*, 2011; Zomkowski *et al.*, 2010). In this study, DCS did not change the behaviour of animals in the TST, however, a potentiating effect was seen when DCS was given jointly with PME or fluoxetine. This apparent potentiation was manifested as a reduction of immobility time, which suggests a participation of the glycine site of the NMDA receptor complex in the antidepressant-like activity of PME. In another study, the influence of D-serine (a full agonist on glycine/NMDA receptors) on the activity of PME in the TST was evaluated. D-serine did not change the immobility time in the TST, however, concomitant administration with PME, fluoxetine and desipramine blocked their anti-immobility actions. It has been reported that D-serine blocked the antidepressant effects of imipramine, fluoxetine and reboxetine, suggesting that activation of the glycine/NMDA receptor complex abolishes the antidepressant effects of both serotonin and noradrenaline-based compounds (Wlaz *et al.*, 2011; Poleszak *et al.*, 2008; 2011). Interaction between NMDA receptor and serotoninergic pathway is more obvious than NMDA receptor and noradrenergic one; since there is emerging evidence that the interaction between excitatory amino acids and serotonin may be important for the control of many brain activities. In fact, the NMDA receptor antagonists release/increase the concentration of serotonin and increase the turnover of this transmitter in the brain (Yan *et*

al., 1997; Szewczyk *et al.*, 2009). Thus, it can be suggested that the antidepressant-like activity of PME (which act via serotoninergic pathway) may occur *via* an effect on the glycine site of NMDA receptor.

Nitric oxide (NO) is a signaling molecule in the brain and has been implicated in neurotransmission, synaptic plasticity, learning, perception of pain, aggression, anxiety and depression (Esplugues, 2002; Savegnago *et al.*, 2008; Sah *et al.*, 2011). It has been reported that suicidal patients showed significantly higher levels of plasma nitric oxide metabolites than in non-suicidal psychiatric patients or in normal control subjects (Lee *et al.*, 2006; Dhir and Kulkarni, 2011). Similarly, depressed patients showed significantly higher plasma nitrate concentrations, suggesting that NO production is increased in depression (Suzuki *et al.*, 2001; Zomkowski *et al.*, 2010). An appreciable number of studies have attributed a significant role to the L-arginine-NO-cGMP pathway in the pathophysiology of depression. (Almeida *et al.*, 2006; Dhir and Kulkarni, 2007). Therefore, the possible participation of this pathway in the antidepressant effect of PME was investigated.

Results of the present study showed that pre-treatment of mice with L-arginine, a NOS substrate significantly inhibited the anti-immobility effect of the extract. Furthermore, a synergistic antidepressant-like effect was observed when PME was administered with N^G nitro-L-arginine-methyl ester (L-NAME), a non-selective nitric oxide synthase inhibitor or methylene blue, an inhibitor of both NOS and sGC. Several studies have demonstrated that NOS inhibitors exert antidepressant-like effects in animal models predictive of antidepressant activity (Heiberg *et al.*, 2002; Mutlu *et al.*, 2009). Thus, these results indicate that the inhibition of NO synthesis may be involved in the antidepressant-like effect of PME in the TST. Several reports have shown that NOS inhibitors augment the behavioural effect of tricyclic antidepressants and selective serotonin reuptake inhibitors, but not noradrenaline reuptake inhibitors in the FST (Harkin *et al.*, 2004; Zomkowski *et al.*, 2010; Ulak *et al.*, 2008). Therefore, the administration of NOS inhibitors could cause an enhanced effect of SSRIs in antidepressant therapy. It has been shown that PME elicits its anti-immobility effect via the 5-HT pathway devoid of the noradrenergic system. Therefore, the possible involvement of the nitric oxide system in the antidepressant-like effect of PME could be attributed to its interaction with the 5-HT system.

Heiberg *et al.* (2002), demonstrated that excessive cGMP levels may produce a depressionlike state and reducing its levels may produce antidepressant-like actions. The

levels of cGMP can be decreased by either inhibiting the soluble guanylate cyclase (by methylene blue) or by decreasing the function of nitric oxide (by inhibiting NOS enzyme). In addition,

cGMP is degraded into guanosine monophosphate (GMP) with the help of phosphodiesterase enzyme (PDE). Thus, inhibiting phosphodiesterase enzyme by using its inhibitors may increase the levels of cGMP and therefore produce a depression-like state (Dhir and Kulkarni, 2011). Sildenafil is a selective PDE5 inhibitor that increases the cGMP level in target tissues (Beavo, 1995; Savegnago *et al.*, 2008). In the present study, the antidepressant-like effect of PME was reversed by pretreatment with sildenafil (a selective PDE5 inhibitor), indicating that the extract exerts its effect in the TST by decreasing cGMP levels. This is consistent with several reports in which sildenafil blocked the antiimmobility effects of compounds with antidepressant activity (Almeida *et al.*, 2006; Moretti *et al.*, 2011; Bettio *et al.*, 2012).

5.5 **CONCLUSION**

Results indicate that *Pseudospondias microcarpa* extract produces an antidepressant-like effect in the FST and TST that is dependent on the serotoninergic system, NMDA receptor complex and the L-arginine-NO-cGMP pathway.

Chapter 6 CHRONIC ANTIDEPRESSANT ACTIVITY

6.1 INTRODUCTION

Depression is a chronic mental disorder mainly characterized by depressed mood and anhedonia, and often coupled to sleep disturbances, low self-esteem, guilt feelings, and suicidal tendencies (Wong and Licinio, 2001; Elizalde *et al.*, 2008).

Exposure to stress is a main environmental risk factor associated with the occurrence of depression (Kessler, 1997; Kendler *et al.*, 1999; Keller *et al.*, 2007). Recent work has indicated that stress exposure may interact with genetic risk factors to increase susceptibility to depression (Caspi *et al.*, 2003; Kaufman *et al.*, 2006). For these reasons, many animal models have attempted to reproduce some core components of major depressive disorder through exposure to stress (Duman, 2010).

Chronic mild stress (CMS) and repeated open-space swim models in rodents have been proposed to model some of the environmental factors that contribute to the induction of depressive disorders in humans (Willner, 1997; 2005; Stone *et al.*, 2008). In recent years, some herbal medicines, with their high safety margins, have proven to be effective in the treatment of depression (Nemeroff, 2007; van der Watt *et al.*, 2008; Zhang *et al.*, 2014).

Pseudospondias microcarpa is one of the plants used for its medicinal properties. The extract (PME) has been shown to possess antidepressant-like effects in the FST and TST. Moreover, it elicits its antidepressant-like effect via the serotoninergic system, NMDA receptor complex and the L-arginine-NO-cGMP pathway.

However, the effect of PME in chronic antidepressant models is still unknown and needs to be explored to identify its potential usefulness in the treatment or prevention of depression disorder. Therefore, this present study assessed the effect of PME in two chronic models of depression (chronic mild stress and repeated open-space swim test). In addition, behavioural tests—coat state, splash test, forced swimming test, tail suspension test, elevated-plus maze (EPM), open field test (OFT), novelty suppressed feeding (NSF), Morris water maze (MWM) and EPM transfer latency—were also performed.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Male ICR mice were used in this experiment and experimental conditions were as described in section 3.2.1.

6.2.2 **Drugs and Chemicals**

Desipramine hydrochloride was purchased from Sigma-Aldrich Inc., St. Louis, MO, USA and fluoxetine hydrochloride (Prozac®), Eli Lilly and Company Ltd., Basingstoke, England.

6.2.3 **Repeated Open-Space Swim Model**

This test is a modification of the acute forced swim paradigm that responds to chronic and not acute or subacute administration of a variety of antidepressants including tricyclics, selective serotonin reuptake inhibitors and monoamine oxidase inhibitors but not anxiolytics or antipsychotics (Sun and Alkon, 2003; 2006; Stone *et al.*, 2007; 2008). In this procedure, mice are swum for 15 min/d for 4 days in rat tub cages (24 cm × 43 cm × 23 cm, w × h × l) filled with lukewarm water (13 cm deep, 32–34 ºC) with these cages divided into 4 imaginary quadrants. This schedule produces a progressive reduction of active swimming along with a concomitant increase in immobility (floating) which persist unaltered for weeks after the last test and generalize to increased immobility in the TST.

Drug treatments begun on the fifth day and were done as described above in the FST. The time of onset of drug action was assessed by swimming the mice at days 5, 8, 11, 14 and 18 after i.p. or oral administration. All swims were videotaped with a digital video camera placed 80 cm above the tub cage. Videotapes were rated for immobility time (drifting with no observable movements of the limbs or tail) and distance swum (number of tank quadrants entered). No special procedures were used to dry or warm the animals as they rapidly dried themselves with no observable episodes of shivering. The distance moved (mobility) includes all the distance moved during the entire 15 min, as caused by active swimming/searching as well as slow drifts. Active swimming is defined as those swimming motions a mouse makes to move around in the pool .

6.2.3.1 ***Body Weight Change***

Body weight of animals was measured in order to assess the influence of repeated administrations of PME, FLX and DES on growth in the repeated open-space swim test.

6.2.3.2 ***Tail Suspension Test***

Twenty four (24) hours after the last swim, animals were assessed in the TST. Briefly, mice were individually suspended by the tail from a horizontal ring-stand bar raised 30 cm above the floor using adhesive tape placed 1 cm from the tip of tail. The mice were positioned

such that the base of their tail was aligned with the horizontal plane. Test sessions lasted for 6 min and were videotaped. Behaviours for the last 4 of the 6-minute period were then analyzed for mobility and immobility times.

6.2.3.3 *Spatial Working Memory (Morris water maze)*

On day 21, the effects of mice behaviour on hippocampal-dependent spatial learning and memory was evaluated with the Morris water maze task (Vorhees and Williams, 2006; Save and Poucet, 2000).

The MWM equipment consisted of a tank that was 120 cm in diameter and 60 cm in height, which was filled with water to the depth of 45 cm and maintained at 23±1 °C. Black nontoxic ink was added to make the water opaque. The tank was divided into four equal quadrants (NE, SE, NW and SW) by two imaginary perpendicular lines crossing the center of the tank. A movable black circular platform (5 cm in diameter) was located in the center of SW quadrant (target quadrant) and submerged 2 cm below the water surface so that a mouse could easily climb and escape from water. Each session was recorded with a video camera (Everio^{TM} model GZ-MG 130U, JVC, Tokyo, Japan) suspended approximately 100 cm above the centre of the maze. Data from the trials were collected for analyses. The environment was kept lightless, maintaining visual extra-maze cue and minimizing the noise disturbance.

The MWM task consisted of two sections: place navigation and spatial probe trial. In the place navigation test, animals were subjected to 4 training trials of 2 min per day for five consecutive days (days 21-25 of treatment). It assessed the animal's motivation and ability to swim and escape from the aversive situation of being placed into the water by associating the platform with the escape. For each trial the platform was located at the center of SW quadrant. The mouse entered the pool facing the wall from a different starting point each time so that the direct route to the platform differed. Briefly, the location of the platform remained constant and mice were allowed to swim for 60 s or until they located the platform. Mice that failed to locate the platform within 60 s were guided manually to the platform and remained for at least 5 s before returning to their home cage. There was a 5 min interval among trials.

Twenty four (day 26) hours after the last training trial in the escape acquisition test, mice were submitted to the probe trial in which the platform was removed. In the 60-s probe

trial, the time in the target quadrant (the quadrant in which the platform was located in the training sessions) was obtained as a measure for spatial memory.

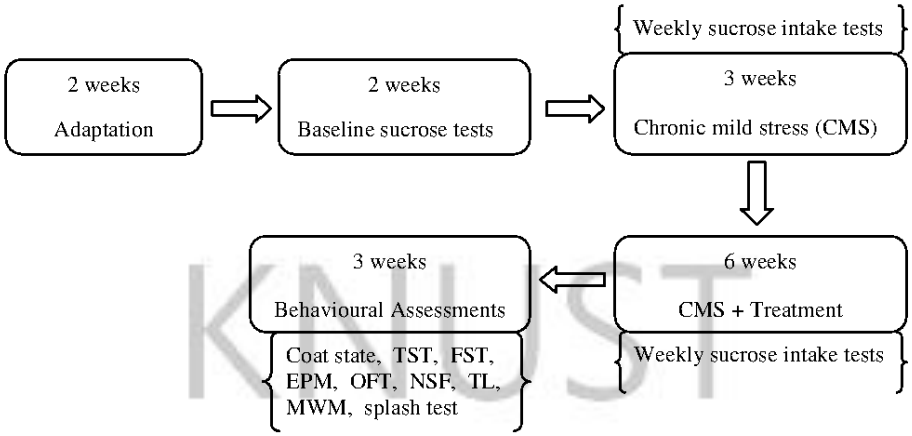
Performance parameters in MWM determined included latency to the platform, quadrant dwell time and swimming speed.

6.2.4 **Chronic Mild Stress Model**

Chronic mild stress (CMS) protocol was adopted from the procedure described by Papp *et al.* (2003). Mice were first housed individually and trained to consume a palatable sucrose solution. Training consisted of six 1 h tests in which 1 % sucrose in water was presented, following 14 h of food and water deprivation. Testing was carried out at 10:00 h and at the end of each test, sucrose intake was measured by weighing pre-weighed bottles. Subsequently, sucrose intake was measured, under similar conditions, at weekly intervals for the duration of the experiment. On the basis of their sucrose intakes in the final baseline test, the animals were divided into two matched groups.

One group of animals was subjected to the chronic stress procedure for a period of 9 consecutive weeks. Each week of the stress regime consisted of: two periods of food or water deprivation; two periods of 45º cage tilt; two periods of intermittent illumination (lights on and off every 2 h); two periods of soiled cage (250 ml water in sawdust bedding); two periods of paired housing; two periods of low intensity stroboscopic illumination (150 flashes/min); and two periods of no stress. All stressors were 10–14 h of duration and were applied individually and continuously, day and night. Control animals were housed in separate rooms and had no contact with the stressed animals. They were deprived of food and water for 14 h preceding each sucrose test, but otherwise food and water were freely available in the home cage.

On the basis of their sucrose intake following initial 3 weeks of stress, both stressed and control animals were each further divided into matched subgroups (n=8 mice per group), and for subsequent 6 weeks they received daily oral administrations of vehicle, PME (30, 100 and 300 mg kg^{-1}) or FLX (3, 10 and 30 mg kg^{-1}) administered at 10.00 am in the morning.



Stressor schedule

Time	Condition
Monday morning	Stroboscopic illumination
Monday evening	Soiled cage
Tuesday morning	Cage cleaning, followed by no stress
Tuesday evening	Food and water deprivation
Wednesday morning	Sucrose test, followed by no food or water deprivation
Wednesday evening	Grouped housing
Thursday morning	Intermittent illumination
Thursday evening	Soiled cage
Friday morning	Cage cleaning, followed by water deprivation
Friday evening	Grouped housing
Saturday morning	Stroboscopic illumination
Saturday evening	Food deprivation
Sunday morning	Cage tilt
Sunday evening	Cage untilting, followed by no stress

Figure 6.1 Schematic diagram representing experimental design of chronic mild stress (CMS) procedure

6.2.4.1 *Weight Variation*

Before and during the unpredictable chronic mild stress, the body weights of the animals were recorded every Monday.

6.2.4.2 *Coat State Assessment*

Mice were inspected on week 6 of PME treatment to observe their coat state. After removing animals from their home cages, the state of the coat of eight different body parts including the head, neck, forepaws, dorsal coat, ventral coat, hind legs, tail, and genital region of each individual mouse were inspected visually and recorded systematically (Yalcin *et al.*, 2005; Piato *et al.*, 2008). For each body area, a score of 0 was attributed for

a coat in good form, and a score of 1 for a dirty or disheveled coat. The resulting score was represented as the average for all body areas.

6.2.4.3 ***Splash Test***

This test was performed after week 6 of treatment to evaluate the grooming behaviour of both stressed and non-stressed mice. Grooming is cleaning the fur of animal by licking or scratching. A 10 % sucrose solution was splashed on the dorsal coat of mice in their home cage and animals were videotaped for 5 min (Ducottet and Belzung, 2004). Frequency of grooming, which refers to the number of licking during the 5 min test period was recorded. Grooming bouts recorded included nose/face grooming (strokes along the snout), head washing (semicircular movements over the top of the head and behind the ears), body grooming (body fur licking) (Kalueff and Tuohimaa, 2004).

6.2.4.4 ***Open-Field Test***

This test was performed in control and CMS mice at the end of the 9-week CMS exposure to study exploratory and anxiety behaviour (Nirmal *et al.*, 2008). The open field apparatus consisted of a white Plexiglas box 50 cm × 50 cm × 20 cm with its floor divided into 16 squares with black lines. Four squares were defined as the center and 12 squares along the walls as the periphery. The apparatus was illuminated with a low intensity diffuse light (45 W) situated 45 cm above the floor level. Entire room, except the open field was kept dark during the experiment. Each animal was placed in the central square and observed for 5 min and the following behaviours were recorded: central activity (number and duration of entries into the central squares with all the four paws) as well as number and duration of rearing (animal standing upright on its hind limbs). Between tests, the apparatus was cleaned with 5 % alcohol.

6.2.4.5 ***Elevated Plus Maze (EPM) Test***

Elevated plus maze (EPM) assesses unconditioned anxiety-like behaviour in rats and mice (Lister, 1987). EPM consisted of two open arms (30 cm × 5 cm), two enclosed arms (30 cm × 5 cm), and a connecting central platform (5 cm × 5 cm). The maze was elevated 38.5 cm above the ground. At the beginning of the 5-min session, each mouse was placed in the central neutral zone, facing one of the close arms. Percentage entries and time in the open arms were recorded. An arm entry was defined as a mouse having entered an arm of the maze with all four legs.

6.2.4.6 *Novelty Suppressed Feeding (NSF) Test*

The test was carried out during a 5 min period as previously described (Bodnoff *et al.*, 1989). Briefly, the testing apparatus consisted of a plastic box (50 cm × 50 cm × 20 cm); the floor was covered with 2 cm of wooden bedding. Twenty-four hours before behavioural testing, animals were deprived of all food in the home cage. At the time of testing, a single pellet of food was placed on a white paper platform positioned in the center of the box. The test began after the animal was placed in a corner of the box. The latency to feed (time elapsed until the mice started to eat) was recorded manually.

6.2.4.7 *Tail Suspension Test*

Procedure for TST was adopted from section 6.1.3.2.

6.2.4.8 *Forced Swimming Test*

Forced swimming test protocol was adopted from the procedure described in section 5.2.3. However, the behavioural parameter measured was immobility time.

6.2.4.9 *Spatial Learning and Memory*

Effects of mice behaviour on spatial learning and memory were evaluated with the Morris water maze task as described in section 6.1.3.3.

6.2.4.10 *EPM Transfer Latency*

Procedure for assessing learning and memory was adopted as previously described (Dhingra *et al.*, 2004; Singh and Parle, 2003). Elevated plus maze served as the exteroceptive behavioural model (wherein the stimulus existed outside the body) to evaluate learning and memory in mice. On the first day, each mouse was placed at one end of the open arms, facing away from central platform. Transfer latency (TL) was taken as the time taken by the mouse to move into any one of the closed arms with all its four legs. Transfer latency was recorded on the first day for each animal. If the animal did not enter into one of the closed arms within 90 s, it was gently pushed into one of the two closed arms and TL was assigned as 90 s. The mouse was allowed to explore the maze for another 2 min and returned to its home cage. Retention of this learned task (memory) was examined 24 h after the first day trial.

6.2.5 **Statistical Analysis**

In all experiments, a sample size of 5-8 animals was utilized. All data are presented as mean±SEM. To compare differences between groups, one-way ANOVA was performed with Newman-Keuls' test as *post hoc*. The time-course curves were subjected to two-way (*treatment* × *time*) repeated measures analysis of variance (ANOVA) with Bonferroni's *post hoc* test. GraphPad Prism for Windows 5 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis. $P<0.05$ (Newman-Keuls' test or Bonferroni's test) was considered statistically significant. Doses for 50 % of the maximal effect (ED_{50}) for each drug were determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation:

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

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.

6.3 **RESULTS**

6.3.1 **Repeated Open-Space Swim Test**

Mice showed a gradual and significant reduction in the distance moved as well as an increase in immobility over trials as they swum in the rat tub cages 15 min/d for four consecutive days. Unlike the behaviour patterns observed in the classical forced swimming test, no climbing on the wall was observed. This might probably be due to the large diameter of the rat tub cages. As the trials progressed, the control mice showed a significant decrease in active swimming. A maximal reduction in their mobility was reached at the 3rd trial in these control mice. At this point, control mice did not make any movements other than those just sufficient to keep its head above the water surface (immobility), a characteristic behaviour that is taken as an indicator of depression in the forced swimming test. As shown in figure 6.2, without antidepressant drug (depressed group with vehicle injection), the open space swim test induced a significant immobility for almost the entire duration of the experiment.

All drug-treated groups displayed significant increase in the distance moved over trials (figure 6.3), when compared with the vehicle-treated group [PME: $F_{3,128}$=12.94, P=0.0002; fluoxetine: $F_{3,128}$=3.752, P=0.0325; desipramine: $F_{3,128}$=3.970, P=0.0272; two-way ANOVA (*treatment* x *time*)]. Moreover, PME and the classical antidepressants significantly increased the reduction in immobility time over the entire duration of the experiment, when compared with the vehicle-treated group [PME: $F_{3,128}$=89.81; P<0.0001; fluoxetine: $F_{3,128}$=9.732, P=0.0007; desipramine: $F_{3,128}$=8.139, P=0.0016; Two-way ANOVA (*treatment* x *time*)]. Bonferroni's *post hoc* analysis revealed a significant antiimmobility effect of PME on day 5 (treatment day 1) (P<0.001). In comparison, treatment with the classical antidepressants, which started 24 h after the four open space swim test trials, was not immediately effective. The first week of treatment did not result in a significant improvement in mobility of the mice (P>0.05, when compared with control). The improvement was observed on day 10 of continued antidepressant treatment (P<0.05) and achieved its peak 2 weeks after the continued treatment (P<0.001).

Oral administration of PME (30-300 mg kg^{-1}) significantly increased the total distance swum ($F_{3,16}$=22.29, P<0.0001) and decreased the immobility time ($F_{3,16}$=119.8, P<0.0001) indicative of antidepressant effect. Similar effects were also observed for fluoxetine (distance swum: $F_{3,16}$=5.671, P=0.0077; immobility: $F_{3,16}$=8.946, P=0.001) and desipramine (distance swum: $F_{3,16}$=4.554, P=0.0172; immobility: $F_{3,16}$=7.562, P=0.0023). PME showed more efficacy than desipramine and fluoxetine though less potent in decresing the immobility time. The extract was however was the most potent of the test compounds with regards to increase in distance travelled [PME (ED_{50}=5.84 mg kg^{-1}); FLX (ED_{50}=15.29 mg kg^{-1}); DES (ED_{50}=15.57 mg kg^{-1})] (figure 6.4).

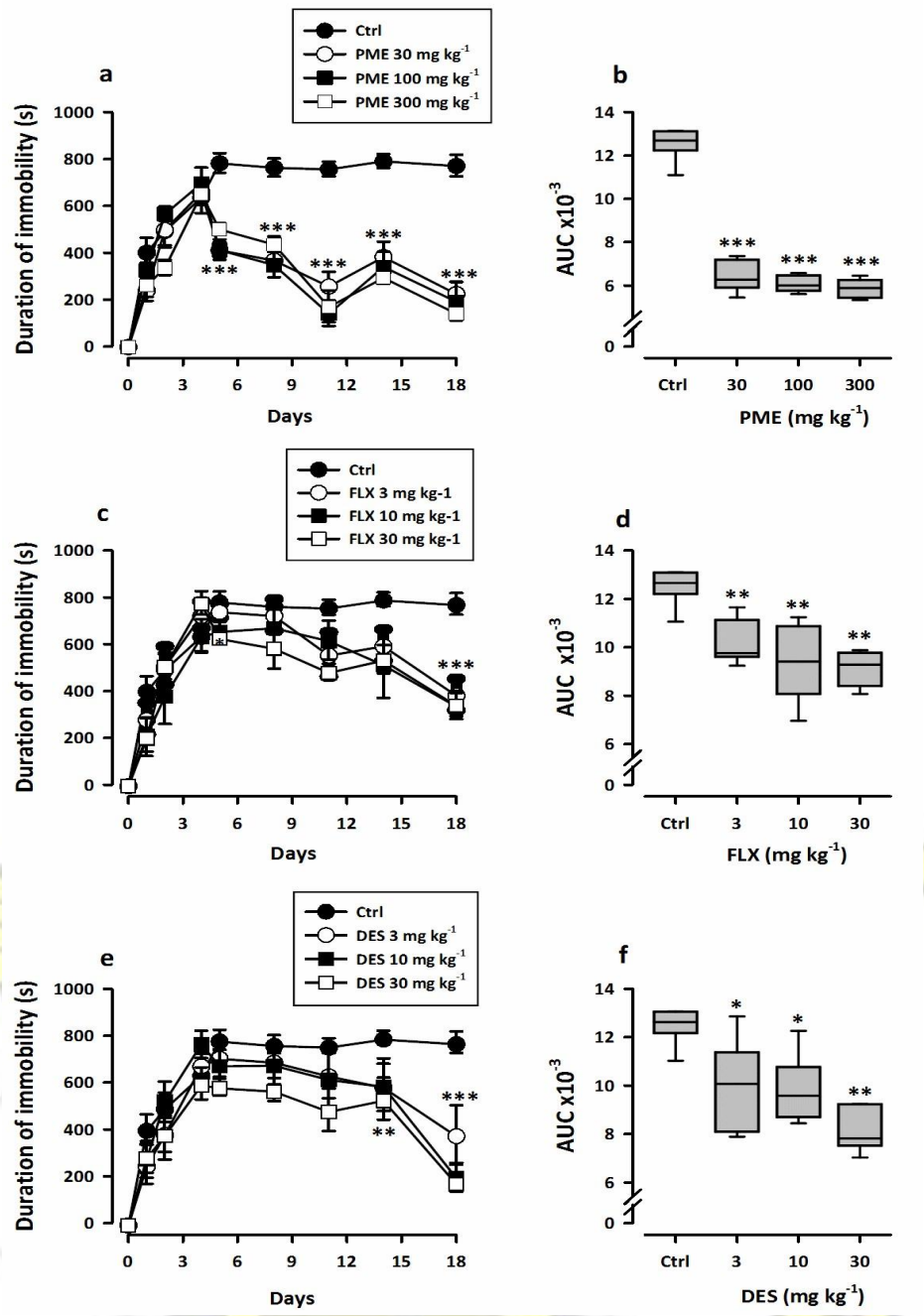


Figure 6.2 Effect of PME (30-300 mg kg^{-1}), fluoxetine, FLX (3-30 mg kg^{-1}) and desipramine, DES (3-30 mg kg^{-1}) treatment on the total duration of immobility in the open space swim test. Data are presented as both time course curves (a, c and e) and mean±SEM (n=5) of their areas under the curves (AUCs) (b, d and f). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box and symbols represent outliers. Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls' test for the AUCs or Two-way ANOVA followed by Bonferroni's *post hoc* test for time-course curves.

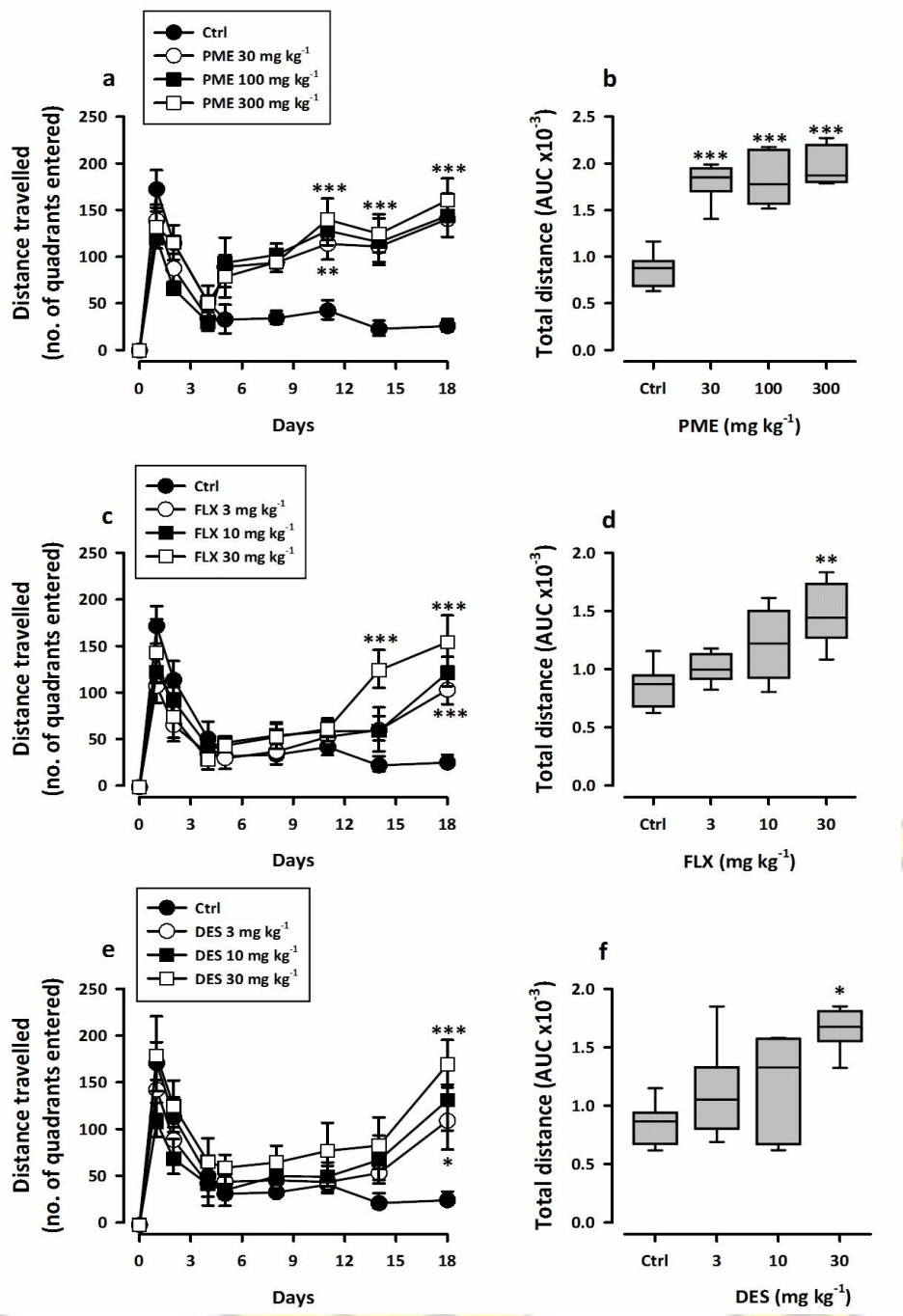


Figure 6.3 Effect of PME (30-300 mg kg^{-1}), fluoxetine, FLX (3-30 mg kg^{-1}) and desipramine, DES (3-30 mg kg^{-1}) treatment on the distance travelled in the open space swim test. Data are presented as both time course curves (a, c and e) and mean±SEM (n=5) of their areas under the curves (AUCs) (b, d and f). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls' *post hoc* test for the AUCs or two-way ANOVA followed by Bonferroni's *post hoc* test for time-course curves.

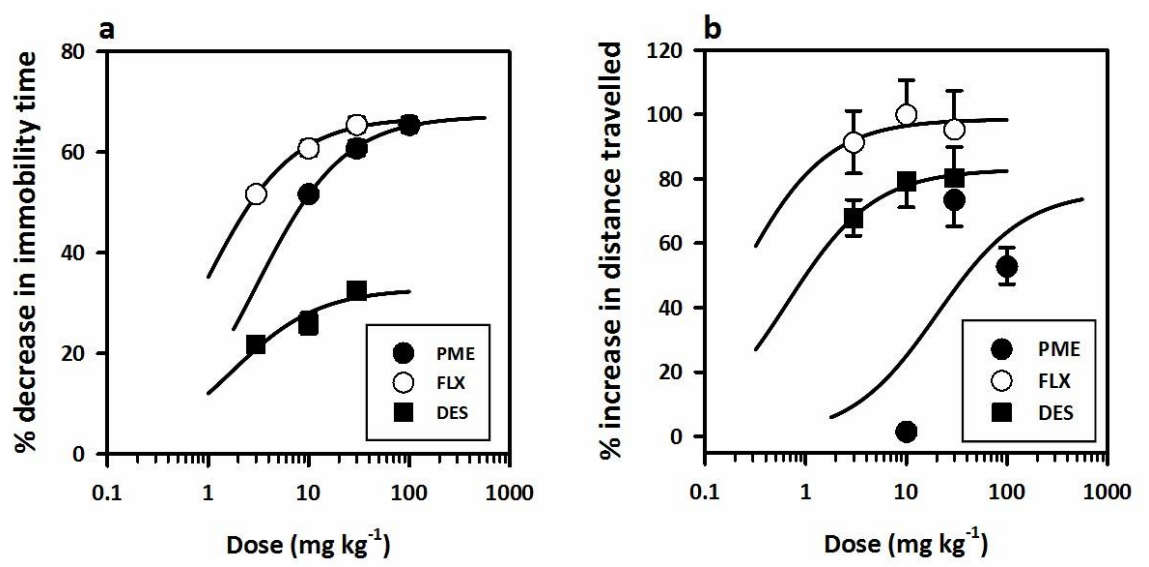


Figure 6.4 Dose response curves for PME (30-300 mg kg^{-1}, *p.o.*), fluoxetine, FLX (3-30 mg kg^{-1}, *p.o.*) and desipramine, DES (3-30 mg kg^{-1}, i.p.) with respect to % decrease in immobility time (a) and % increase in swimming time (b) in the open space swim test in mice. Each point represents the mean±SEM (n=5).

6.3.1.1 ***Body Weight Change***

Two-way ANOVA revealed that repeated administrations of PME, FLX or DES had no influence on the body weight gain compared with the control animals [PME: $F_{3,80}$=0.0492, P=0.985; fluoxetine: $F_{3,80}$=1.551, P=0.2401; desipramine: $F_{3,80}$=0.368, P=0.7771; two-way ANOVA (*treatment* x *time*)].

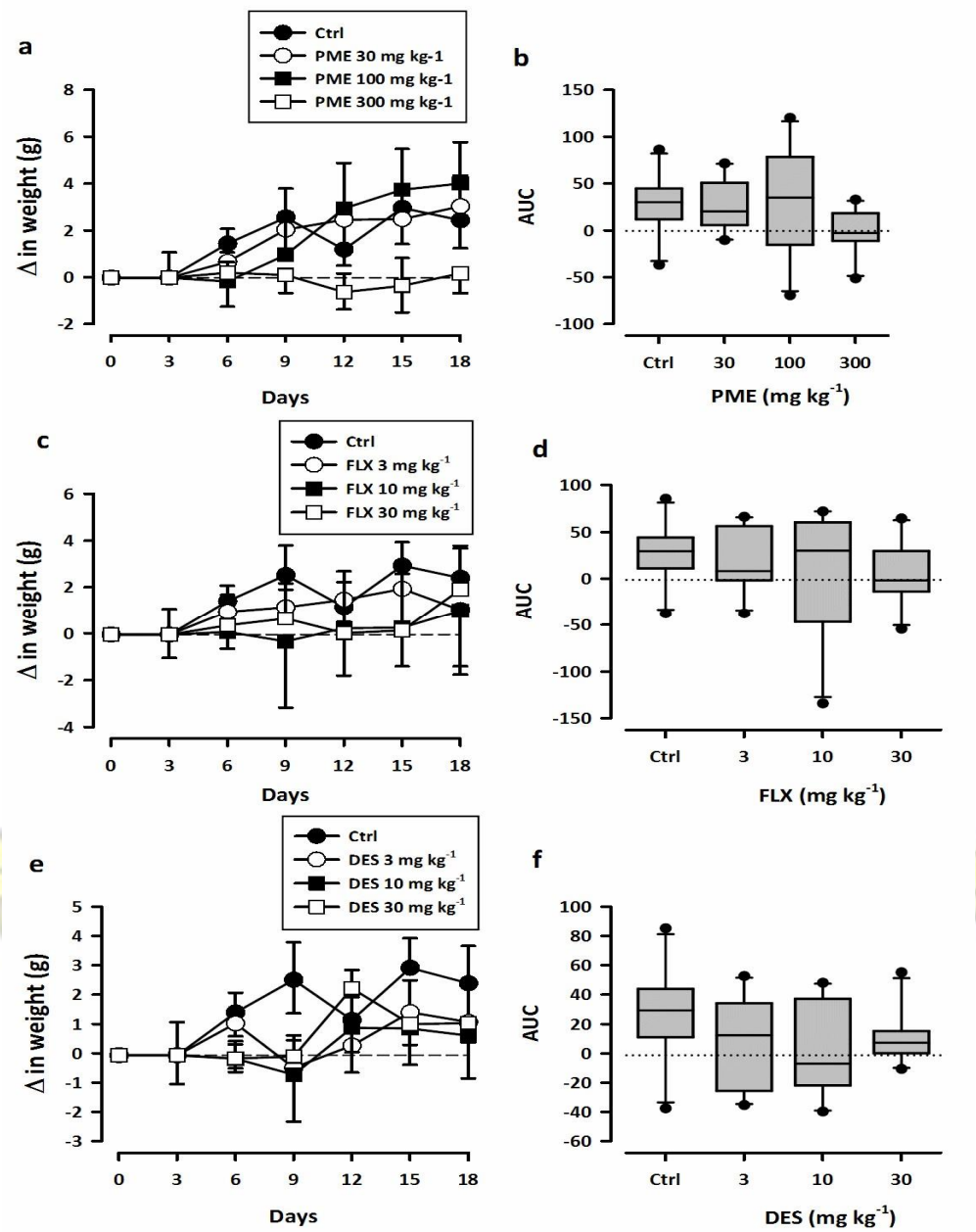


Figure 6.5 Effect of PME (30-300 mg kg^{-1}), fluoxetine, FLX (3-30 mg kg^{-1}) and desipramine, DES (3-30 mg kg^{-1}) treatments on weight change of mice in the open space swim test. Data are presented as both time course curves (a, c and e) and mean±SEM (n=5) of their areas under the curves (AUCs) (b, d and f). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box and symbols represent outliers.

6.3.1.2 *Tail suspension Test*

The effects of mice behaviours in the TST 24 h after behavioural assessment in the repeated open-space swim procedure are shown in figure 6.6. ANOVA revealed a significant reduction in the immobility effect of all drug-treated groups when compared to control

[PME: $F_{3,16}$=4.881, P=0.0135; fluoxetine: $F_{3,16}$=4.391, P=0.0195; desipramine: $F_{3,16}$=8.358, P=0.0014]. Dose-response curves are shown in figure 6.7.

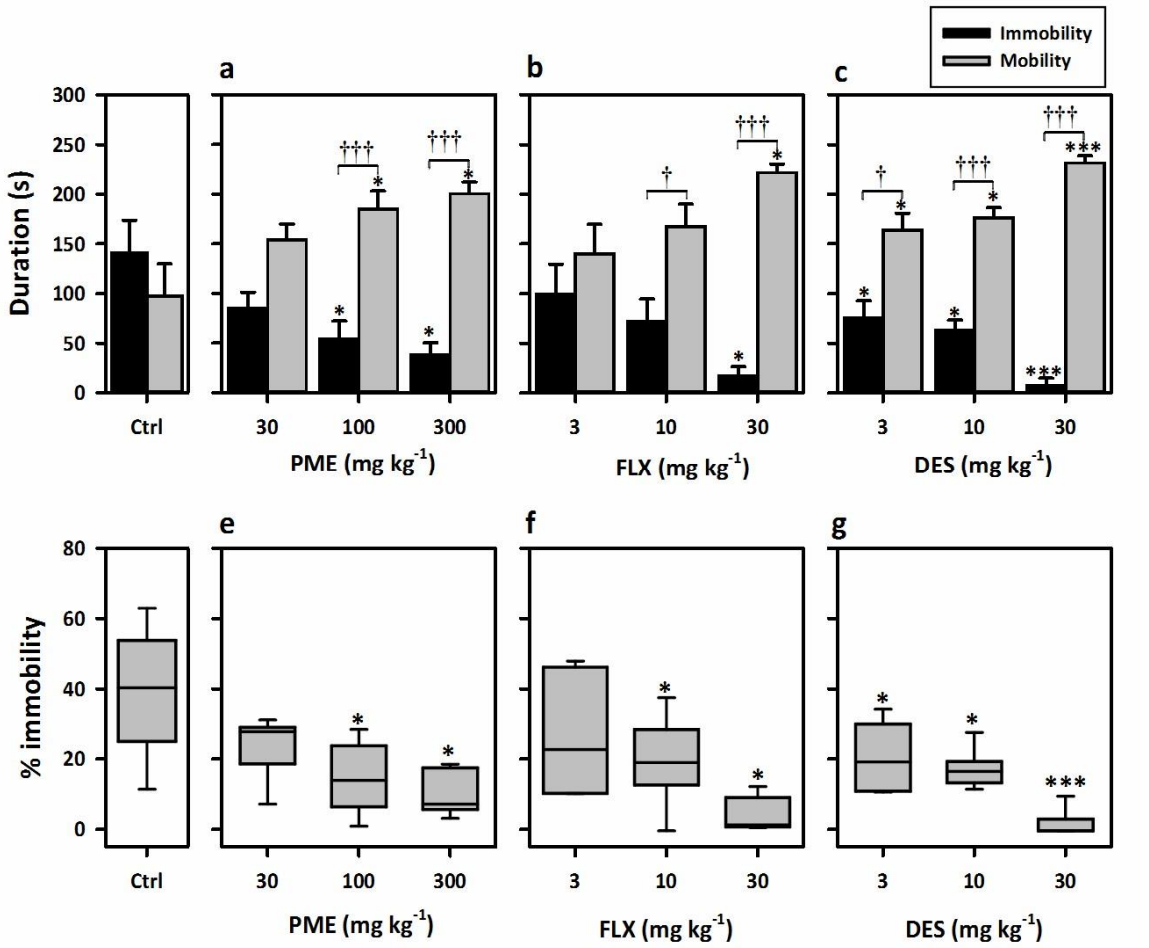


Figure 6.6 Effects of PME (30-300 mg kg^{-1}), fluoxetine, FLX (3-30 mg kg^{-1}) and desipramine, DES (3-30 mg kg^{-1}) treatment on immobility and mobility duration (a, b and c) and % immobility (e, f and g) in the TST performed after the repeated open space swim procedure. Significantly different from control: *P<0.05, ***P<0.001 (One-way ANOVA followed by Newman-Keuls' test) and significant difference when immobility and mobility were compared: †P<0.05, †††P<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test). Data are presented as group mean±SEM (n=5).

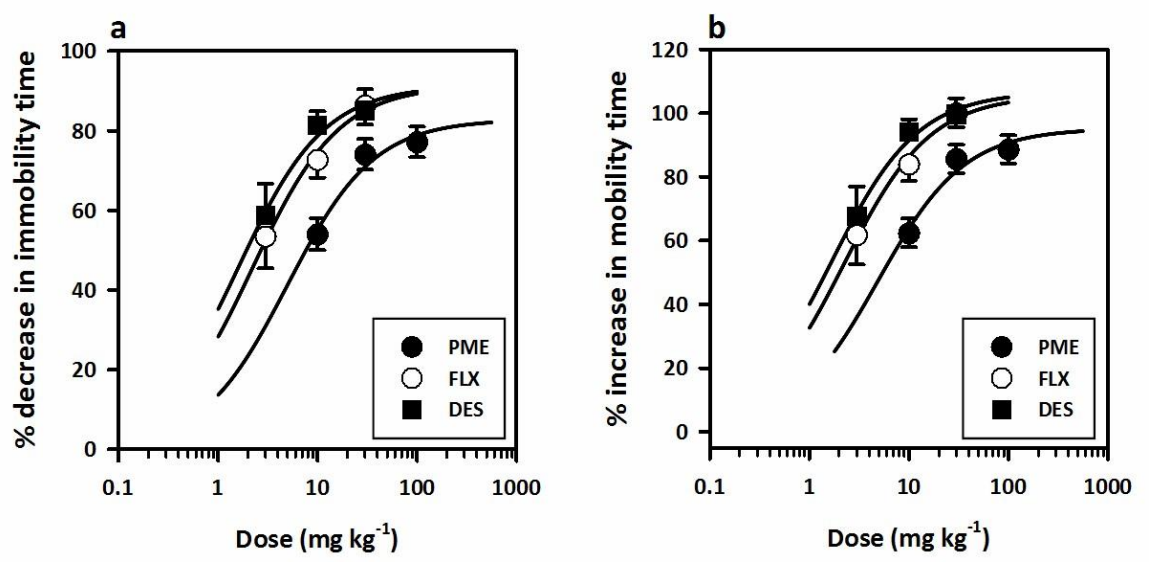


Figure 6.7 Dose response curves for PME (30-300 mg kg^{-1}, *p.o.*), fluoxetine, FLX (3-30 mg kg^{-1}, *p.o.*) and desipramine, DES (3-30 mg kg^{-1}, i.p.) with respect to % decrease in immobility time (a) and % increase in mobility time (b) in the TST performed after the repeated open space swim procedure in mice. Each point represents the mean±SEM (n=5).

6.3.1.3 ***Spatial Learning and Memory***

A day after the behavioural assessment in the TST, the effects of induced depressive behaviour was tested on spatial learning in mice, using the Morris water maze (MWM). All groups showed no significant changes in escape latency during the first day as compared to the depressed control group (P>0.05). However, the depressive behaviour-induced reduction in learning was eliminated by the administration of PME, fluoxetine and desipramine.

The change in latency to escape to the platform in all drug treated groups of mice decreased significantly following the training sessions, indicating that all mice showed some degrees of learning [PME: $F_{4,100}$=12.86, P<0.0001 (figure 6.8a); fluoxetine: $F_{4,100}$=9.572, P<0.0002 (figure 6.8c); desipramine: $F_{4,100}$=16.46, P<0.0001 (figure 6.8e); Two-way ANOVA (*treatment* x *time*)]. One-way ANOVA revealed a significant decrease in the change in escape latency for PME ($F_{4,24}$=12.07, P<0.0001; figure 6.8b), fluoxetine ($F_{4,24}$=7.555, P=0.0007; figure 6.8d) and desipramine ($F_{4,24}$=10.93, P<0.0001; figure 6.8f) when compared with depressed mice. Moreover, a *post hoc* analysis revealed significant difference from the third trial for all treated groups (P<0.001) confirming good learning in the mice after the depressive behaviour induction. Although a decreased change in escape latency was observed for naive group when compared to the depressed mice, this was not

statistically significant. Memory retention was evaluated after the training. Spatial probe trial tests 24 h after the last training trial revealed that the mice after the depressive behaviour induction did not show significant preference for the target quadrant (quadrant 4), where the platform was previously placed during the training trials. In figure 6.10, Administration of PME ($F_{4,24}$=13.33, P<0.0001, fluoxetine ($F_{4,24}$=6.885, P=0.0012) and desipramine ($F_{4,24}$=14.81, P<0.0001) significantly increased the percentage time spent in the target quadrant indicative of an improved memory. Newman-Keuls' *post hoc* analysis also showed that naïve mice significantly increased percentage time spent in the target quadrant as compared to depressed mice (P<0.05). Desipramine was the most potent in improving memory, followed by fluoxetine then the extract (figure 6.9). In addition, the swimming speeds (figure 6.11) of each group remained constant throughout the test with no significant differences between groups (P>0.05).

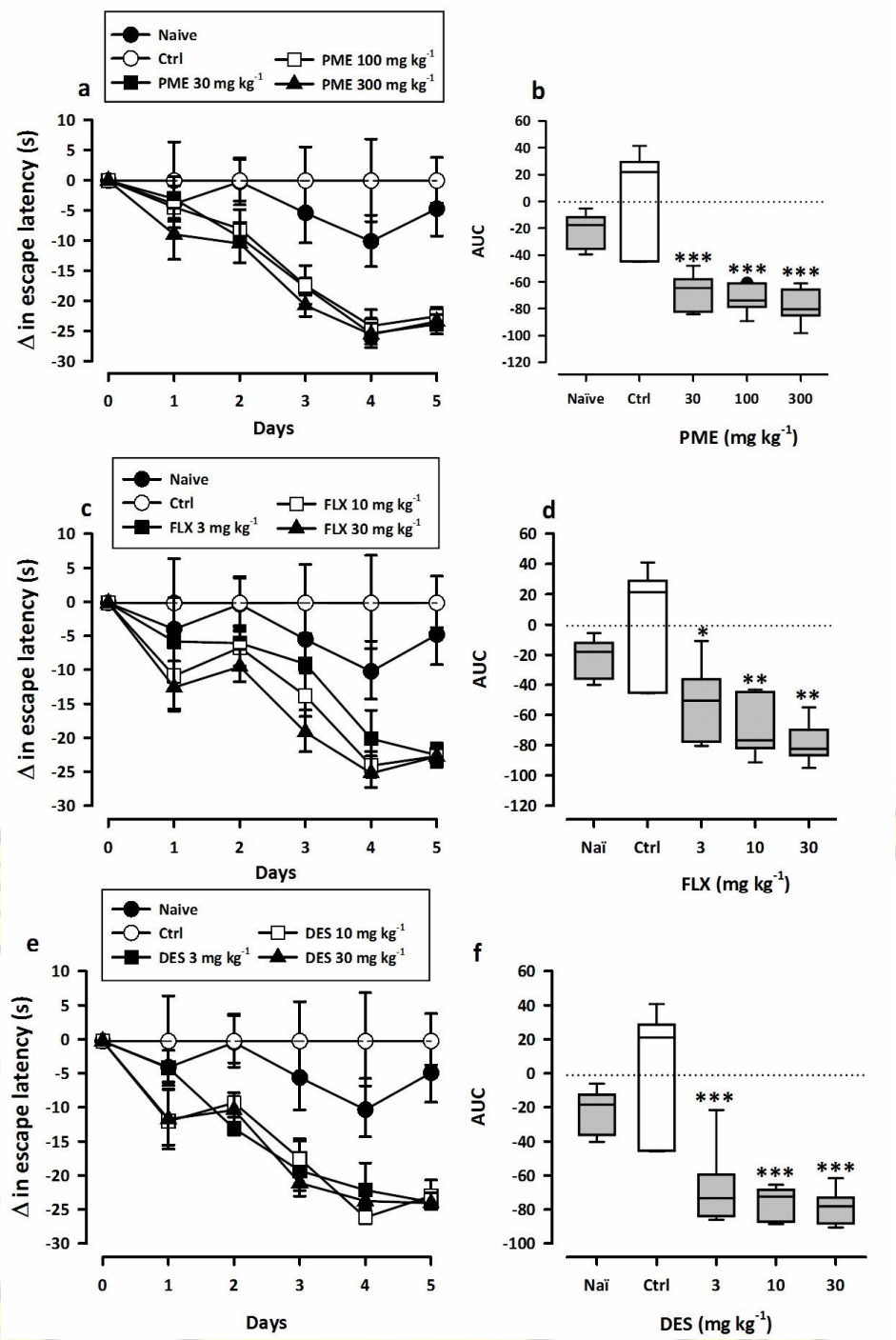


Figure 6.8 Effects of PME (30-300 mg kg^{-1}), fluoxetine, FLX (3-30 mg kg^{-1}) and desipramine, DES (3-30 mg kg^{-1}) treatments on escape latency from the place navigation session in the Morris water maze test. Data are presented as both time course curves (a, c and e) and mean±SEM (n=5) of their areas under the curves (AUCs) (b, d and f). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.001 (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

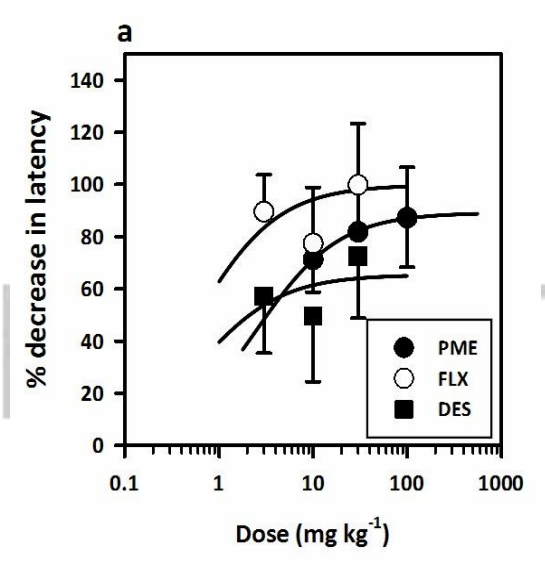


Figure 6.9 Dose response curves for PME (30-300 mg kg^{-1}, *p.o.*), fluoxetine, FLX (3-30 mg kg^{-1}, *p.o*) and desipramine, DES (3-30 mg kg^{-1}, i.p.) with respect to % decrease in escape latency (a) MWM test in mice. Each point represents the mean±SEM (n=5).

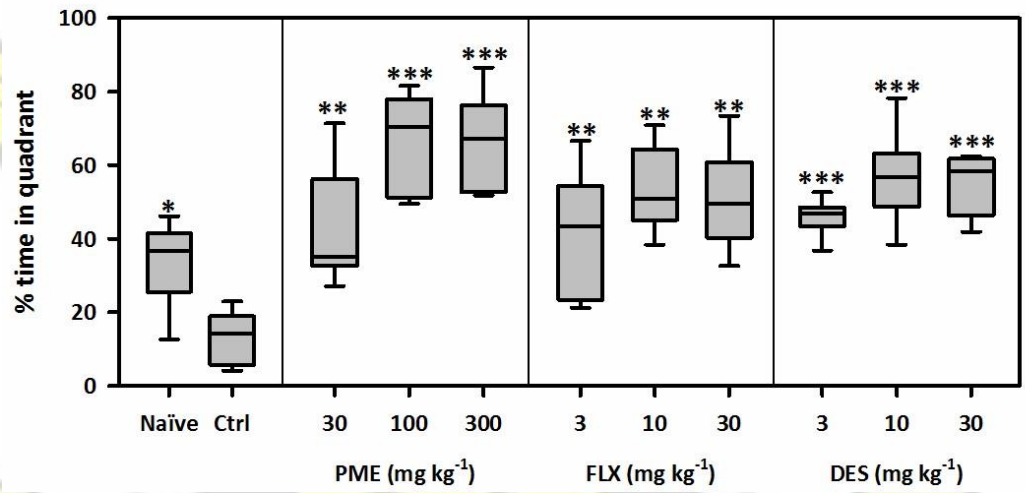


Figure 6.10 Effect of PME (30-300 mg kg^{-1}), fluoxetine, FLX (3-30 mg kg^{-1}) and desipramine, DES (3-30 mg kg^{-1}) treatments on % time in quadrant from the probe trial session in the Morris water maze test. Data are presented as group mean±SEM (n=5). The lower and upper margins of the boxes represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.001 (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

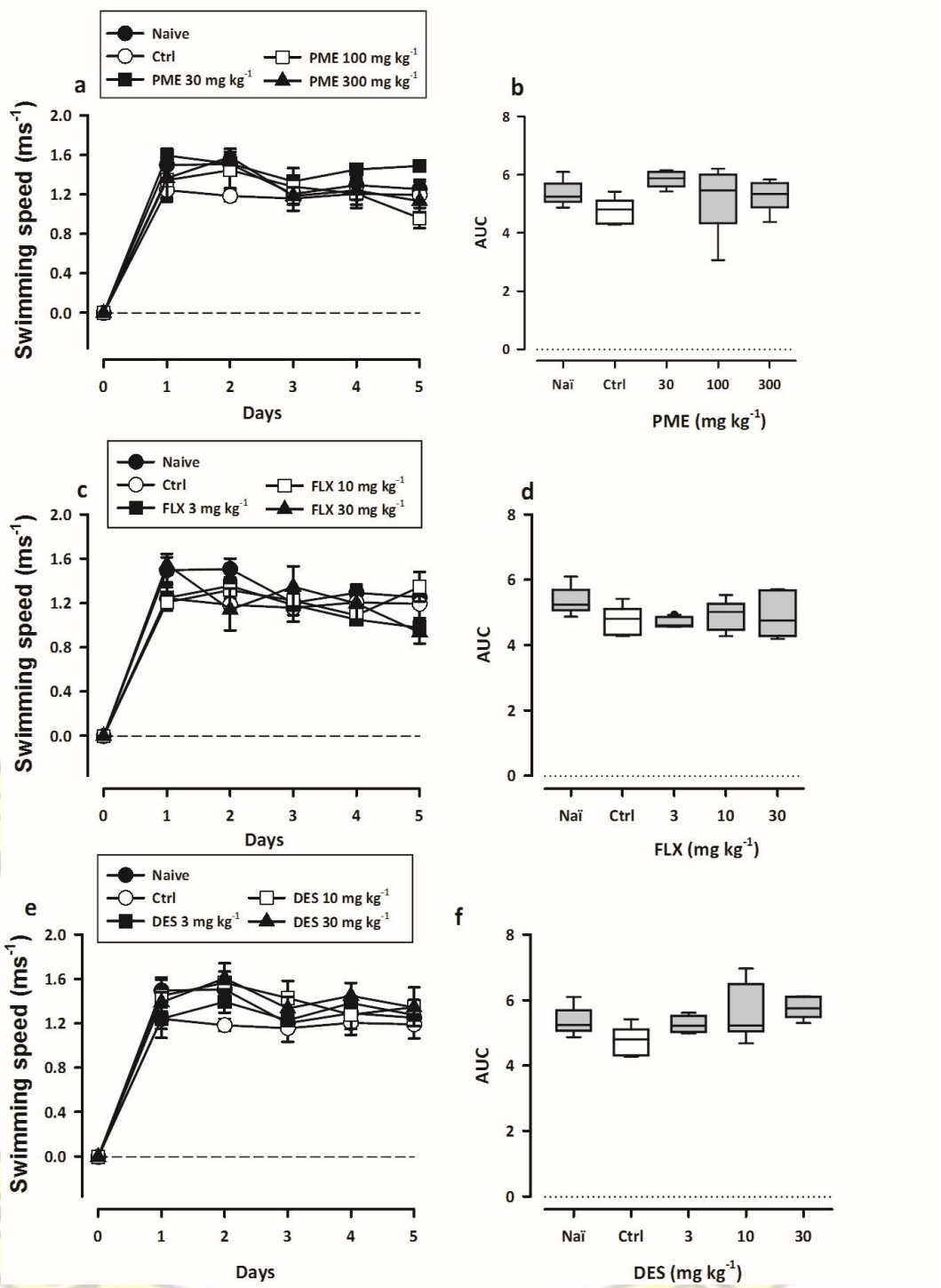


Figure 6.11 Effects of PME (30-300 mg kg^{-1}), fluoxetine, FLX (3-30 mg kg^{-1}) and desipramine, DES (3-30 mg kg^{-1}) treatments on swimming speed from the place navigation session in the Morris water maze test. Data are presented as both time course curves (a, c and e) and mean±SEM (n=5) of their areas under the curves (AUCs) (b, d and f). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box.

6.3.2 **Sucrose Intake Test**

From the initial baseline test, all animals drank approximately 1.68–3.72 g of sucrose solution (data not shown). Exposure of mice to various stressors in the CMS regime for 3 weeks (week 0) resulted in a significant decrease in the consumption of 1 % sucrose solution relative to the control group ($P<0.001$). The sucrose intake in the controls however remained at the same level (approximately 2.60 g). The difference between control and stressed animals treated with vehicle, persisted at similar level for the remainder of the 6week treatment period ($P<0.001$).

Chronic treatment with PME and FLX had no significant effect on sucrose intake in control animals ($P<0.05$). However, as shown in figure 6.12a, administration of PME increased sucrose consumption in stressed animals, resulting in significant effects of treatment ($F_{3,196}=34.48$, $P<0.0001$), interaction ($F_{18,196}=2.160$, $P=0.0054$) and time ($F_{6,196}=16.17$, $P<0.0001$). As compared to Week 0 scores, the increases in sucrose intake in stressed animals administered with PME reached statistical significance after 2 weeks of treatment as shown in figure 6.12a (30 mg kg^{-1}; $P<0.05$, 100 mg kg^{-1}; $P<0.05$ and 300 mg kg^{-1}; $P<0.001$). This effect was maintained for the remainder of the treatment period and by Week 3 sufficient recovery from the stress-induced deficit in sucrose consumption was observed as compared to the vehicle-treated controls. From the AUC, a one-way ANOVA demonstrated a significant increase in sucrose intake ($F_{3,28}=32.29$, $P<0.0001$; figure 6.12b) for PME with Newman-Keuls *post hoc* analysis showing significance at all the doses used (all $P<0.001$).

In the stressed animals, administration of fluoxetine as shown in figure 6.12c also increased sucrose consumption resulting in significant effects of treatment ($F_{3,196}=13.40$, $P<0.0001$) and time ($F_{6,196}=13.98$, $P<0.0001$). However, unlike PME, increased sucrose consumption in stress animals reached significance after 4 weeks of fluoxetine treatment (all $P<0.05$). In analyzing the AUC for fluoxetine-treated stress animals, a one-way ANOVA demonstrated a significant increase in sucrose intake ($F_{3,28}=19.31$, $P<0.0001$; figure 6.12d) as compared to the stressed control.

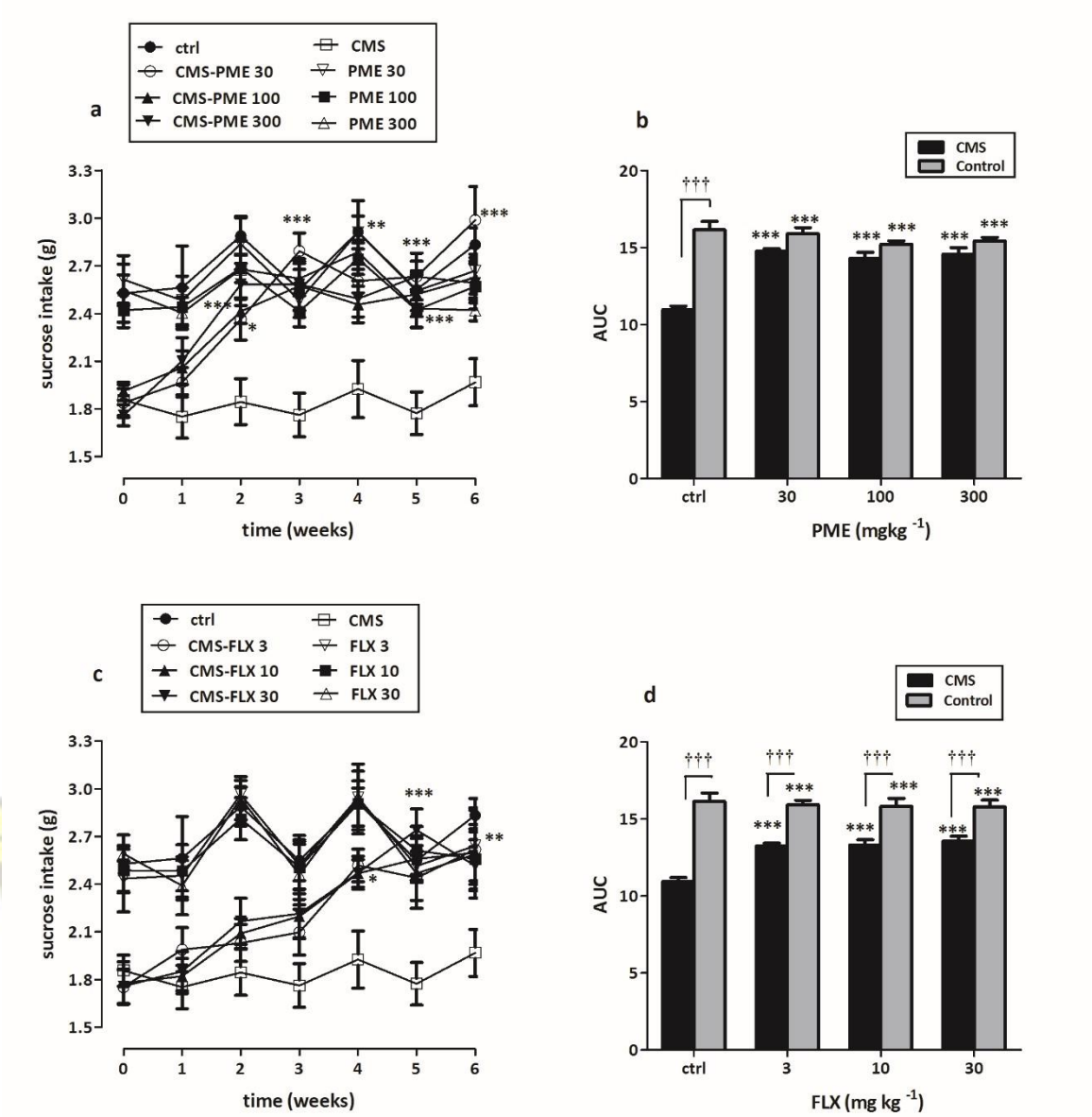


Figure 6.12 Sucrose intake of control and CMS exposed mice chronically treated with saline, PME (30-300 mg kg^{-1}) or FLX (3-30 mg kg^{-1}). Data are presented as both time course curves (a and c) and mean±SEM (n=8) of their areas under the curves (AUCs) (b and d). Significantly different from stress control: **P*<0.05 ***P*<0.01, ****P*<0.001 (Newman-Keuls' *post hoc* test for the AUCs or two-way ANOVA followed by Bonferroni's *post hoc* test for time-course curves.) and significant difference when CMS and control groups were compared: †††*P*<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

6.3.2.1 ***Weight Change***

Weight change in saline-control mice increased gradually until it peaked on the third week. Mice subjected to the CMS paradigm for 3 weeks (week 0) resulted in a significant

decrease in weight change relative to the control group (P<0.001). Administration of the extract to stressed animals resulted in a significant increase in body weight as compared to the CMS group ($F_{4,280}$=136.7, P<0.0001; figure 6.13a) with a Bonferroni's *post hoc* analysis revealing significance after week 2 and 3 for 100 and 300 mg kg^{-1} respectively (both at P<0.05). Fluoxetine administration also improved weight change ($F_{4,280}$=153.6, P<0.0001; figure 6.13e) in stressed mice reaching significance after week 3 (P<0.001 for 10 mg kg^{-1} and P<0.05 for 30 mg kg^{-1}) and week 6 (P<0.01 for 3 mg kg^{-1}). From the AUCs, it was observed that with increasing dose, chronic treatment with PME increased (figure 6.13d) whereas FLX decreased (figure 6.13h) weight change in control mice. However, this change was not significant to the saline-control group.

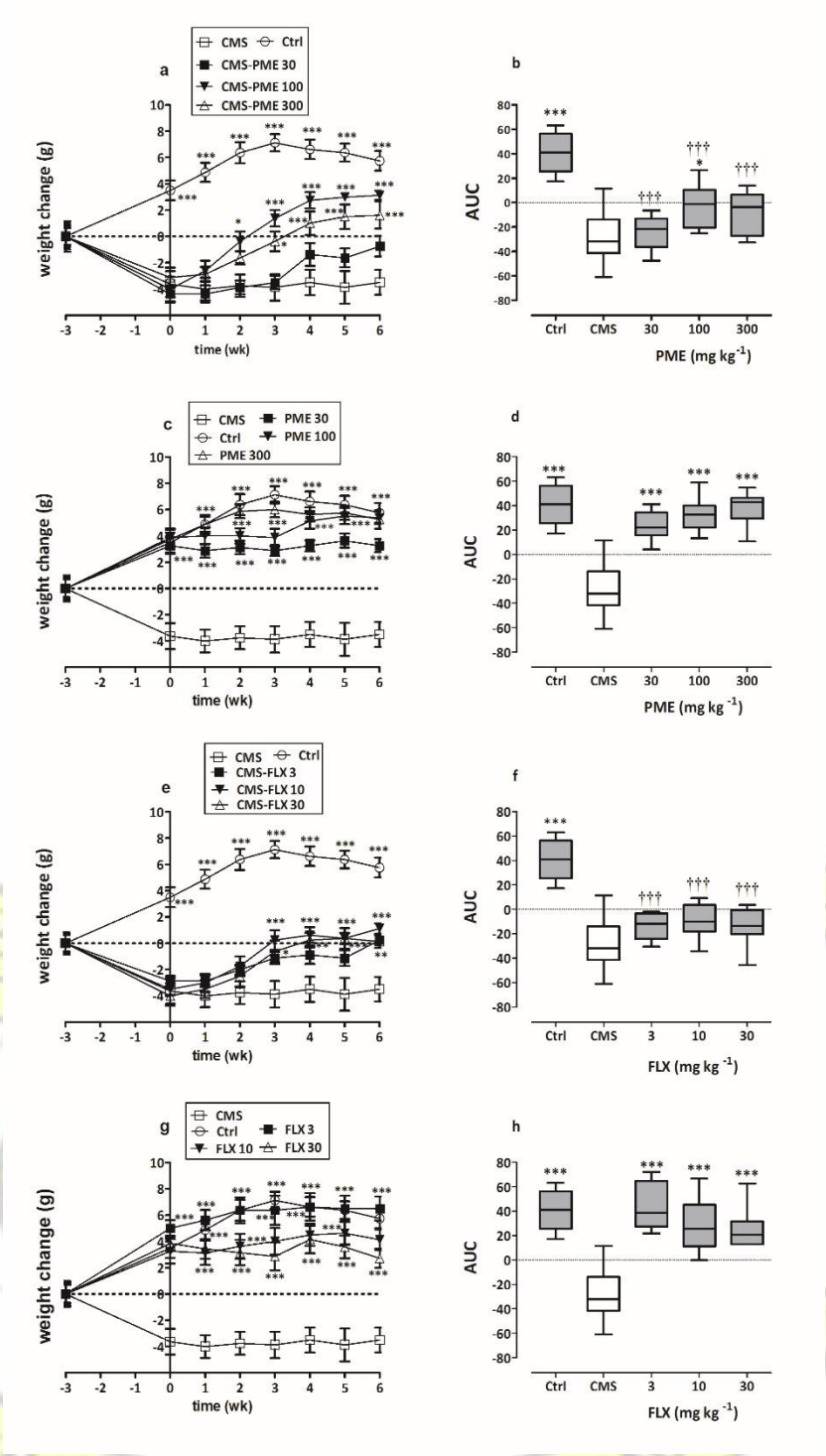


Figure 6.13 Effect of PME (30-300 mg kg^{-1}) and fluoxetine, FLX (3-30 mg kg^{-1}) treatments on weight change in control and CMS-exposed mice. Data are presented as both time course curves (a, c, e and g) and mean±SEM (n=8) of their areas under the curves (AUCs) (b, d, f and h). Significantly different from stress control group: **P*<0.05, ****P*<0.001 (Newman-Keuls' *post hoc* test for the AUCs or two-way ANOVA followed by Bonferroni's *post hoc* test for time-course curves.) and significant difference when compared to saline control: †††*P*<0.001 (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

6.3.2.2 *Coat State Assessment*

Coat state of both stressed and non-stressed mice were scored 9 weeks after the beginning of the unpredictable chronic mild stress regimen. As shown in figure 6.14, a significant difference was observed between non-stressed vehicle and stressed vehicle groups at the end of the unpredictable chronic mild stress regimen (P<0.001). PME ($F_{4,35}$=19.70, P<0.0001) and FLX ($F_{4,35}$=12.01, P<0.0001) significantly reversed the degradation of the coat state induced by chronic mild stress in stressed mice when compared to CMS group. No significant difference was observed between drug-treated control mice and the vehiclecontrol group (P>0.05).

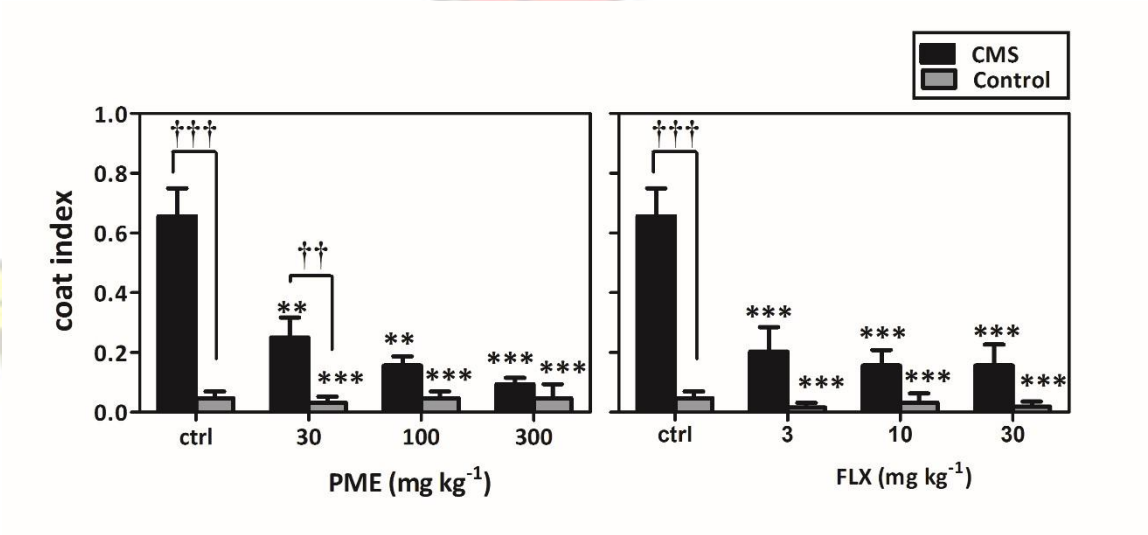


Figure 6.14 Effect of PME (30-300 mg kg^{-1}) and fluoxetine, FLX (3-30 mg kg^{-1}) treatments on coat state in control and CMS-exposed mice. Data are presented as mean±SEM (n=8). Significantly different from stress control: ** P<0.01, ***P<0.001 (One-way ANOVA followed by Newman-Keuls' test) and significant difference when CMS and control groups were compared: ††P<0.01, †††P<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

6.3.2.3 *Splash Test*

Total grooming frequency of both stressed and non-stressed mice were scored after 6 weeks of treatment. A significant difference was observed between non-stressed vehicle and stressed vehicle groups at the end of the unpredictable chronic mild stress regimen (P<0.05). Treatment of stressed mice with PME ($F_{4,35}$=3.145, P=0.026) and FLX ($F_{4,35}$=2.891, P=0.036) significantly increased the total grooming frequency in the splash test when compared to the CMS group. No significant difference was observed between drug-treated control mice and the vehicle-control group (P>0.05) (figure 6.15).

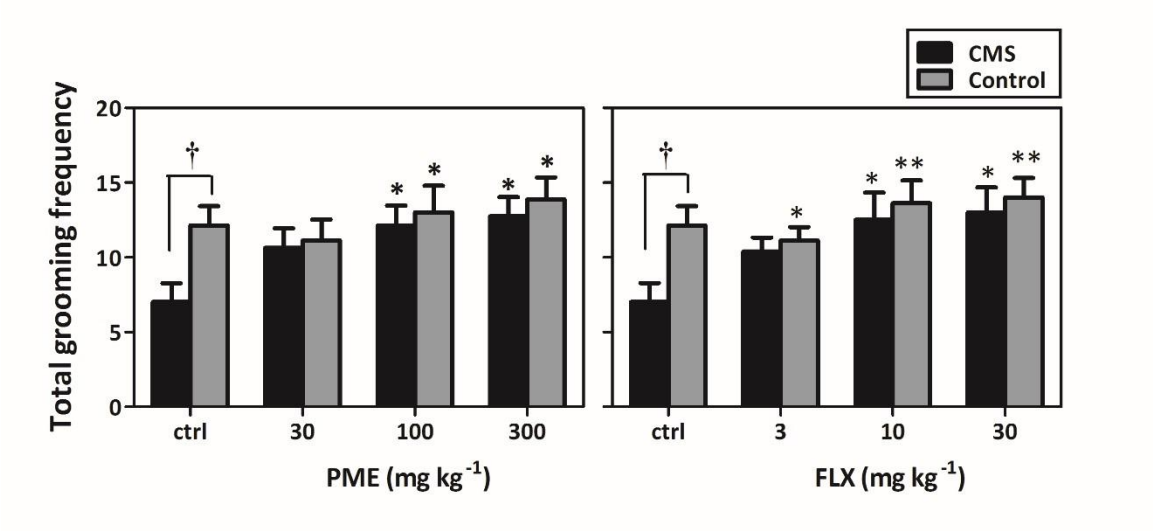


Figure 6.15 Effects of a 6 week treatment with PME (30-300 mg kg^{-1}) and fluoxetine, FLX (3-30 mg kg^{-1}) on the total frequency of the grooming behaviour during the splash test after the end of the chronic mild stress regimen. Data are expressed as group mean±SEM (n=8). Significantly different from CMS-control: **P*<0.05, ***P*<0.01 (One-way ANOVA followed by Newman-Keuls' *post hoc* test) and significant difference when CMS and control groups were compared: †*P*<0.05 (Two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

6.3.2.4 ***Open Field Test***

In table 6.1, Newman-Keuls' *post hoc* analysis showed that exposure of mice to CMS paradigm decreased central ambulation (*P*<0.001) and rearing (*P*<0.001) when compared to the naïve control group. However, chronic administration of PME to CMS-exposed mice significantly reversed the stress-induced behavioural alteration as observed by increased central ambulation ($F_{4,35}$=6.73, *P*=0.0004; Table 6.1) and rearing ($F_{4,35}$=6.012, *P*=0.0009; Table 6.1) as compared to the CMS group. Moreover, in table 6.1, daily administration of FLX to CMS mice exhibited an increase in central ambulation ($F_{4,35}$=6.819, *P*=0.0004) and rearing ($F_{4,35}$=4.278, *P*=0.0064).

The total activity (number of crossings) which is a measure of exploratory behaviour in the open field test was significantly decreased in CMS mice as compared to the saline-control group (*P*<0.05). After 6 weeks of PME treatment, a *post hoc* analysis showed no significant difference in total activity (*P*>0.05; compared to CMS or saline control groups).

Chronic treatment with fluoxetine also had no effect on total activity (*P*>0.05) (Table 6.1).

Table 6.1 Effects of PME and fluoxetine treatment on the behaviour of control and CMS mice in the open field test

Groups	**Dose**	**Central**	**Total**	**Rearing**

	(mg kg^{-1})	ambulation	activity	frequency
Ctrl		25.25±3.30***	122.3±10.25***	22.75±1.82***
CMS		8.38±1.51	74.0±5.32	7.00±1.13
PME (Ctrl)	30	24.63±2.59***	115.4±6.04***	19.75±2.22***
	100	25.50±1.75***	116.3±3.18***	20.13±2.22***
	300	26.63±3.01***	119.4±4.42***	22.88±1.89***
PME(CMS)	30	14.25±1.84*†	85.50±7.00	15.13±2.35*
	100	15.25±2.54*	87.50±13.71	19.00±4.21**
	300	18.13±2.29*	86.38±10.79	17.88±1.06**
FLX (Ctrl)	3	24.00±4.42***	112.50±11.28**	18.88±2.72**
	10	24.88±2.01***	114.10±4.99**	19.63±3.02**
	30	28.43±2.34***	129.00±10.33**	23.57±2.82***
FLX (CMS)	3	12.63±2.12††	84.50±11.60	17.13±4.16*
	10	15.88±2.32*	90.38±4.76	17.88±2.40*
	30	18.88±2.62*	88.13±16.69	17.00±3.30*

Key: Ctrl=control, CMS=chronic mild stress

Data are expressed as group mean±SEM (n=8). Significantly different from stress control: *P<0.05, **P<0.01, ***P<0.001 (One-way ANOVA followed by Newman-Keuls' test) and significant difference when CMS and control groups were compared: †P<0.05 (Two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

6.3.2.5 *Elevated Plus Maze Test*

In the EPM test, mice exposed to CMS were more anxious than the control group as observed by a decrease in % open arm entries and % time spent in open arms. This anxietylike behaviour was however reversed by daily administrations of PME where % open arm entries ($F_{4,35}$=3.592, P=0.014; figure 6.16a) and % time spent in open arms ($F_{4,35}$=9.967, P=0.010; figure 6.16c) were significantly increased. Newman-Keuls post hoc analysis also revealed an anxiolytic-like activity in PME treated non-stressed mice as compared to the saline control group by significantly increasing % entries and % time spent in the open arms at 300 mg kg^{-1} (both at P<0.05).

Fluoxetine administration in CMS mice also yielded similar results as PME [% open arm entries ($F_{4,35}$=3.554, P=0.015; figure 6.16b) and % time spent in open arms ($F_{4,35}$=9.497, P=0.0003; figure 6.16d)]. Chronic administration of FLX to non-stressed mice also showed an anxiolytic-like effect as compared to the saline control group [% open arm entries ($F_{4,34}$=9.923, P<0.0001) and % time spent in open arms ($F_{4,34}$=24.31, P<0.0001)].

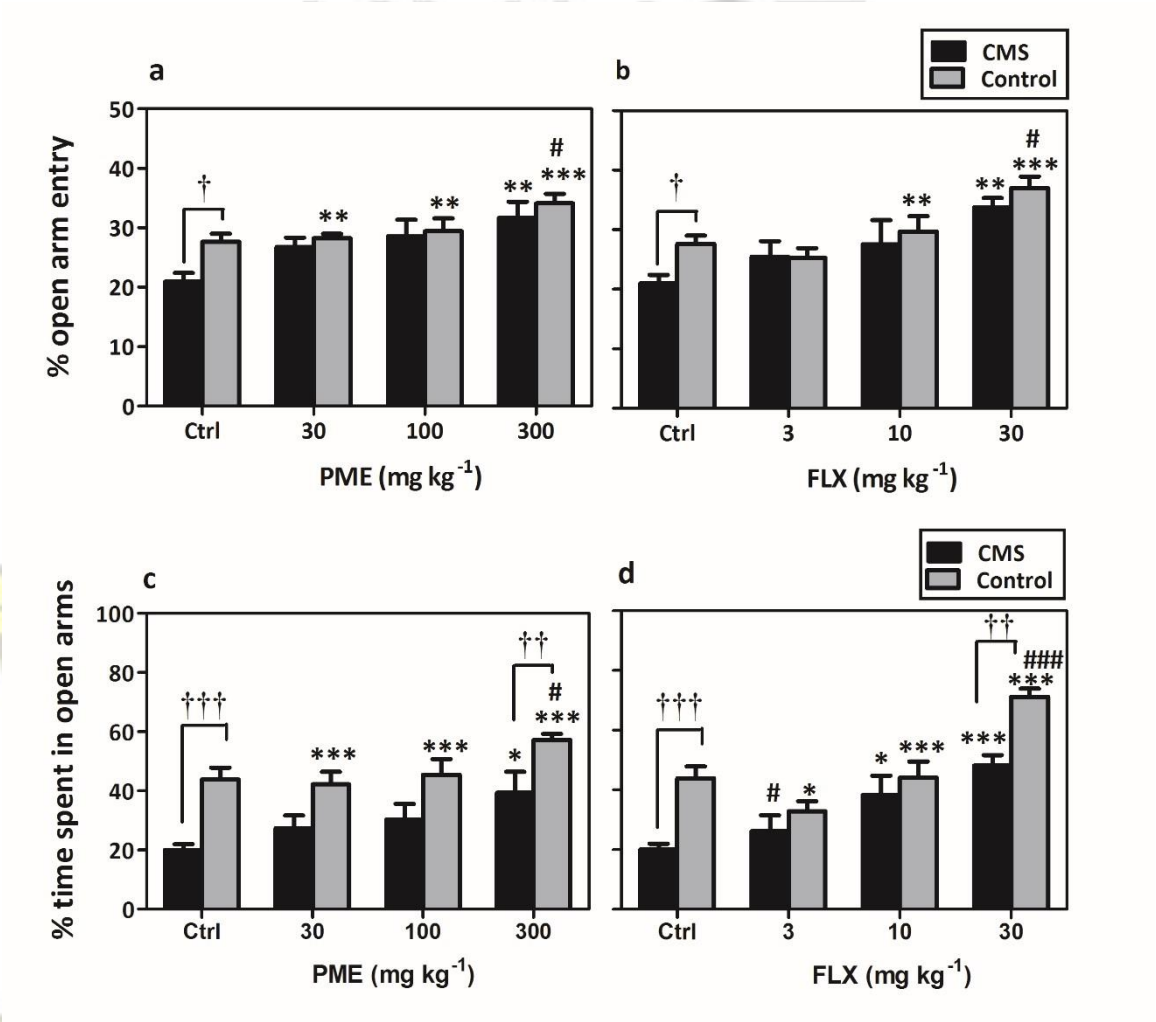


Figure 6.16 Effect of PME (30-300 mg kg^{-1}) and fluoxetine, FLX (3-30 mg kg^{-1}) treatments on the % open arm entries (a and b) and % time spent in open arms (c and d) in control and CMS-exposed mice. Data are presented as mean±SEM (n=8). Significantly different from stress control: * P<0.05, ** P<0.01, ***P<0.001 (one-way ANOVA followed by Newman-Keuls' test) and significant difference when CMS and control groups were compared: ††P<0.01, †††P<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test). #P<0.05: compared to saline-control group.

6.3.2.6 ***Forced Swimming Test***

Figure 6.17 shows the effect of PME and FLX treatment on the duration of immobility in mice FST. A significant increase in immobility time (P<0.01) was observed in mice subjected to CMS compared to the naïve controls. Chronic treatment with PME (30, 100 and 300 mg kg^{-1}, *p.o.*), however, significantly decreased the immobility duration

($F_{4,352}$=4.631, P=0.0042) in stressed mice as compared to the CMS-control group. Daily administration of PME in non-stressed mice decreased immobility time as compared to the naïve-control group with *post hoc* analysis revealing significance at 300 mg kg^{-1} (P<0.05). The antidepressant drug fluoxetine (3, 10 and 30 mg kg^{-1}, *p.o.*) also significantly reversed immobility duration ($F_{4,35}$=7.632, P=0.0001) in CMS mice when compared to the stressed group.

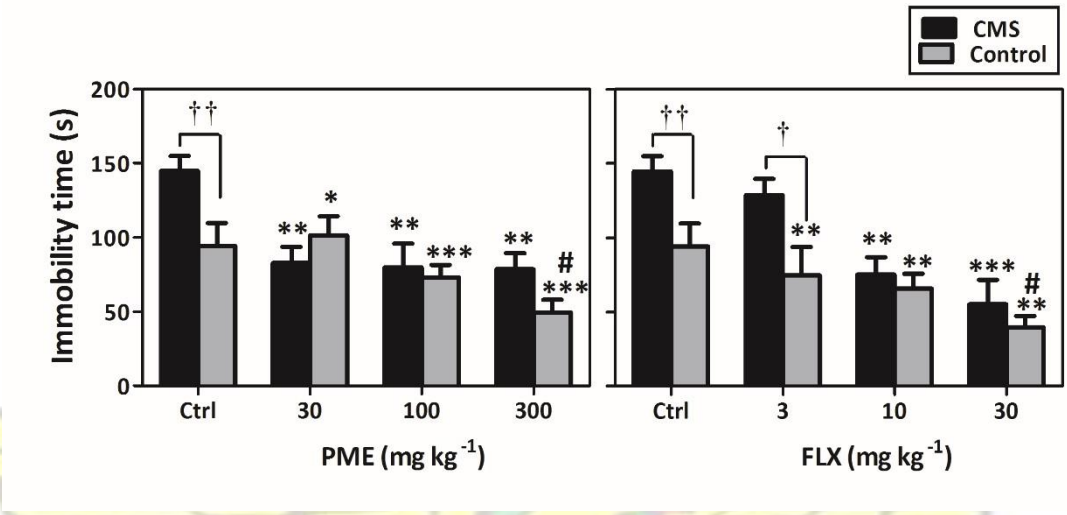


Figure 6.17 Effect of PME (30-300 mg kg^{-1}) and fluoxetine (3-30 mg kg^{-1}) treatments on immobility time in control and CMS-exposed mice in the FST. Data are presented as mean±SEM (n=8). Significantly different from stress control: * P<0.05, ** P<0.01, ***P<0.001 (one-way ANOVA followed by NewmanKeuls' test) and significant difference when CMS and control groups were compared: †P<0.05, ††P<0.01 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc*). #P<0.05: compared to saline control group.

6.3.2.7 *Tail Suspension Test*

CMS-induced depressive mice exhibited a significant increase in immobility duration as compared to the control group (P<0.05). However, administration of PME for six weeks significantly reversed the increased immobility time ($F_{4,35}$=3.872, P=0.010; figure 6.18) induced by CMS in mice. In non-stressed mice, PME significantly decreased immobility time as compared to the naïve-control group with *post hoc* analysis revealing significance at 300 mg kg^{-1} (P<0.05). Fluoxetine treatment also significantly decreased immobility duration ($F_{4,35}$=2.932, P=0.0343; figure 6.18) in CMS mice when compared to the stressed

group. A *post hoc* analysis showed a significant decrease in immobility time at 30 mg kg^{-1} (P<0.05) when FLX control mice were compared to saline-control group.

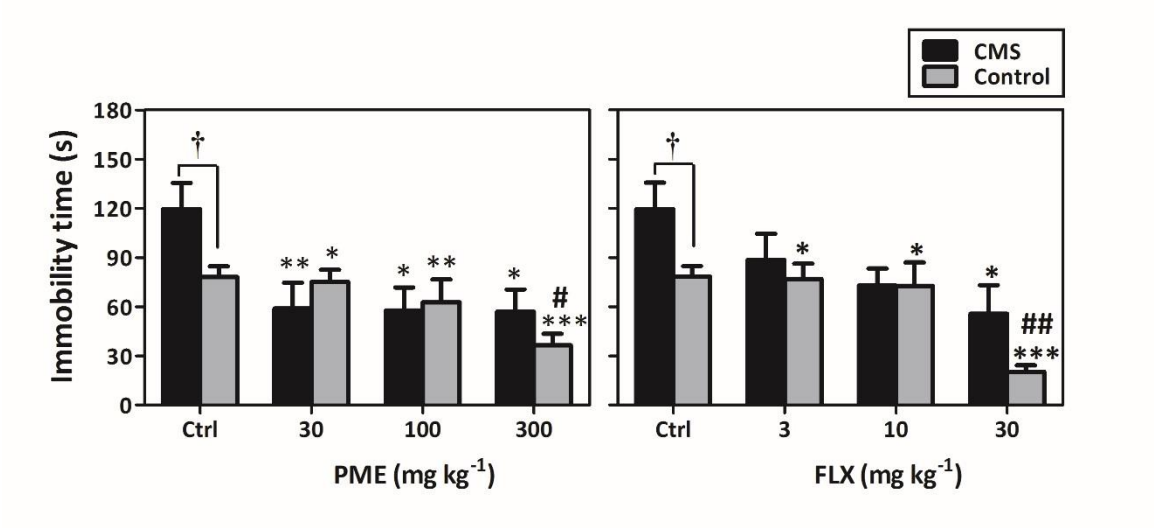


Figure 6.18 Effect of PME (30-300 mg kg^{-1}) and fluoxetine (3-30 mg kg^{-1}) treatments on immobility time in control and CMS-exposed mice in the TST. Data are presented as mean±SEM (n=8). Significantly different from stress control: *P<0.05, **P<0.01, ***P<0.001 (one-way ANOVA followed by NewmanKeuls' test) and significant difference when CMS and control groups were compared: †P<0.05 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test). #P<0.05, ##P<0.01: compared to saline-control group.

6.3.2.8 ***Novelty Suppressed Feeding Test***

In figure 6.19, exposure of mice to the CMS paradigm significantly prolonged feeding latency (P<0.05, compared to control). However, chronic treatment with either PME ($F_{4,35}$=3.42, P=0.018) or fluoxetine ($F_{4,35}$=3.024, P=0.030) significantly decreased latency to feed in the novel arena as compared to the CMS group. No difference was observed between treatment groups and saline control group (P>0.05).

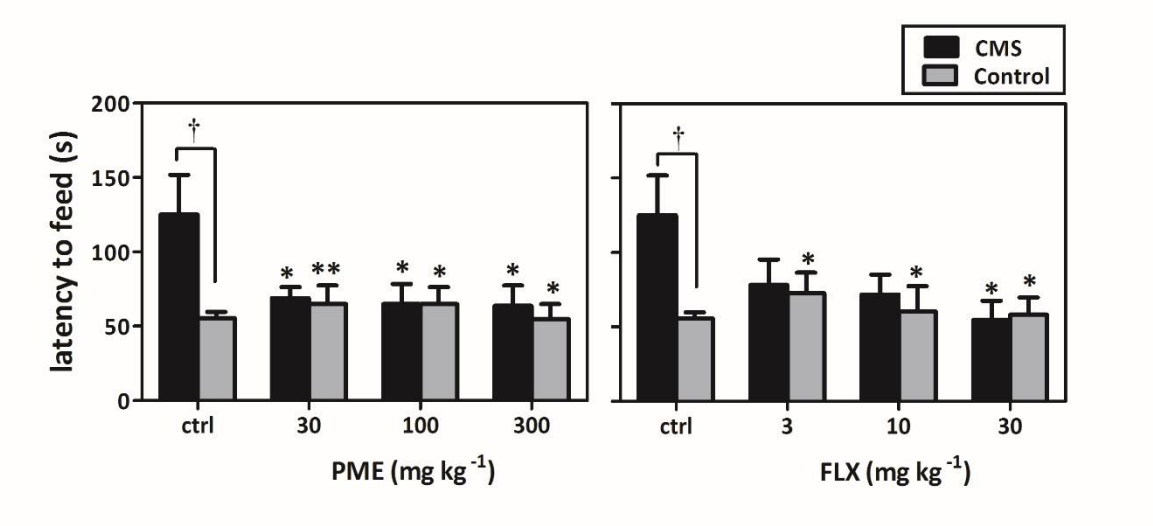


Figure 6.19 Effect of PME (30-300 mg kg^{-1}) and fluoxetine (3-30 mg kg^{-1}) treatments on the latency to feed in control and CMS-exposed mice in the NSF paradigm. Data are presented as mean±SEM n=8). Significantly different from stress control: * P<0.05, ** P<0.01 (one-way ANOVA followed by NewmanKeuls' *post hoc* test) and significant difference when CMS and control groups were compared: †P<0.05 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

6.3.2.9 ***Morris Water Maze Test***

Morris water maze (MWM) was performed in order to assess spatial learning and memory in CMS and control mice. As trial days progressed, mice exposed to CMS increased escape latency (decreased learning behaviour) as compared to the control group, reaching significance on the third day (P<0.05). However, administration of PME and fluoxetine attenuated the learning deficit induced by CMS. All groups showed no significant changes in escape latency during the first day as compared to the depressed control group (P>0.05).

Change in escape latency in all drug treated stressed mice decreased significantly following the training sessions, indicating that all mice showed some degrees of learning [PME: $F_{4,209}$=11.31, P<0.0001 (figure 6.20a) and fluoxetine: $F_{4,209}$=12.72, P<0.0001 (figure 6.20e); Two-way ANOVA (*treatment* x *time*)]. Moreover, a *post hoc* analysis revealed significant difference from the third trial for all treated groups (P<0.05) confirming good learning in mice exposed to various stressors. One-way ANOVA revealed a significant decrease in the change in escape latency for PME ($F_{4,35}$=6.693, P=0.0004; figure 6.20b) and fluoxetine ($F_{4,35}$=5.898, P=0.001; figure 6.20f) indicative of an improvement in learning behaviour as compared with CMS control mice.

In addition, spatial probe trial test was performed 24 h after the last training session to assess memory retention in mice. CMS mice showed an impaired cognitive performance

compared with control animals by significantly decreasing preference for the target quadrant (where the platform was previously placed during the training trials) (P<0.01). in figure 6.21, administration of PME ($F_{4,35}$=4.421, P=0.0054) and fluoxetine ($F_{4,35}$=4.496, P=0.0049) significantly increased the percentage time spent in the target quadrant indicative of an improved memory in stressed mice. Chronic PME and fluoxetine treatment showed no effects on spatial learning and memory in the non-stressed animals compared to control mice (P>0.05).

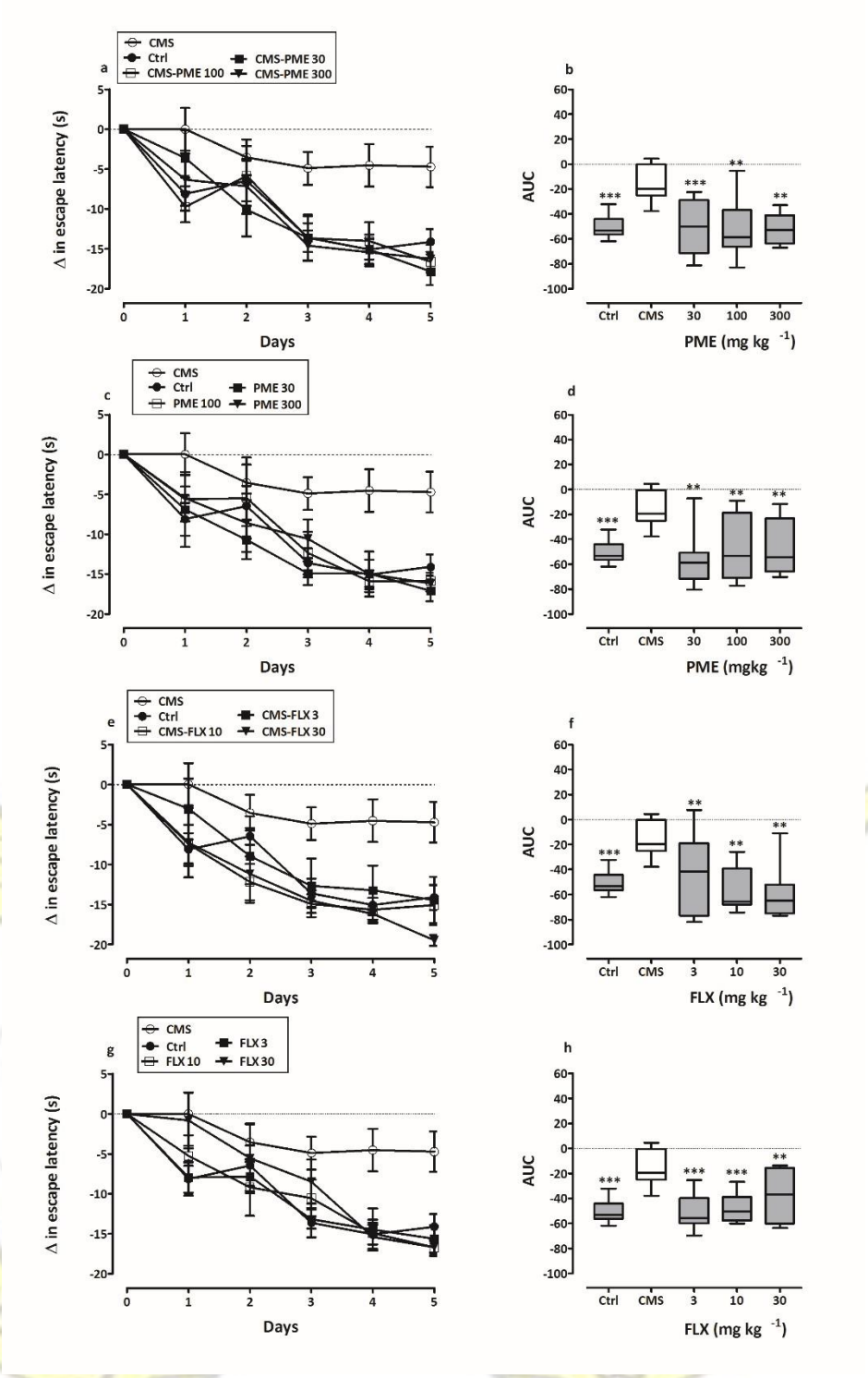


Figure 6.20 Performance of control mice and CMS-exposed mice chronically treated with PME (30-300 mg kg^{-1}) or fluoxetine, FLX (3-30 mg kg^{-1}) in the Morris water maze test. Data are presented as both time course curves (a, c, e and g) and mean±SEM (n=8) of their areas under the curves (AUCs) (b, d, f and h). Significantly different from stress control group: ***P*<0.01, ****P*<0.001 (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

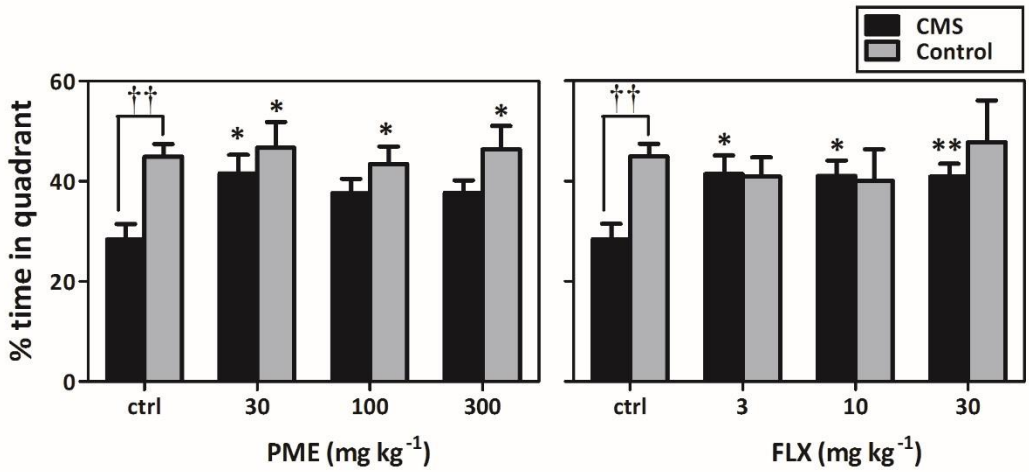


Figure 6.21 Effect of PME (30-300 mg kg^{-1}) and fluoxetine (3-30 mg kg^{-1}) treatments on the % time spent in target quadrant in control and CMS-exposed mice in the probe trial test. Data are presented as mean±SEM (n=8). Significantly different from stress control: * $P<0.05$, ** $P<0.01$ (One-way ANOVA followed by Newman-Keuls' test) and significant difference when CMS and control groups were compared: ††$P<0.01$ (Two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

6.3.2.10 *EPM Transfer Latency*

CMS-induced depressive mice significantly increased transfer latency (TL) on day 1 (learning) in the EPM as compared to the control group (P<0.05). However, treatment with PME ($F_{4,35}$=2.907, P=0.035; figure 6.22a) and FLX ($F_{4,35}$=3.136, P=0.026; figure 6.22b) significantly decreased TL in CMS mice as compared to the CMS control group. On day 2 (memory), the decreased TL observed in CMS mice was significantly reversed by chronic treatment with PME ($F_{4,35}$=3.181, P=0.0249; figure 6.22c) and FLX ($F_{4,35}$=2.836, P=0.038; figure 6.22d).

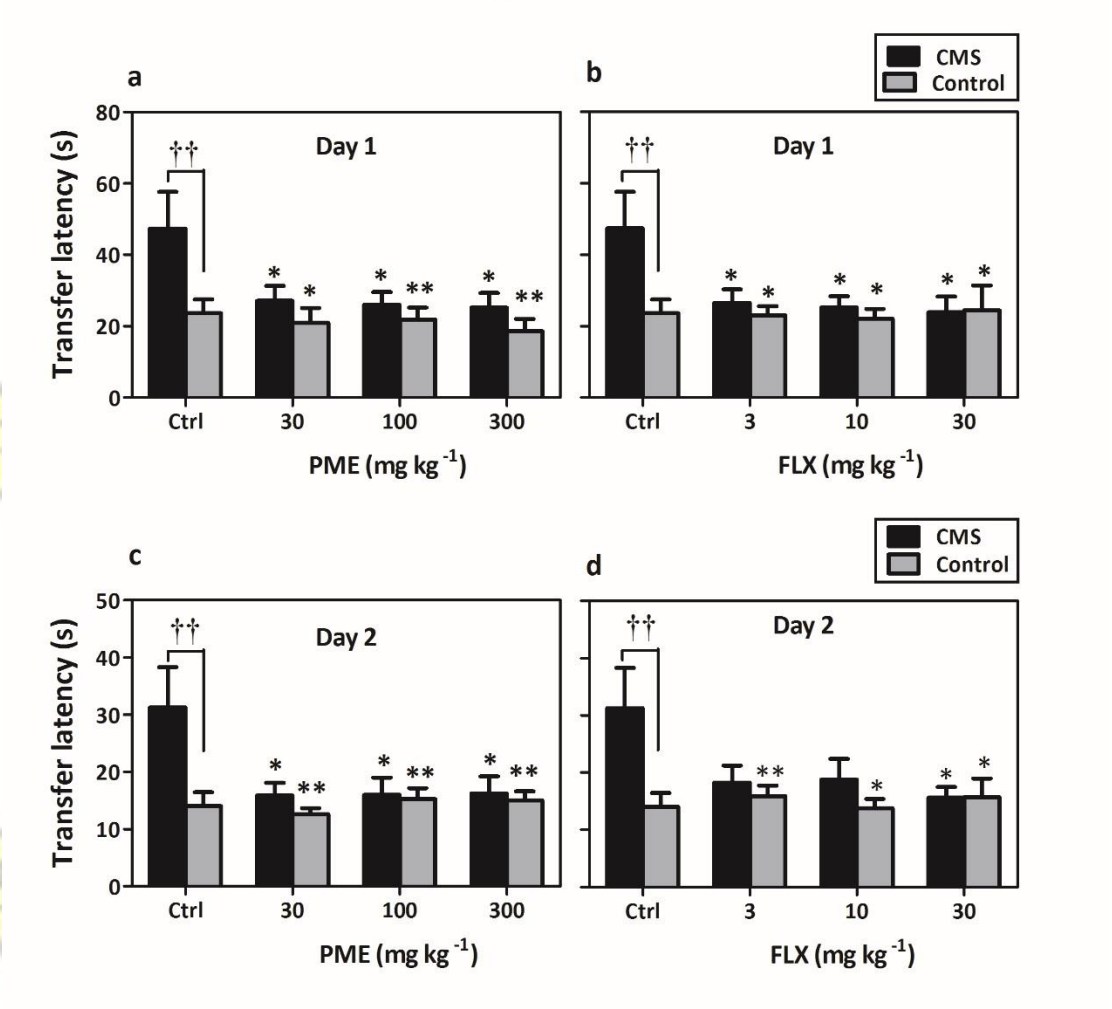


Figure 6.22 Performance of control mice and CMS-exposed mice chronically treated with PME (30-300 mg kg^{-1}) or FLX (3-30 mg kg^{-1}) in the EPM transfer latency test. Data are presented as mean±SEM (n=8). Significantly different from stress control: * P<0.05, ** P<0.01 (one-way ANOVA followed by NewmanKeuls' *post hoc* test) and significant difference when CMS and control groups were compared: ††P<0.01 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

6.4 DISCUSSION

Depression is a chronic condition, thus the effect of the extract in chronic models of depression was investigated. Results from the present study showed that depressive behaviour as induced in the repeated open-space swim test significantly impairs learning and memory. In addition, the long-term behavioural effects of mice exposed to CMS induced persistent altered behavioural profile including anhedonia, depression in the forced swimming test and TST and anxiety (EPM, OFT and NSF). Moreover, cognitive impairments in the MWM and transfer latency tests were observed. These behavioural alterations induced by chronic stress was prevented by treatment with *P. microcarpa*, suggesting that this plant could protect against the detrimental effects of chronic depression.

Mice showed a progressive decrease of active swimming and a corresponding increase in floating behaviour with repeated open-space swims that persisted for several weeks with occasional repeated swims. Moreover, the increased inactivity was reversed selectively by the extract and the classical antidepressant drugs (desipramine and fluoxetine), in that they inhibited the increased floating behaviour. Further evidence for a depressing effect of repeated open-space swimming came from the tail suspension test. Mice subjected to this procedure were found to show a higher level of immobility on the tail suspension test. This immobility is known to be characteristic of rodent models of depression. However, this effect was significantly reversed by chronic treatment with the extract and the classical antidepressants, fluoxetine and desipramine.

Results of this study also show that administration of an established antidepressant eliminates the lag time for its behavioural effects in a model of chronic behavioural depression based on repeated open-space swimming in mice. Previous experiments have demonstrated that the reversal of immobility and inactivity in this mouse model by several different types of antidepressant drugs, given peripherally, requires 10–14 days administration (Sun and Alkon, 2004; Stone *et al.*, 2008; Lin *et al.*, 2011). In confirmation of this, 2 weeks of daily doses of a classical antidepressant treatment, starting 24 h after the depressive behaviour was induced, were needed to produce a significant improvement of mobility in the open space swim test, a response mimicking the time course in human antidepressant therapy. In addition, the results indicate that the open space swim test induces a depressive behaviour that is not only lasting but also similar in the time course

of clinical effectiveness during antidepressant treatment in humans. In contrast, a single administration of the extract was found to produce an immediate and significant reduction in immobility together with an increase in distance swum. This finding indicates that PME has a rapid and sustained antidepressant action giving it an advantage over the classical antidepressants.

Research has shown that a single sub-anesthetic dose of ketamine (NMDA receptor antagonist), when given intravenously induces a rapid (within two hours) and sustained (1–2 weeks) antidepressant effect in patients with treatment-resistant major depression (Zarate *et al.*, 2006; Bunney and Bunney, 2012). It has been hypothesized that the rapid onset of ketamine and its sustained effects are the result synaptic potentiation resulting from an increase in AMPA relative to NMDA glutamatergic throughput and early neuroplastic changes respectively (Du *et al.*, 2006; Machado-Vieira *et al.*, 2008; Zarate *et al.*, 2010). Ketamine is known to increase presynaptic release of glutamate (Moghaddam *et al.*, 1997), and this preferentially favours AMPA receptors over NMDA as the latter type receptors are blocked (Maeng *et al.*, 2008). This results shows an enhanced glutamatergic throughput of AMPA relative to NMDA which leads to synaptic potentiation and antidepressant effects (Maeng *et al.*, 2008). In addition, AMPA potentiators (AMPAkines) have antidepressantlike properties in animal models (Li *et al.*, 2001). Similar to the results obtained for ketamine, glycine site NMDA receptor antagonist or partial agonists also possess rapid and sustained antidepressant effects in animal models as well as in clinical trials (Zhu *et al.*, 2013; Shin *et al.*, 2014).

The antidepressant-like activity of PME has been shown to involve the glycine site of the NMDA receptor complex. Therefore, the rapid and sustained antidepressant effect observed for PME in the repeated open-space forced swim procedure could possibly be through its interaction with NMDA receptors. Thus,it could be suggested that modulation of the glutamatergic system may be a critical therapeutic target for obtaining rapid antidepressant actions.

Chronic administration of various uncontrollable stressors in an unpredictable manner is a well-documented animal model for the preclinical evaluation of antidepressants (Willner *et al.*, 1992; Kumar *et al.*, 2011). The chronic mild stress (CMS) model has been shown to induce lower consumption of sucrose (sweet food) postulated to reflect anhedonia (the loss of interest or pleasure) in animals, one of the core symptoms required for diagnosis of a

major depressive episode in humans (Katz, 1982; Willner, 1997; Wang *et al.*, 2009; Wiborg, 2013). Interestingly, treatment with PME or fluoxetine was able to reverse the anhedonic behaviour. Anhedonia is a symptom of depression, thus it can be suggested that the administration of PME or fluoxetine exerted an antidepressant effect. Results of this study confirm earlier reports that chronic sequential exposure to a variety of mild stressors causes a substantial decrease in the consumption of 1 % sucrose solution, and that this deficit can be effectively reversed by chronic treatment with traditional antidepressant drugs (Papp *et al.*, 2003; Muscat *et al.*, 1992; Bessa *et al.*, 2009b).

Several reports have shown that reversal of CMS-induced anhedonia typically requires 3–4 weeks of treatment, which closely resembles the clinical time course of antidepressant action (D'Aquila *et al.*, 1997; Kubera *et al.*, 2001; Strekalova *et al.*, 2006; Elizalde *et al.*, 2008). Moreover, it has been observed that action of antidepressants in the CMS is similar to their clinical activity, with regards to their efficacy (full recovery at the end of treatment period) and specificity (lack of significant effects in control animals) (Willner, 1997; Papp *et al.*, 2003). In the present study, sucrose intakes in stressed animals receiving PME was reversed within the first 2 weeks of treatment, compared to 4 weeks required by fluoxetine. This therefore indicates a rapid onset of action for PME than that usually observed following chronic administration of traditional antidepressants. Furthermore, the rapid onset of action of PME was sustained for the entire treatment period. This rapid and sustained antidepressant-like effect of PME further confirms the previous observeation in the open space-swim model of chronic depression. As indicated, this rapid and sustained effect could be due to the interaction of the extract with NMDA receptors since various reports have shown glycine site NMDA antagonists possess rapid antidepressant effects in the CMS (Zhu *et al.*, 2013; Sowa-Kucma *et al.*, 2008; Shin *et al.*, 2014).

The FST and TST are two commonly used behavioural tests to predict efficacy of antidepressant treatments. An increase in immobility time in these models is indicative of a depression-like effect. (Bourin *et al.*, 2005; Cryan *et al.*, 2005a; Petit-Demouliere *et al.*, 2005). In this study, CMS increased immobility time of mice in the FST and TST indicating depression in these animals. This is consistent with previous findings in which mice increased immobility time after chronic exposure to various stressors in the CMS (Vitale *et al.*, 2009; Kumar *et al.*, 2011; Zhang *et al.*, 2014; Liu *et al.*, 2013). However, treatment with PME and FLX significantly reversed the increased immobility induced by CMS which

indicates antidepressant-like effect. Moreover, it was observed that PME decreased the duration of immobility in mice FST and TST (Section 5.3).

Signs of anxiety are often present in both depressed patients and animal models of depression and many antidepressant drugs have anxiolytic properties (Nutt, 2005; Bessa *et al.*, 2009a). Several studies have also shown that exposure of mice to the CMS paradigm induces anxiety (Nirmal *et al.*, 2008; Ma *et al.*, 2011). Therefore, the EPM, OFT and novelty-suppressed feeding paradigms were performed in order to assess anxiety. The open field test is a classical approach/avoidance paradigm in which the novel environment concurrently evokes both anxiety and exploration (Prut and Belzung, 2003; Dulawa *et al.*, 2004). An increase in activity or time spent in the center of the open field indicates reductions in anxiety and/or increases in exploration (Dulawa *et al.*, 2004). In this test, CMS mice exhibited decreased central entries and rearing which indicates increased anxiety and reduced exploration. Moreover, CMS application in the present study decreased activity in the OFT study after repeated stressor exposure, which is in agreement with previous findings (Mattioli *et al.*, 2009; Rygula *et al.*, 2006; Wang *et al.*, 2009). Treatment with PME and fluoxetine reversed the altered open field behaviour by significantly increasing central ambulation and rearing probably due to its anxiolytic effect. Furthermore, the decreased locomotor activity caused by CMS was prevented by long-term treatment with PME and FLX. This finding is consistent with previous reports that strongly suggests chronic fluoxetine treatment decreases anxiety and/or increases exploration in mice (Dulawa *et al.*, 1999; 2004).

Coat state assessment has been indirectly used to evaluate animal grooming activity (Mineur *et al.*, 2003; Yalcin *et al.*, 2005). Chronically stressed depressed mice generally display poor coat status, whereas antidepressant treatments tend to reverse this phenotype (Smolinsky *et al.*, 2009; Ducottet *et al.*, 2003). The splash test is both a direct measure of grooming, and an indirect evaluation of sucrose intake (Willner, 2005). As expected, the CMS regimen used in this study induced degradation of the coat state and decreased grooming behaviour in the splash test in stressed mice. However, treatment with PME and fluoxetine increased total grooming frequency and improved coat state indicating antidepressant activity. Results of this study are consistent with reports in which antidepressants are able to prevent the effects of the unpredictable chronic mild stress on the state of the coat and/or the splash test (Yalcin *et al.*, 2005; Detanico *et al.*, 2009; Piato *et al.*, 2008).

As a widely used model, the EPM provides an independent measure of anxiety-like behaviour (percentage of entries or time spent on the open arms) in rodents (File, 2001; Carobrez and Bertoglio, 2005; Rodgers, 2010), and increased anxiety-like behaviour in this test has been observed after CMS (Griebel *et al.*, 2002; Maslova *et al.*, 2002; Bessa *et al.*, 2009b). Animals exhibiting anxiety-like behaviour naturally avoid the open arms of the EPM, and anxiolytic compounds typically increase open arm exploration (Carobrez and Bertoglio, 2005; Spiacci Jr *et al.*, 2008). In the present study, animals exposed to CMS displayed significant decrease in the percentage entries and time spent in the open arms of the EPM, suggesting an anxiety-like state. However, chronic treatment with PME and FLX significantly reversed this anxiogenic behaviour indicating an anxiolytic-like effect.

Novelty suppressed feeding test was employed to further assess the anxiolytic action of PME in CMS and control mice. Hyponeophagia refers to the inhibition of feeding produced by exposure to novelty. Anxiolytic treatments decrease the latency and increase consumption, while anxiogenic treatments increase the latency and decrease consumption in the novel environment (Dulawa and Hen, 2005). CMS animals increased (anxiogenic effect) whereas PME and FLX decreased (anxiolytic effect) feeding latency. Data obtained is in line with previous reports showing increased hyponeophagia in rodents exposed to unpredictable chronic mild stress (Bessa *et al.*, 2009b; Sun *et al.*, 2013). Reversal of the CMS-induced hyponeophagia by chronic treatment with FLX is in agreement with previous studies in which chronic treatment with antidepressants is required to produce anxiolytic effects (Dulawa *et al.*, 2004; Santarelli *et al.*, 2003; Bodnoff *et al.*, 1989).

The concurrence and interrelationship of depression and cognitive impairment in humans are striking features of these two disorders. Several possible interactions may exist between depression and dementia, for instance increasing cognitive impairments may induce depression, and dementia may also occur as a symptom of depression (Payne *et al.*, 1998; Zubenko *et al.*, 2003; Song *et al.*, 2006). Thus, the effects of PME on cognitive function in the Morris water maze task was asssessed in the chronic models of depression.

The Morris water maze (MWM) task is a well-validated method for evaluating learning and memory. It can reliably express hippocampus-related acquisition and the persistence of spatial memory (Ibi *et al.*, 2008; Clark *et al.*, 2007). Learning and memory is measured as a decreased latency to discover the hidden platform across sessions or as time spent in the area of the platform during a test session in which the platform has been removed

(Kenney and Gould, 2008). Data from this study indicates that the depressive-behaviour induced by the repeated open-space swim test impairs hippocampal-dependent spatial learning and memory performance. This finding is in agreement with a study showing similar deficits in spatial learning and memory following repeated open-space forced swim procedure (Sun and Alkon, 2004). The learning ability was mainly reflected by the performances in place navigation section. Results in this study revealed that with the increase of training days, escape latencies were consistently decreased and there was prominent improvement during the last three days between treated and depressed groups. Furthermore, the persistence of spatial memory was mainly reflected through mouse performances in spatial probe trial. It was observed that numbers of platform crossing and quadrant dwell time were markedly decreased in depressed mice. However, treatment with the extract and the classical antidepressants ameliorated these defects efficiently. In the elevated plus maze, an increase in transfer latency on day 1 and day 2 is indicative of impaired learning and memory respectively, whereas a decrease shows improved cognitive function. Learning and memory performance in both Morris water maze and elevated plus maze were significantly impaired in CMS animals. Results of this study provide direct evidence that CMS significantly impairs spatial learning and memory (Elizalde *et al.*, 2008; Bessa *et al.*, 2009b). However, repeated treatment with PME and FLX significantly improved cognitive performance in both mazes indicating therapeutic efficacy against chronic stress-induced memory impairment. Lines of evidences have suggested that impaired cognition is an element of depression and that antidepressant therapy may improve the cognitive function (Meneses, 2002; Yau *et al.*, 2002; Song *et al.*, 2006).

Several reports have shown that the 5-HT system plays an important role in cognitive functions, such as learning and memory, demonstrated through the activation or blockade of 5-HT receptor subtypes as well as its reuptake sites (Buhot *et al.*, 2000; Meneses, 2007; King *et al.*, 2008; Mørk *et al.*, 2013). For example, it was observed that fluoxetine and tianeptine reversed memory impairments induced by scopolamine (a cholinergic antagonist) and dizocilpine (a glutamatergic antagonists) (Meneses and Hong, 1995; Meneses, 2002). Furthermore, although blockade of NMDA receptors leads to impairment of neuronal plasticity (learning) (Collingridge and Bliss, 1995), studies have also indicated cognitive enhancing effects (Si *et al.*, 2004; Parsons *et al.*, 2007; Cole *et al.*, 2013). For instance, memantine, a non-competitive NMDA receptor antagonist, has demonstrated cognitive and behavioural improvements in both humans (Gauthier *et al.*, 2005; Peskind *et*

al., 2006; Schulz *et al.*, 2011) and animals (Pietá Dias *et al.*, 2007; Minkeviciene *et al.*, 2008; Borre *et al.*, 2012). Therefore, the reversal of chronic depression-induced memory deficits by PME could possibly be due to its interaction with the 5-HT system and NMDA receptor complex.

Although pretreatment with PME and the classical antidepressants improves spatial learning and memory of depressed mice in the repeated open-space procedure, it is necessary to deduce whether altered acquisition reflects impairment of learning or memory. Analysis of swimming velocity to reach the platform revealed no differences between depressed and treated animals, ruling out any non-specific effects of induced-depressive behaviour on spatial acquisition and memory. This finding demonstrate that improvement of spatial learning and memory by PME and the classical antidepressants in depressed mice are not due to any nonspecific changes in gross motor activity or motivational state.

Significant weight gain or loss is a common depressive symptom (Berton and Nestler, 2006; Wang *et al.*, 2013). The present study showed that chronic mild stress could cause significant weight loss in mice. Previous studies have reported that chronic stress alters the rate of weight gain, particularly in male rats (Duncko *et al.*, 2001; Faraday, 2002; Dalla *et al.*, 2005). This reduction in body weight gain is a reliable index of the stress experience (Willner *et al.*, 1992; Konkle *et al.*, 2003; Mattioli *et al.*, 2009). Chronic administration of PME however increased body weight gain in the stressed mice without having any effect in control animals. It was observed that FLX treatment also improved weight gain in stressed animals. However, although not significant, FLX had the potential to decrease body weight of unstressed mice with increasing dose. Previous reports have demonstrated that sub chronic or chronic fluoxetine treatment reduced weight gain in non-depressed rodents (Yen *et al.*, 1987; Wong *et al.*, 1988; Yen and Fuller, 1992; First *et al.*, 2011) or humans (Levine *et al.*, 1987; Ferguson and Feighner, 1987; Goldstein *et al.*, 1993).

6.5 CONCLUSION

The present study provides evidence that chronic administration of *P. microcarpa* extract results in inhibition of the behavioural changes induced by chronic exposure to mild stressors. Moreover, it exhibits rapid and sustained antidepressant effect probably through its interaction with NMDA receptor complex. The finding of the present study also suggest that modulation of the glutamatergic system may be a critical therapeutic target for obtaining rapid antidepressant actions.

Chapter 7 ANXIOLYTIC ACTIVITY

7.1 INTRODUCTION

Anxiety disorders represent a frequent and clinically important comorbid disorder in epilepsy patients (Vazquez and Devinsky, 2003). Available evidence strongly suggests that epilepsy patients have a higher prevalence of anxiety disorders than controls, in both hospital and community samples (Scicutella and Ettinger, 2002; Vazquez and Devinsky, 2003). GABA is the principal inhibitory neurotransmitter of the brain and drugs that stimulate $GABA_A$ receptors, such as benzodiazepines (BDZs) and barbiturates, have anxiolytic and antiseizure effects via $GABA_A$-mediated reduction of neuronal excitability, which effectively raises the seizure threshold (Mula *et al.*, 2007). In the Irwin test, mice treated with PME showed signs of sedation suggesting possible central depressant effects. Furthermore, presence of sedation in the Irwin test suggests anxiolytic, antipsychotic, or anticonvulsant activity (Roux *et al.*, 2005). In addition, the extract has anticonvulsant effect probably by interacting with GABA receptors and could possess anxiolytic-like properties. Therefore, this study further explored the anxiolytic effect of PME in various classical models of anxiety.

7.2 MATERIALS AND METHODS

7.2.1 Animals

Male ICR mice were used in this experiment and experimental conditions were as described in section 3.2.1.

7.2.2 Drugs and Chemicals

Pentylenetetrazole (Sigma-Aldrich Inc., St. Louis, MO, USA); diazepam (INTAS, Gujarat, India).

7.2.3 Elevated Plus Maze (EPM) Test

This test has been widely validated for measuring anxiolytic and anxiogenic-like activities in rodents (Pellow *et al.*, 1985; Lister, 1987). The apparatus was made of Plexiglas and consists of two open (30 cm × 5 cm) and two closed (30 cm × 5 cm × 15 cm) arms, extending from a central platform (5 cm × 5 cm) and elevated to a height of 60 cm above the floor in a lit room (~750 lux). A rim of Plexiglas (0.5 cm in height) surrounded the

perimeter of the open arms to provide additional grip and thus prevented the mice falling off (Rodgers and Johnson, 1995).

Mice were randomly assigned to ten experimental groups (n=5): vehicle-control, PME (30, 100 or 300 mg kg^{-1}, *p.o.*), diazepam (0.1, 0.3 or 1.0 mg kg^{-1}, i.p.) and pentylenetetrazole (3, 10 or 30 mg kg^{-1}, i.p.). Diazepam and pentylenetetrazole served as reference anxiolytic and anxiogenic drugs respectively. Thirty minutes after intraperitoneal injection and 1 h after oral administration, mice were placed individually in succession in the central platform of the maze for 5 minutes and their behaviour videotaped with a camcorder (Everio™, model GZ-MG1300, JVC, Tokyo) placed above the maze. Behavioural parameters were scored from the videos using the public domain software JWatcher™ Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sidney, Australia available at http://www.jwatcher.ucla.edu/.) as follows: (1) Number of entries and time spent in each arm i.e. closed and open arms, (2) Number and duration of protected and unprotected stretch-attend postures, (3) Number and duration of protected and unprotected head dipping.

Entry into an arm was defined as the animal placing all four paws into the arm. Protected head dipping was defined as the mouse stretching to dip its head into the open space and observing the environment with the body remaining in a closed arm or the central platform while in unprotected head dipping, the mouse dips its head into the open space and observing the environment with the body being in an open arm. Protected stretch-attend postures were defined as the mouse stretching forward and retracting without moving forward its feet whilst in the closed arm or central platform of the maze whereas unprotected stretch-attend postures were defined as the mouse stretching forward and retracting without moving forward its feet whilst in the open arm.

To compute total distances travelled by the mice, the software Behaviour Collect (http://cas.bellarmine.edu/tietjen /DownLoads/computer_programs_for_data_colle.htm) was used to obtain raw XY data from the videos. These data were then exported into Microsoft® Office Excel 2007 and further analyzed. Distance between two X-Y coordinate pairs was calculated from the formula: $\sqrt{[(X_1-X_2)^2 + (Y_1-Y_2)^2]}$.

7.2.4 **Light/Dark Box (LDB) Test**

Anxiety-related behaviour was further tested in the light–dark exploration test as described by Crawley and Goodwin. (1980) with modifications. The apparatus was a wooden box (36 cm long × 33 cm wide × 30 cm deep) divided into two compartments by a wooden board with a small opening (8 cm × 8 cm) connecting the compartments. The larger compartment comprised two-thirds of the apparatus, painted white, open and illuminated by a 60-W lamp placed 50 cm above the compartment. The smaller compartment was painted black and had a cover that was closed during testing. Male ICR mice were divided into ten groups (n=5) and treated with PME, diazepam, PTZ and the vehicle as described above for the elevated plus-maze test. At the beginning of the experiments, mice were placed individually at a far corner of the dark compartment facing the light compartment and videotaped with a digital video camera for a period of 5 minutes. Behaviours of the animals from the videos were analyzed for the following parameters: (1) The latency to emerge from the dark compartment with all four paws into the light compartment, (2) Total time spent in each compartment, and (3) Total number of transitions between the compartments.

7.2.5 **Open-Field Test**

The test was based on that described previously by other workers (Sakina *et al.*, 1990; Woode *et al.*, 2010). Testing was conducted in clear Plexiglas boxes (40 cm × 40 cm × 30 cm) whose floor was divided into 16 equal squares by black lines. For behavioural analysis, the arena of the open field was designated as (i) corner (one of the four corner squares); (ii) periphery (the squares along the walls); or center (the four inner squares). The animals were divided into ten groups of five animals each, and received treatments as described above in the elevated plus maze test. Thirty minutes after i.p. and 1 h after oral administration of the test compounds, mice were placed individually in the centre of the open field and allowed to explore freely for 5 minutes. Each session was recorded with a video camera suspended approximately 100 cm above the arena. Behavioural assessment in the open field was analyzed for number of entries as well as duration of stay in the individual zones. Total distances travelled by the mice were determined as described above in the EPM test.

7.2.6 **Social Interaction Test**

Test for sociability and preference for social novelty was conducted as previously described (Moy *et al.*, 2004; Crawley, 2004). The apparatus comprised a rectangular, threechambered box. Each chamber was 20 cm × 40 cm × 22 cm and the dividing walls were made from clear Plexiglas, with small square openings (5 cm × 3 cm) allowing access into each chamber. An animal was placed in the middle chamber with the dividers closed to allow it to explore the middle chamber for five minutes. After this five-minute habituation period, an unfamiliar male (stranger 1) that had no prior contact with the subject mouse was placed in one of the side chambers. Placement of stranger 1 in the left or right side chambers was systematically alternated between trials. The stranger mouse was enclosed in a small, circular wire cage (11 cm high, with a bottom diameter of 9 cm and bars spaced 0.5 cm apart) that allowed nose contact between the bars, but prevented the stranger mouse from initiating any social contact and limited the possibility of aggressive interactions. The subject mouse was first placed in the middle chamber and allowed to explore the entire social test box for 10-min which was video-taped. Measures were taken of time spent and entries into the chamber containing the unfamiliar mouse in a wire cage (stranger side) and the chamber containing only the empty wire cage on the opposite side of the apparatus (empty side) for 10 minutes. An entry was defined as all four paws in one chamber. The duration and number of direct (active) contacts between the subject mouse and the containment cup housing or not housing the Stranger 1 mouse, for each chamber was also analyzed. Direct contact between the subject mouse and the containment cup, or stretching of the body of the subject mouse in an area 3-5 cm around the cup is counted as an active contact or sniffing.

A 10-minute test to quantitate preference for social novelty began immediately after the 10minute test for sociability. The original stranger mouse (stranger 1) remained in its wire cage on one side of the apparatus. A new unfamiliar mouse (stranger 2) was placed in the wire cage on the opposite side, which was previously empty during the sociability test. Identical measures as previously described were scored: time spent in each chamber, entries between chambers and time spent sniffing each wire cage. Stranger 1 and stranger 2 animals originated from different home cages and had never been in physical contact with the subject mice or each other. After each testing day, the wire cages and apparatus were wiped down with 70 % ethanol and allowed to air-dry. In this test, male ICR mice

were divided into ten groups (n=7) and treated with PME, diazepam, PTZ and the vehicle as described above for the elevated plus-maze test.

7.2.7 **Stress-Induced Hyperthermia (SIH)**

This test is based on the principle that mice have a natural hyperthermic response to stress, which reflects the level of anxiety. Test procedure for the modified stress-induced hyperthermia was adopted from Van der Heyden *et al.* (1997). The test involves two measures of rectal temperature repeated in the same animal with a 10-minute interval. Mice were singly housed in smaller cages (26 cm x 21 cm x 14 cm) for 24 h before testing with free access to food and water.

On the morning of the experiment, animals were first divided into 7 groups (n=10) and treated with PME (30, 100 or 300 mg kg^{-1}, *p.o.*), diazepam (0.1, 0.3 or 1.0 mg kg^{-1}, i.p.) or normal saline. Thirty minutes after intraperitoneal injection and 1 h after oral administration, each animal was removed from the holding cage and rectal temperature was measured to the nearest 0.1 °C by an ELLAB instruments (Copenhagen, Denmark) thermometer via a lubricated thermistor probe (2 mm diameter) inserted 20 mm into the rectum while the mouse was hand held near the base of the tail. The probe was left in place until steady readings were obtained. This temperature was recorded as the baseline rectal temperature (T_1). The animal was immediately placed back to the holding cage and after a 10-min interval the 2nd rectal temperature (T_2) was taken using the same procedure as in measuring T_1. Stress-induced hyperthermia was assessed as the difference between the second measurement and the first measurement. The first measurement was used to evaluate whether the test compound by itself would have a potential effect on basal body temperature.

7.2.8 **Beam Walk Test**

The test was carried out as described previously (Stanley *et al.*, 2005). Mice were trained to walk from a start platform along a ruler (80 cm long, 3 cm wide) elevated 30 cm above the bench by wooden supports to a goal box (enclosed hamster house). Three trials were performed for each mouse, and were designed such that the mice tested would be aware that there was a goal box that could be reached. A ruler was used because the mice found this easy to cross and, at the same time, it induced minimum anxiety.

On the day of the experiment, mice were randomly divided into eight groups (n=5): salinetreated control group; diazepam group (0.1, 0.3, 1 or 3 mg kg^{-1} i.p.) and PME group (30, 100, or 300 mg kg^{-1}, *p.o.*). Mice were placed on the beam at one end and allowed to walk to the goal box. Mice that fell were returned to the position they fell from, with a maximum time of 60 s allowed on the beam. Measurements taken were number of foot slips (one or both hind limbs slipped from the beam) and the number of falls.

7.2.9 **Statistical Analysis**

A sample size of 5-8 animals was utilized. All data are presented as mean±SEM. To compare differences between groups, one-way ANOVA was performed with NewmanKeuls' test as *post hoc* and two-way ANOVA followed by Bonferroni's test as *post hoc*. GraphPad Prism for Windows 5 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis. $P<0.05$ (Newman-Keuls' test or Bonferroni's test) was considered statistically significant.

7.3 **RESULTS**

7.3.1 **Elevated Plus Maze Test**

Oral administration of PME significantly increased number and percentage number of open arm entries as revealed by one-way ANOVA ($F_{3,16}=4.799$, $P=0.0143$; figure 7.1a and $F_{3,16}=6.153$, $P=0.0055$; figure 7.1d respectively) indicative of an anxiolytic effect. One-way ANOVA revealed no significance for duration and percentage duration in the open arms but *post hoc* (Newman-Keuls) analysis however showed statistical significance at 300 mg kg^{-1} ($P<0.05$). PME also decreased the time spent in the closed arms with statistical significance occurring at 100 and 300 mg kg^{-1} ($P<0.05$). Two-way ANOVA [*treatment group* × *arm type* (open or closed)] showed significant arm type effect where the number of open arm entries ($F_{1,24}=18.28$, $P=0.0027$; figure 7.1a) and the open arm time ($F_{1,24}=22.31$, $P=0.0015$; figure 7.2a) increased significantly compared to the closed arm entries and closed arm time.

Administration of diazepam caused significant and dose-dependent increase in number of open arms entries ($F_{3,16}=7.753$, $P=0.0020$; figure 7.1b) and the percentage number of open arm entries ($F_{3,16}=7.132$, $P=0.0029$; figure 7.2e). Diazepam also caused a significant and

dose-dependent increase in the amount of time spent in the open arms ($F_{3,16}$=4.606, P=0.0166). Two-way ANOVA, [*treatment* × *arm type* (open or closed)] showed significant arm type effect where the number of open arm entries ($F_{1,24}$=24.86, P=0.0011) and the open arm time ($F_{1,24}$=45.41, P=0.0001) increased significantly compared to the closed arm entries and closed arm time.

Pentylenetetrazole (3-30 mg kg^{-1}) significantly increased open arm avoidance by decreasing the number of entries ($F_{3,16}$=7.197, P=0.0028; figure 7.1c), percentage number of entries ($F_{3,16}$=8.119, P=0.0016; figure 7.1f) and percentage time spent ($F_{3,16}$=4.859, P=0.0137) in the open arms of the EPM. A two-way ANOVA, [*treatment group* × *arm type* (open or closed)] showed significant arm type effect where the number of open arm entries ($F_{1,24}$=30.31, P=0.0006; figure 7.1c) and the open arm time ($F_{1,24}$=16.53, P=0.0036; figure 7.2f) decreased significantly compared to the closed arm entries and closed arm time.

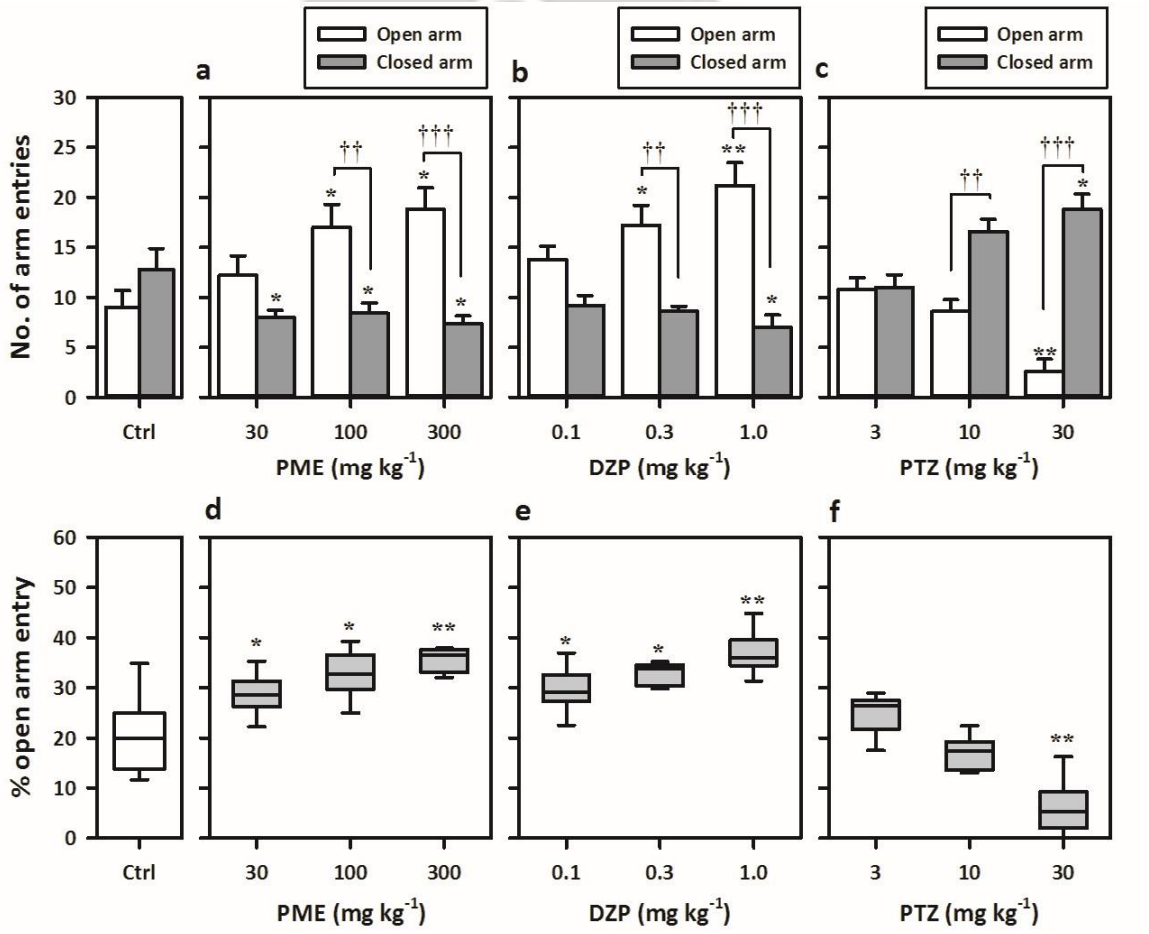


Figure 7.1 Effects of PME (30-300 mg kg^{-1}), diazepam (0.1-1.0 mg kg^{-1}) and pentylenetetrazole (3-30 mg kg^{-1}) on mice behaviour on the EPM over a 5 min test period. Data are presented as group mean±SEM (n=5). The lower and upper margins of the boxes (d, e and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal

line within the box. Significantly different from control: *P<0.05, **P<0.01 (One-way ANOVA followed by Newman-Keuls' *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).

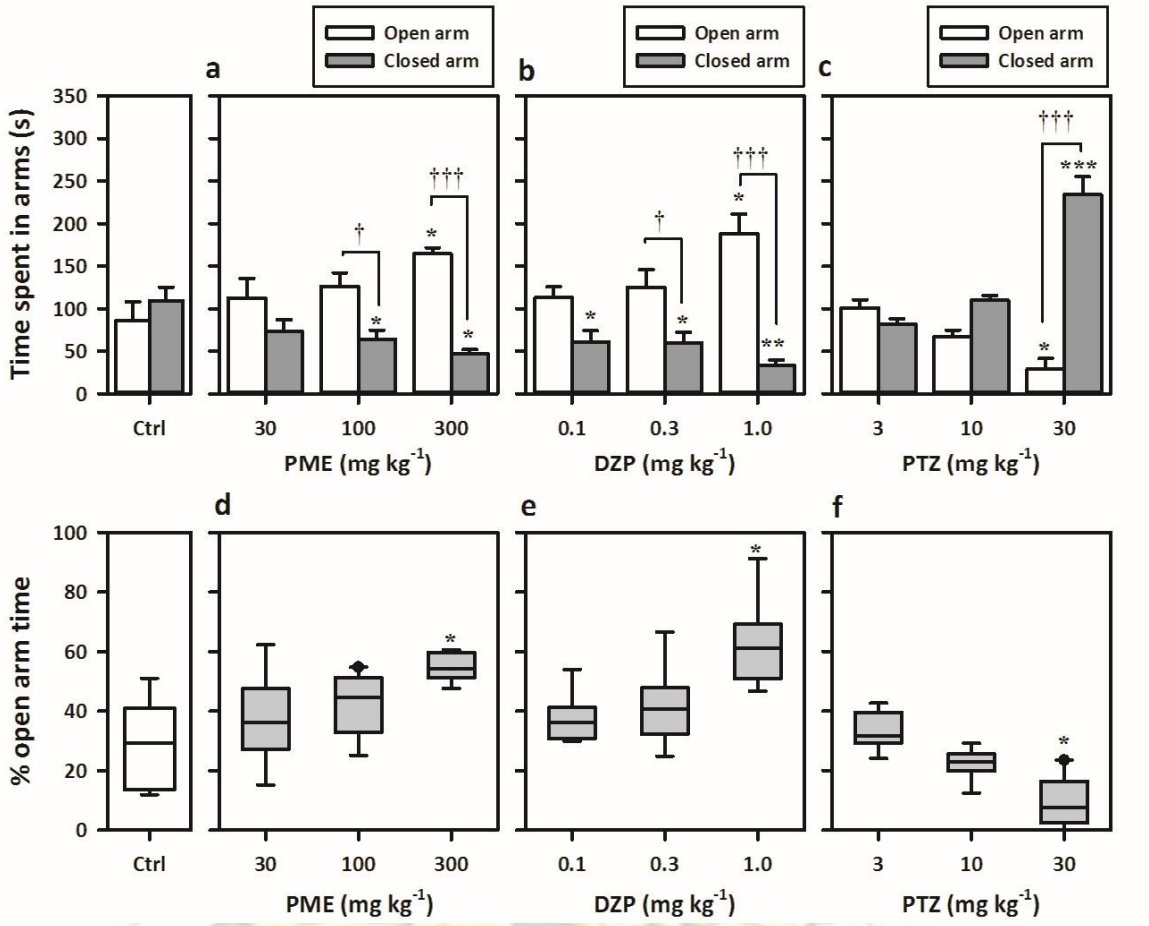


Figure 7.2 Effects of PME (30-300 mg kg^{-1}), diazepam (0.1-1.0 mg kg^{-1}) and pentylenetetrazole (3-30 mg kg^{-1}) on mice behaviour on the EPM over a 5 min test period. Data are presented as group mean±SEM (n=5). The lower and upper margins of the boxes (d, e and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Outlying points are shown individually. Significantly different from control: *P<0.05, **P<0.01, ***P<0.01 (one-way ANOVA followed by Newman-Keuls' *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).

Risk assesstment behaviour

For the risk assessment parameters, PME caused significant increase in frequency and duration of unprotected stretch-attend postures (USAPs) and total head dips (HDs). Oneway ANOVA showed that pretreatment of mice with PME significantly increased number ($F_{3,16}$=6.011, P=0.0061; figure 7.3a) and duration ($F_{3,16}$=10.10, P=0.0006; figure 7.4a) of USAPs. The extract also significantly reduced percentage number ($F_{3,16}$=6.001, P=0.0123; figure 7.3d) and percentage duration ($F_{3,16}$=15.98, P<0.0001; figure 7.4d) of

PSAPs in mice. A two-way ANOVA, [*treatment group* × *SAP type* (protected or unprotected SAP)] showed significant SAP type effect where the number ($F_{1,24}$=37.67, P=0.0004; figure 7.3a) and duration ($F_{1,24}$=93.43, P<0.0001; 7.4a) of USAPs by mice increased significantly compared to the protected stretch-attend postures (PSAPs). For head-dips, PME significantly reduced the number ($F_{3,16}$=9.406, P=0.0008; figure 7.5a), duration
($F_{3,16}$=17.31, P<0.0001; figure 7.6a) and percentage duration ($F_{3,16}$=4.497, P=0.018; figure 7.6d) of PHDs. A two-way ANOVA, [*treatment group* × *HD type* (protected or unprotected HD)] showed significant HD type effect where the number ($F_{1,24}$=32.46, P=0.0005; figure 7.5a) and duration ($F_{1,24}$=12.33, P=0.0079; figure 7.6a) of UHDs by mice increased significantly compared to the protected head dips (PHDs).

Diazepam significantly increased number ($F_{3,16}$=4.071, P=0.0251; figure 7.3b) and duration ($F_{3,16}$=6.631, P=0.004; figure 7.4b) of UPSAPs. It also significantly reduced number ($F_{3,16}$=4.706, P=0.0154; figure 7.3b), percentage number ($F_{3,16}$=10.41, P=0.0005; figure 7.3e), duration ($F_{3,16}$=5.333, P=0.0097; figure 7.4b) and percentage duration ($F_{3,16}$=19.27, P<0.0001; figure 7.4e) of PSAPs. A two-way ANOVA, [*treatment group* × *SAP type* (protected or unprotected SAP)] showed significant SAP type effect where the number ($F_{1,24}$=66.05, P<0.0001; figure 7.3b) and duration ($F_{1,24}$=72.20, P<0.0001; figure 7.4b) of USAPs by mice increased significantly compared to the protected stretch-attend postures (PSAPs). Furthermore, diazepam significantly reduced the number ($F_{3,16}$=9.958, P=0.0006; figure 7.5b), percentage number ($F_{3,16}$=6.634, P=0.004; figure 7.5e), duration ($F_{3,16}$=14.01, P<0.0001; figure 7.6b) and percentage duration ($F_{3,16}$=7.937, P=0.0018; figure 7.6e) of PHDs. A two-way ANOVA, [*treatment group* × *HD type* (protected or unprotected HD)] showed significant HD type effect where the number ($F_{1,24}$=37.73, P=0.0003; figure 7.5b) and duration ($F_{1,24}$=21.61, P=0.0016; figure 7.6b) of UHDs by mice increased significantly compared to the protected head dips (PHDs).

Pentylenetetrazole significantly increased the number ($F_{3,16}$=6.262 P=0.0051; figure 7.3c), percentage number ($F_{3,16}$=7.814, P=0.0020; figure 7.3f), duration ($F_{3,16}$=55.96, P<0.0001; figure 7.4c) and percentage duration ($F_{3,16}$=8.989, P=0.001; figure 7.4f) of PSAPs. It also significantly decreased number ($F_{3,16}$=3.933, P=0.0281; figure 7.3c) of USAPs. A two-way ANOVA, [*treatment group* × *SAP type* (protected or unprotected SAP)] showed significant

SAP type effect where the number ($F_{1,24}$=29.27, P=0.0006; figure 7.3c) and duration ($F_{1,24}$=95.70, P<0.0001; figure 7.4c) of PSAPs by mice increased significantly compared to the unprotected stretch-attend postures (USAPs). One-way ANOVA revealed that PTZ significantly increased the percentage number ($F_{3,16}$=20.20, P<0.0001; figure 7.5f), duration ($F_{3,16}$=7.844, P=0.0019; figure 7.6c) and percentage duration ($F_{3,16}$=16.19, P<0.0001; figure 7.6f) of PHDs. It also significantly decreased number ($F_{3,16}$=8.407, P=0.0014; figure 7.5c) and duration ($F_{3,16}$=4.951, P=0.0128; figure 7.6c) of UHDs. A twoway ANOVA, [*treatment group* × *HD type* (protected or unprotected HD)] showed significant HD type effect where the duration ($F_{1,24}$=9.413, P=0.0154; figure 7.6c) of PHDs by mice increased significantly compared to the UHDs. A two-way ANOVA, [*treatment group* × *HD type* (protected or unprotected HD)] did not show any significant HD type effect for the number of HDs. However, *post hoc* analysis (Bonferroni's test) revealed a significant difference at the dose of 30 mg kg^{-1} (P<0.01; figure 7.5c).

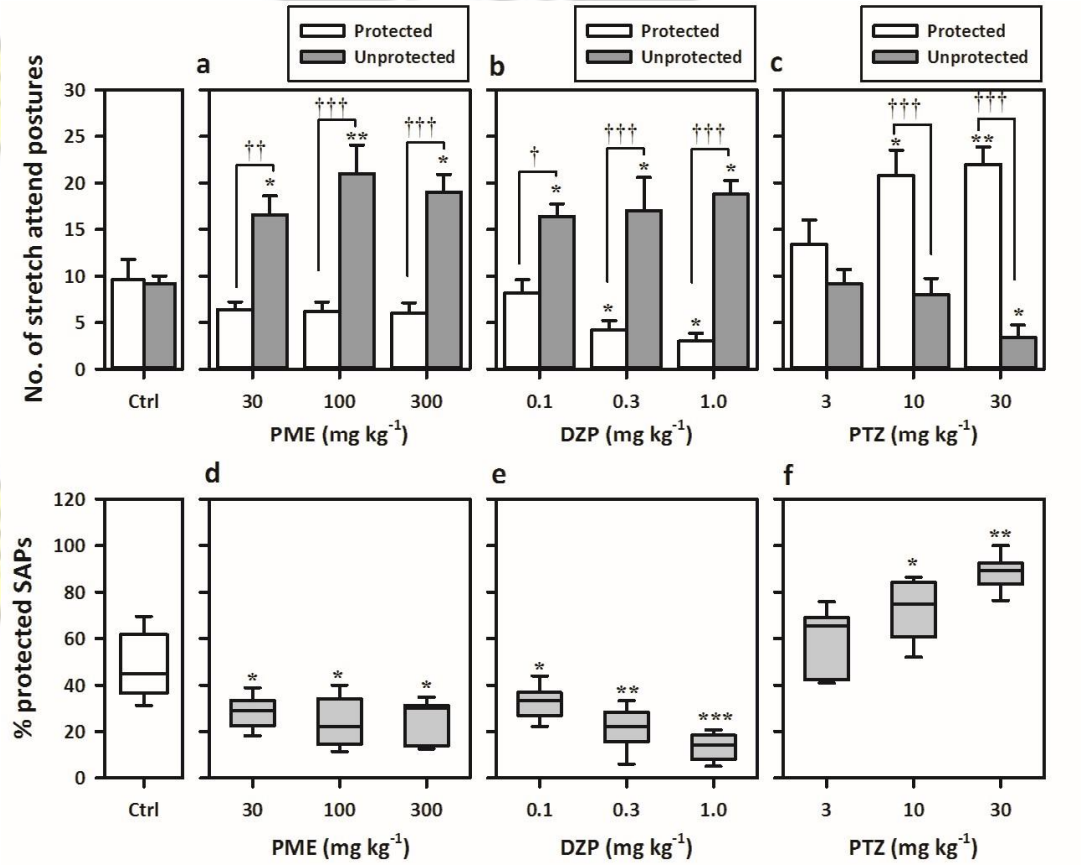


Figure 7.3 Effects of PME (30-300 mg kg^{-1}), diazepam (0.1-1.0 mg kg^{-1}) and pentylenetetrazole (3-30 mg kg^{-1}) on risk assessment behaviours (protected and unprotected stretch-attend postures) over a 5 min test period in mice on the EPM. Data are expressed as group mean±SEM (n=5). The lower and upper margins of the boxes (d, e and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Significant

difference: *P<0.05, **P<0.01, ***P<0.001 compared to control group (one-way ANOVA followed by Newman-Keuls' *post hoc* test) and †P<0.05, ††P<0.01, †††P<0.001 when protected and unprotected stretch-attend postures are compared (two-way ANOVA followed by Bonferroni's *post hoc* test).

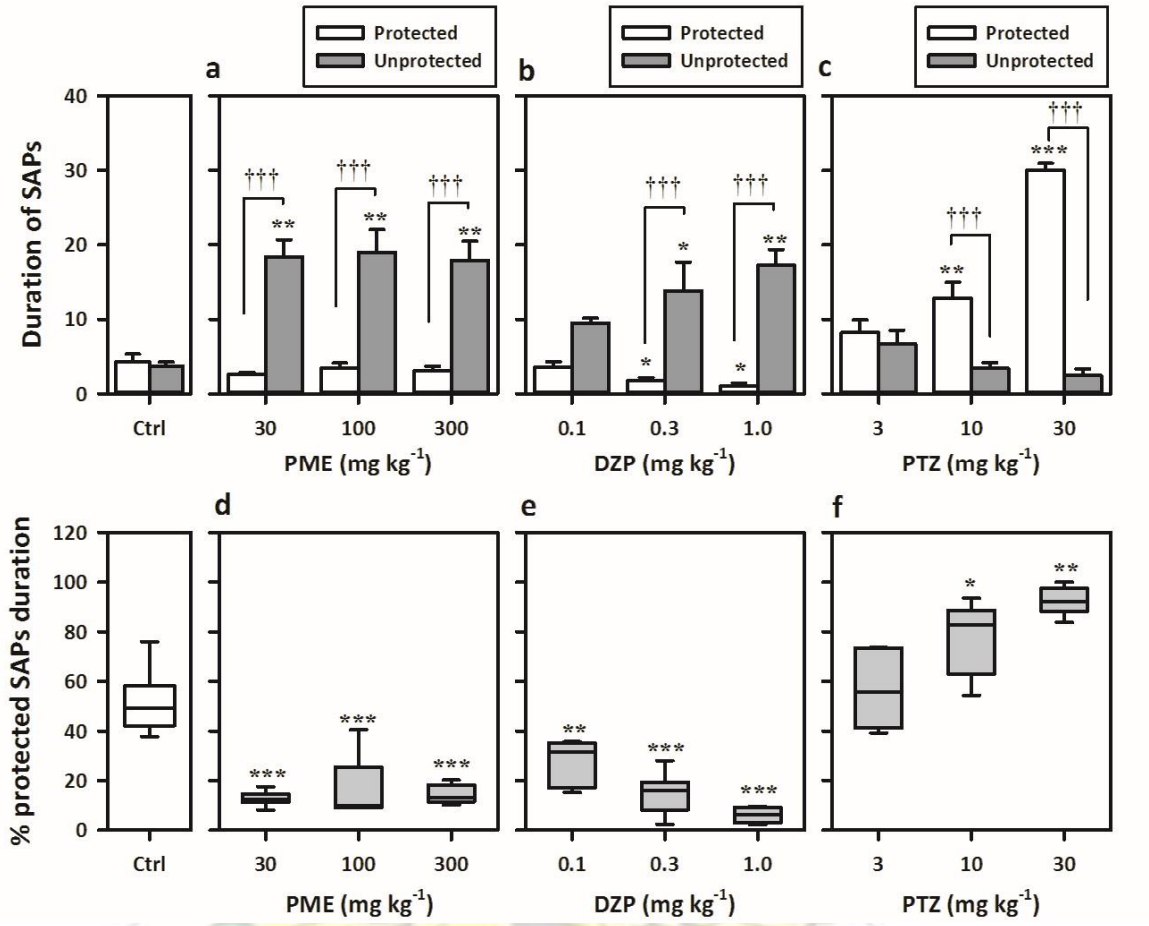


Figure 7.4 Effects of PME (30-300 mg kg^{-1}), diazepam (0.1-1.0 mg kg^{-1}) and pentylenetetrazole (3-30 mg kg^{-1}) on risk assessment behaviours (duration of protected and unprotected stretch-attend postures) over a 5 min test period in mice on the EPM. Data are expressed as group mean±SEM (n=5). The lower and upper margins of the boxes (d, e and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Significant difference: *P<0.05, **P<0.01, ***P<0.001 compared to control group (one-way ANOVA followed by Newman-Keuls' test) and †††P<0.001 when duration of protected and unprotected stretch-attend postures are compared (two-way ANOVA followed by Bonferroni's *post hoc* test).

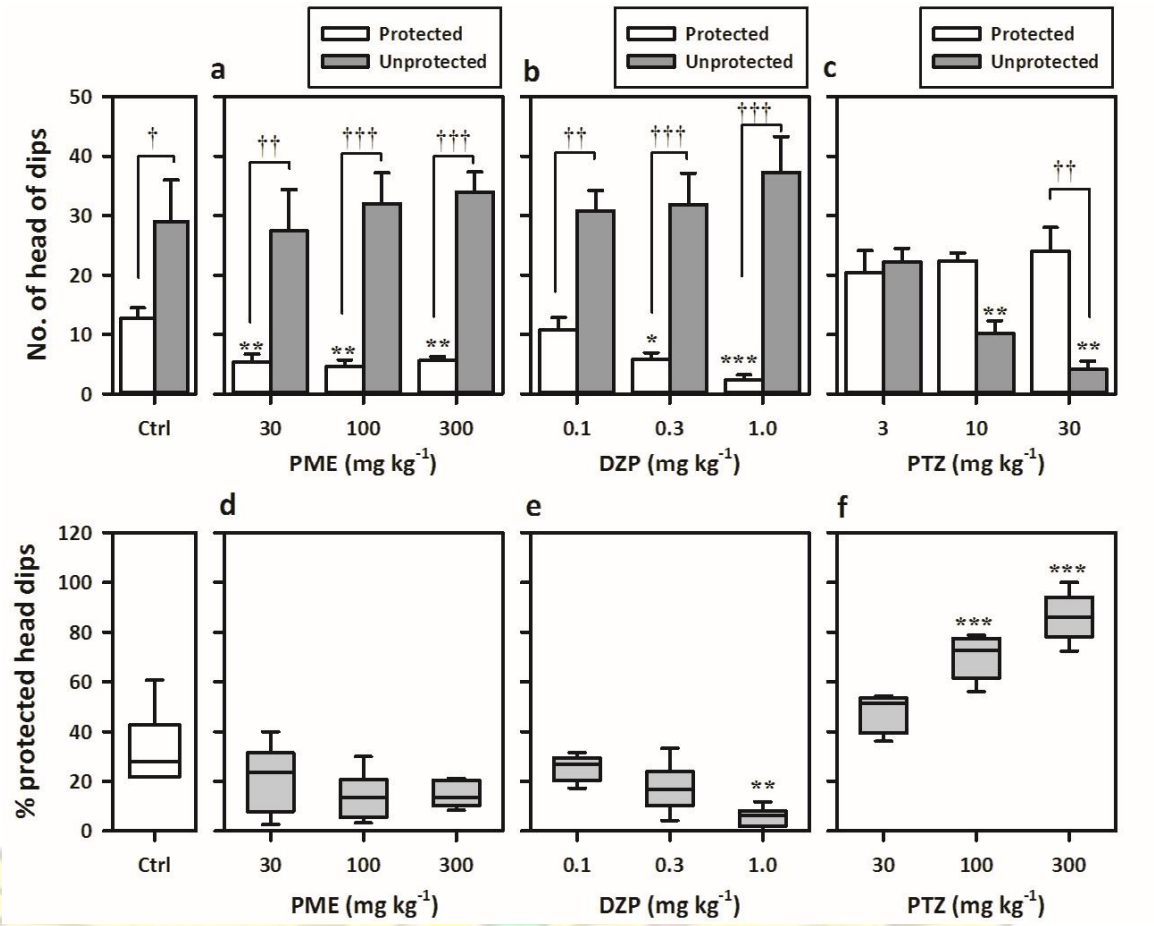


Figure 7.5 Effects of PME (30-300 mg kg^{-1}), diazepam (0.1-1.0 mg kg^{-1}) and pentylenetetrazole (3-30 mg kg^{-1}) on risk assessment behaviours (protected and unprotected head dips) over a 5 min test period in mice on the EPM. Data are expressed as group mean±SEM (n=5). The lower and upper margins of the boxes (d, e and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Significant difference: **P*<0.05, ***P*<0.01, ****P*<0.001 compared to control group (one-way ANOVA followed by NewmanKeuls' *post hoc* test) and †*P*<0.05, ††*P*<0.01, †††*P*<0.001 when protected and unprotected head dips are compared (two-way ANOVA followed by Bonferroni's *post hoc* test).

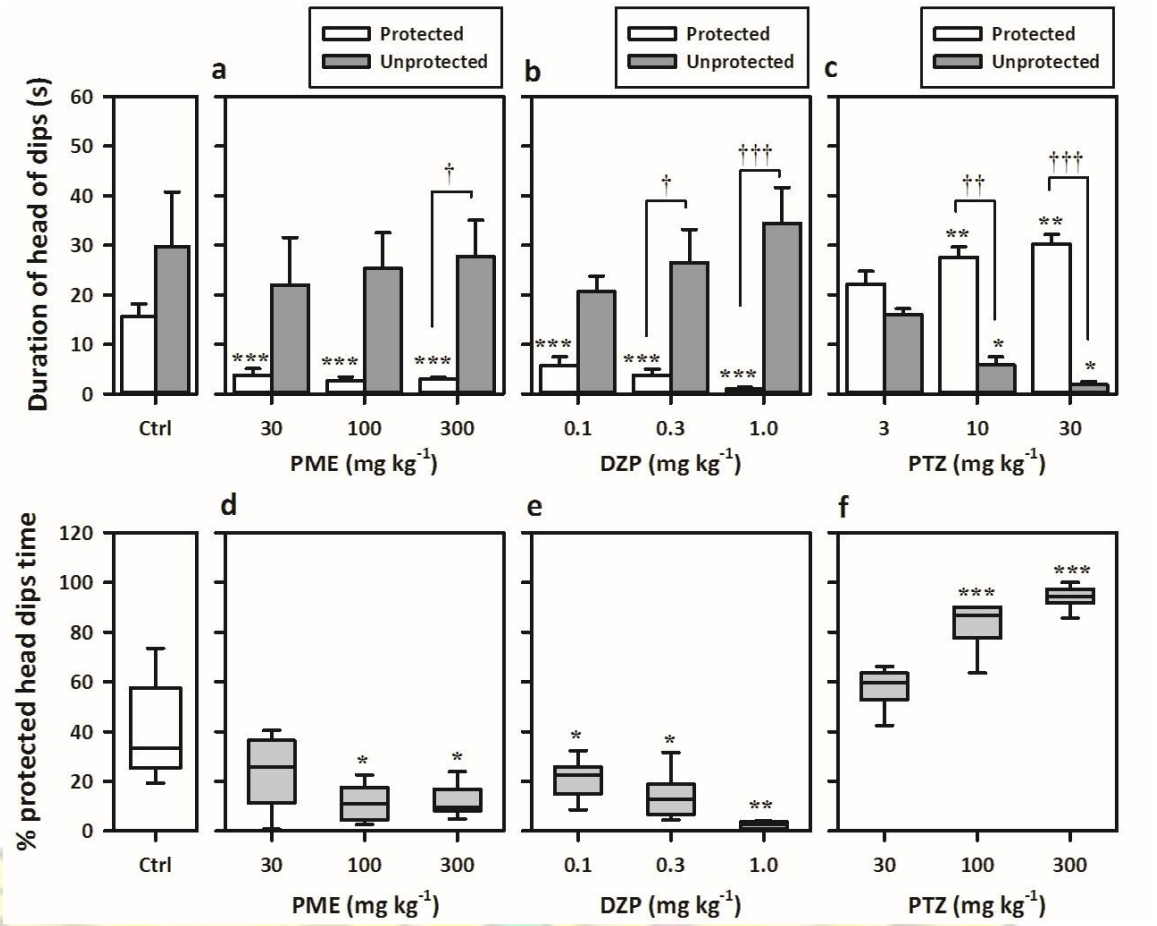


Figure 7.6 Effects of PME (30-300 mg kg^{-1}), diazepam (0.1-1.0 mg kg^{-1}) and pentylenetetrazole (3-30 mg kg^{-1}) on risk assessment behaviours (duration of protected and unprotected head dips) over a 5 min test period in mice on the EPM. Data are expressed as group mean±SEM (n=5). The lower and upper margins of the boxes (d, e and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Significant difference: **P*<0.05, ***P*<0.01, ****P*<0.001 compared to control group (One-way ANOVA followed by Newman-Keuls' *post hoc* test) and †*P*<0.05, ††*P*<0.01, †††*P*<0.001 when protected and unprotected head dips are compared (two-way ANOVA followed by Bonferroni's *post hoc* test).

Total distance travelled

Treatment of mice with the extract ($F_{3,16}$=0.5352, *P*=0.6645) and diazepam ($F_{3,16}$=2.707, *P*=0.0799) did not have any significant effect on the total distance travelled in the EPM compared to the vehicle treated animals. However, in line with its anxiogenic nature, treatment with PTZ caused a significant ($F_{3,16}$=19.60, *P*<0.0001) decrease in the total distance travelled. Comparing the 3D line plots in figure 7.7, animals treated with PME and diazepam seemed to have made a greater number of visits into the open arms than the closed arms of the EPM which is indicative of their anxiolytic properties. In contrast, PTZtreated animals however made more closed arm entries than open arm entries.

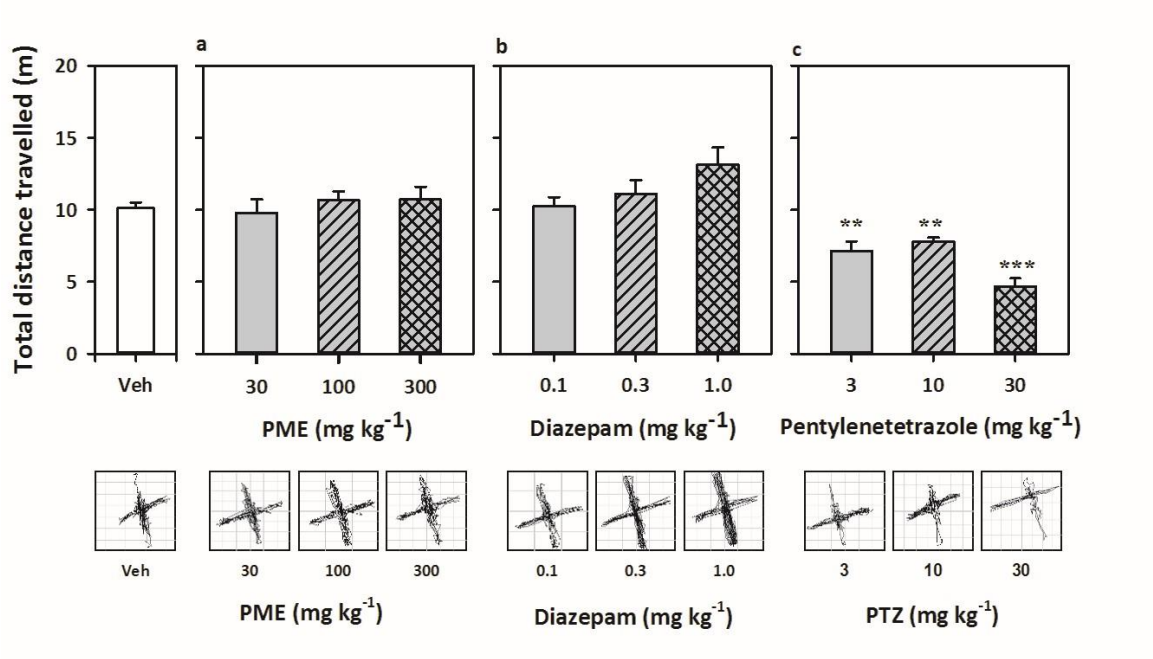


Figure 7.7 Effects of PME (30-300 mg kg^{-1}), diazepam (0.1-1.0 mg kg^{-1}) and pentylenetetrazole (3-30 mg kg^{-1}) on total distance travelled on the EPM. Data are presented as group mean±SEM (n=5). ***P*<0.01, ****P*<0.001 compared to control group (one-way ANOVA followed by Newman-Keuls' *post hoc* test). Line plots (lower panels) 3D plots were generated from the time and XY data obtained using Sigma Plot Version (Systat Software Inc., Point Richmond, CA, USA).

7.3.2 **Light Dark Box**

In the light-dark box test, oral administration of PME (30-300 mg kg^{-1}) significantly decreased the latency into the lit compartment and also increased time spent in the lit compartment as revealed by one-way ANOVA ($F_{3,16}$=3.977, P=0.0271 and $F_{3,16}$=3.458, P=0.0415) respectively without affecting inter-compartmental transitions (figure 7.8). Twoway ANOVA (*treatment* × *box type*, i.e. lit or dark) revealed no significant box type effect ($F_{1,24}$=2.667, P=0.1411; figure 7.8d) when duration in lit box was compared to the dark box. However, Bonferroni's *post hoc* analysis showed a significance at the dose of 300 mg kg^{-1} (P<0.001).

Diazepam produced effects that were similar to those produced by PME. As shown in figures 7.8b and 7.8e, diazepam decreased the latency into the lit compartment and increased time spent in the lit compartment ($F_{3,16}$=5.884, P=0.0066 and $F_{3,16}$=4.894, P=0.0134 respectively). A two-way ANOVA (*treatment* × *box type*, i.e. lit or dark) revealed significant box type effect ($F_{1,24}$=12.74, P=0.0073; figure 7.8e) where mice spent more time in the lit box compared to the dark box.

In contrast to PME and diazepam, PTZ significantly decreased the time spent by mice in the lit box ($F_{3,16}$=8.715, P=0.0012; figure 7.8f) and increased significantly the time spent in the dark box ($F_{3,16}$=8.711, P=0.0012; figure 7.8f). PTZ also significantly decreased the number of inter-compartment transitions ($F_{3,16}$=7.163, P=0.0029; figure 7.8c) and increased significantly latency to enter the lit compartment ($F_{3,16}$=4.832, P=0.014; figure 7.8c). In addition, two-way analysis of the effects of PTZ revealed significant box type effect ($F_{1,24}$=1011, P<0.0001; figure 7.8f) with the *post hoc* analysis (Bonferroni's test) revealing that all treatment groups spent significantly less time in the lit compartment compared to the time spent in the dark compartment (all at P<0.001).

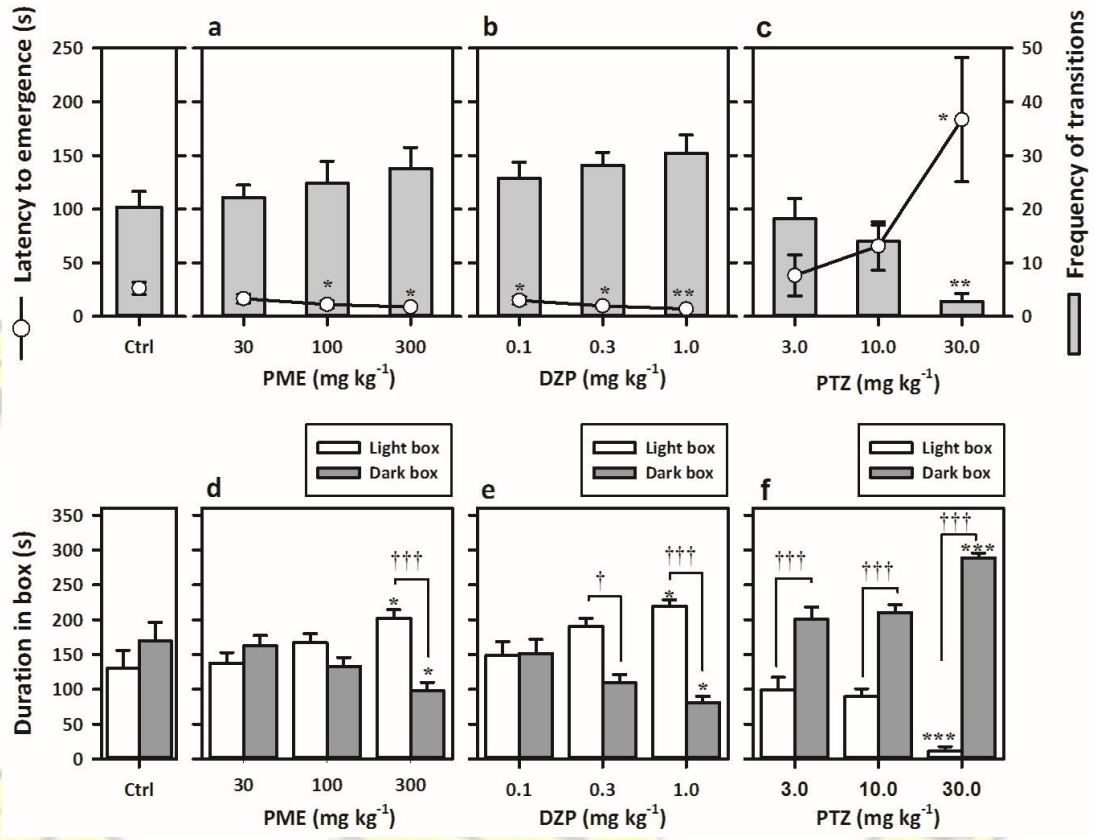


Figure 7.8 Effects of PME, diazepam and pentylenetetrazole on mice behaviour in the light/dark box over a 5-min period. Data are expressed as group Mean±SEM (n=5). *P<0.05, **P<0.01, ***P<0.001 compared to control group (one-way ANOVA followed by Newman-Keuls' test). †P<0.05, †††P<0.001 when light compartment was compared with dark compartment (two-way ANOVA followed by Bonferroni's *post hoc* test).

7.3.3 **Open Field Test**

Figure 7.9-7.11 represents the effect of acute administration of PME (30-300 mg kg^{-1} *p.o.*), the anxiolytic drug diazepam (0.1-1 mg kg^{-1}, i.p.) and the anxiogenic agent pentylenetetrazole (3-30 mg kg^{-1}, i.p.) on mice behaviours in the open field test. *P. microcarpa* treated mice exhibited anxiolytic-like activity by significantly increasing the

number of entries into the central zone ($F_{3,16}$=5.269, P=0.0102). Two-way ANOVA revealed a significant difference in both the number ($F_{2,36}$=114.4, P<0.0001; figure 7.9 a) and duration ($F_{2,36}$=194.9, P<0.0001; figure 7.10a) of entries into the various zones.

Treatment of mice with the anxiolytic drug, diazepam, produced effects that were similar to those produced by PME. Consistent with its anxiolytic nature, it significantly increased the number of entries in the center of the arena ($F_{3,16}$=6.894, P=0.0034; 7.9b) and significantly decreased time spent in the corner ($F_{3,16}$=6.280, P=0.0051; figure 7.10b). There was a significant difference in both the number ($F_{2,36}$=114.4, P<0.0001; figure 7.9b) and duration ($F_{2,36}$=114.4, P<0.0001; figure 7.10b) of entries into the various zones.

In contrast to PME and diazepam, pentylenetetrazole treatment significantly decreased number of entries ($F_{3,16}$=5.945, P=0.0063; figure 7.9c) and percentage number of entries ($F_{3,16}$=10.81, P=0.0004; figure 7.9f) into the central zone. It also significantly decreased both the time ($F_{3,16}$=23.31, P<0.0001; figure 7.10c) and percentage time ($F_{3,16}$=23.31, P<0.0001; figure 7.10f) spent in the central zone of the open field arena. One-way ANOVA also revealed an increase in time spent in the corner ($F_{3,16}$=3.458, P=0.0415; figure 7.10c) with no significant difference observed at all the doses used. Two-way ANOVA showed a significant difference in both the number ($F_{2,36}$=139.8, P<0.0001; figure 7.9c) and duration ($F_{2,36}$=650.6, P<0.0001; figure 7.10c) of entries into the various zones.

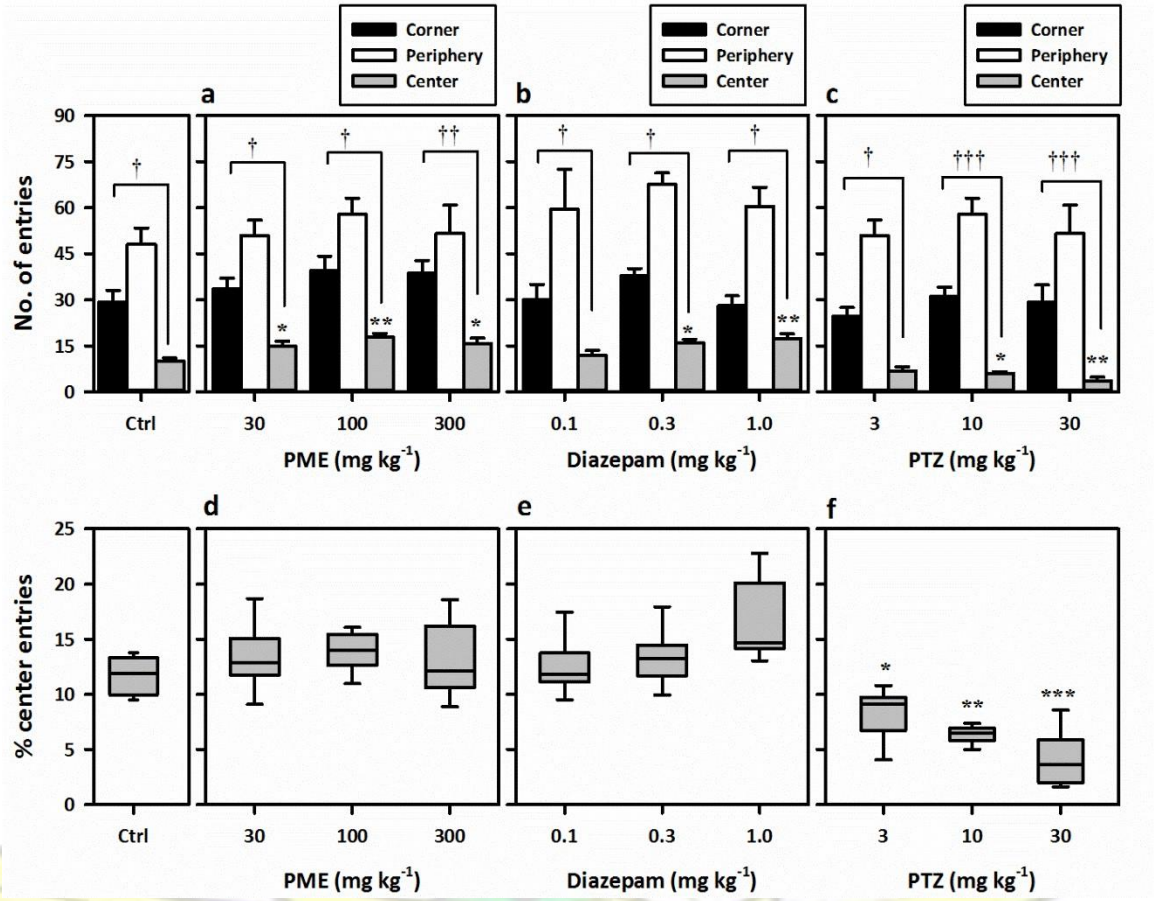


Figure 7.9 Effects of acute PME (30-300 mg kg^{-1}), diazepam (0.1-1.0 mg kg^{-1}) and pentylenetetrazole (3-30 mg kg^{-1}) treatment on the number of zonal entries for PME (a), diazepam (b), PTZ (c) and % entries into central zone for PME (d), diazepam (e), PTZ (f) in the open field test. Data are presented as group means±SEM (n=5). The lower and upper margins of the boxes (d, e and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls' *post hoc* test and significant difference when the zonal entries where compared to each other: †*P*<0.05, ††*P*<0.01, †††*P*<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

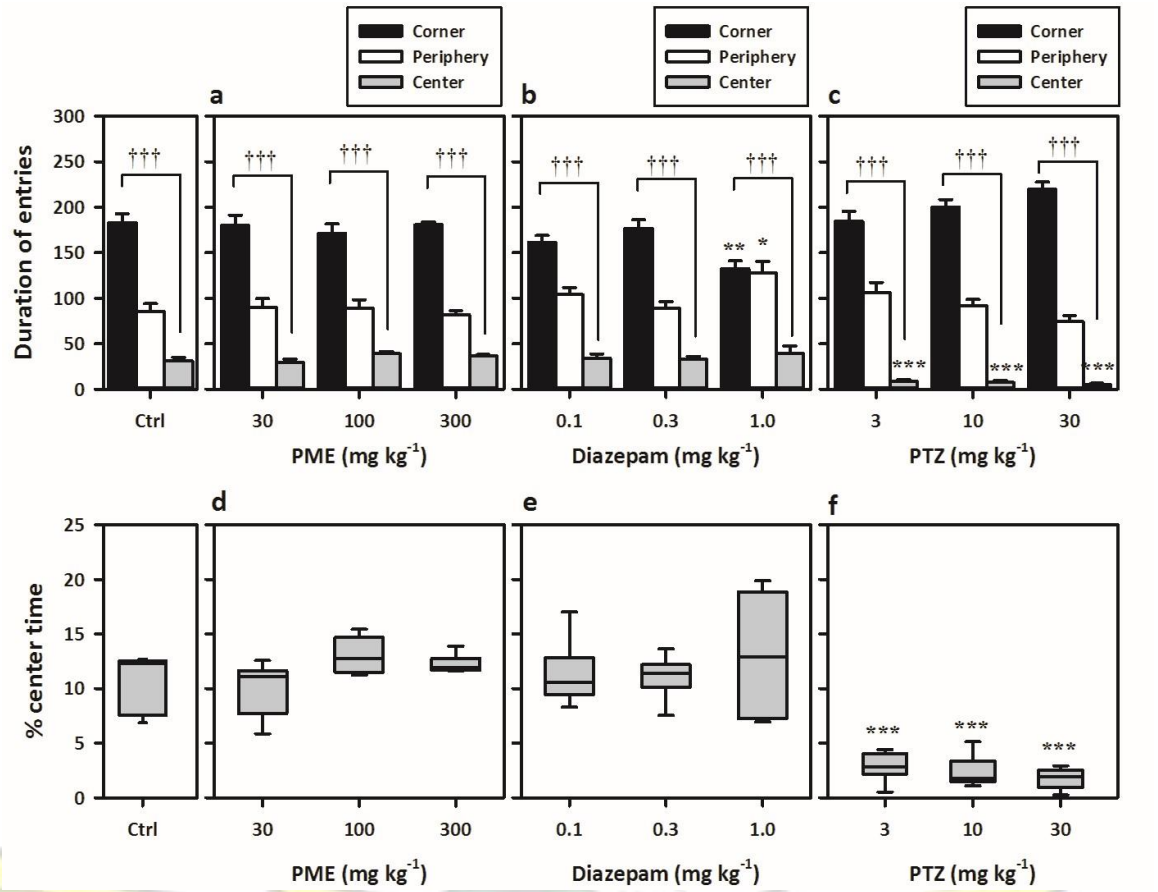


Figure 7.10 Effects of acute PME (30-300 mg kg^{-1}), diazepam (0.1-1.0 mg kg^{-1}) and pentylenetetrazole (330 mg kg^{-1}) treatment on the total time spent in the zones for PME (a), diazepam (b), PTZ (c) and % time in central zone for PME (d), diazepam (e), PTZ (f) in the open field test. Data are presented as group means±SEM (n=5). The lower and upper margins of the boxes (d, e and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test and significant difference when the zonal entries where compared to each other: †††*P*<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

Total distance travelled

All treatment groups did not show any significant differences compared to the vehicletreated group in the total distance travelled in the arena—PME ($F_{3,16}$=1.012, *P*=0.4132), diazepam ($F_{3,16}$=1.012, *P*=0.4132) and pentylenetetrazole ($F_{3,16}$=1.012, *P*=0.4132). However, PME and Diazepam showed an increasing trend whereas PTZ exhibited

decreasing trend in the total distance travelled. The 3D line plots generated from the time and XY data shows that animals treated with PME and diazepam made much more visits to the center of the arena, indicating a decrease in thigmotactic behaviour compared to the

vehicle treated animals. However, treatment with PTZ made the mice highly thigmotactic spending most of the time along the walls of the open field arena (figure 7.11).

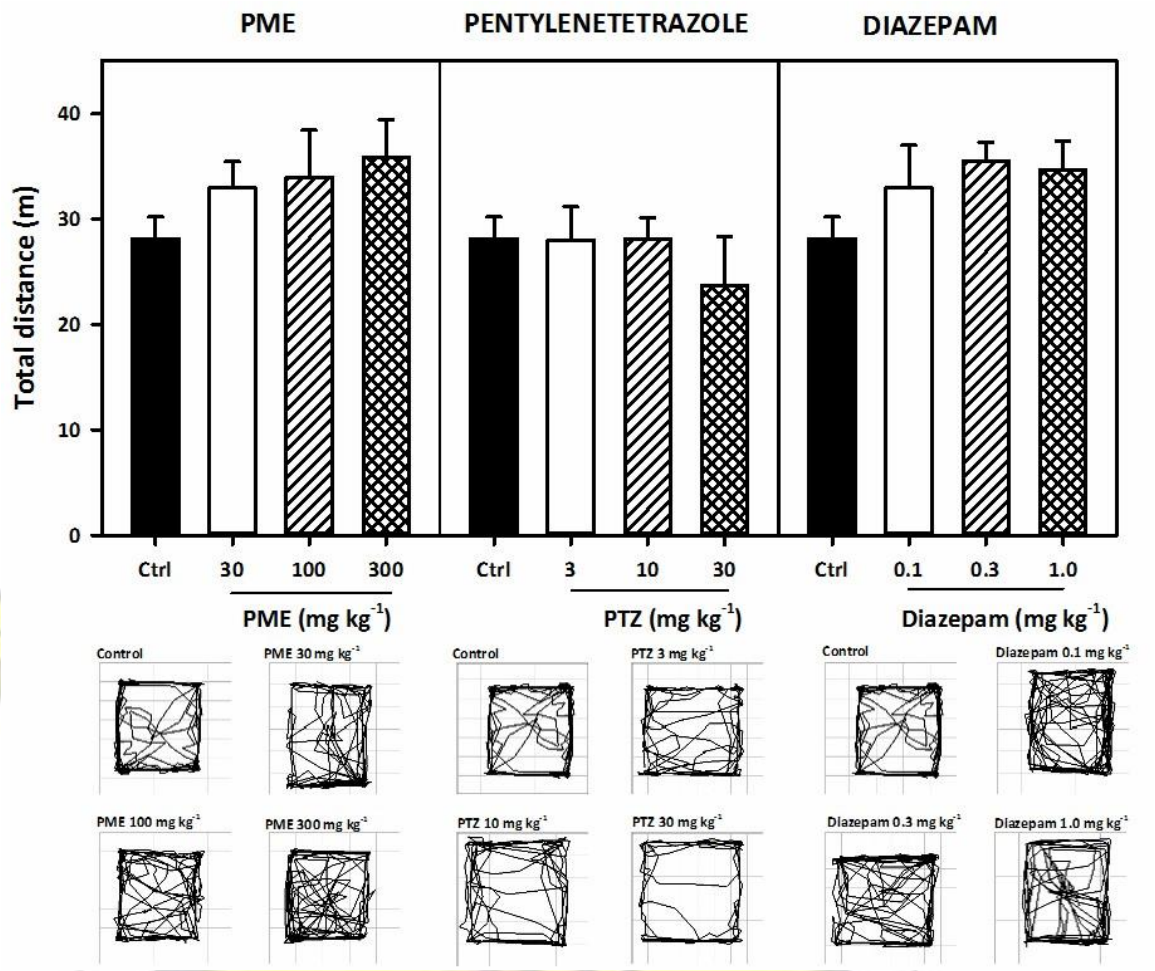


Figure 7.11 Effects of PME (30-300 mg kg^{-1}), diazepam (0.1-1.0 mg kg^{-1}) and pentylenetetrazole (3-30 mg kg^{-1}) on total distance travelled in the open field test. Data are presented as group mean±SEM (n=5). Line plots (lower panels) 3D plots were generated from the time and XY data obtained using SigmaPlot Version 10 (Systat Software Inc., Point Richmond, CA, USA).

7.3.4 **Social Interaction Test**

In the sociability test, mice treated with PME and DZP demonstrated a significant preference for spending time in the chamber containing stranger 1 than the empty chamber [figure 7.12; overall repeated measures ANOVA following significant main effect of side on duration: PME ($F_{2,72}$=136.2, P<0.0001) and DZP ($F_{2,72}$=132.9, P<0.0001)]. Subjects generally spent more time in either side of the apparatus than in the middle chamber. PMEtreated mice spent more time sniffing the wire cage containing the unfamiliar mouse

than the empty wire cage ($F_{1,48}$=140.3, P<0.0001; figure 7.12g) with Bonferroni's *post hoc* analysis revealing significance at all the doses used (all P<0.001). A similar effect was observed for DZP ($F_{1,48}$=90.06, P<0.0001; figure 7.13h). PME and DZP treated mice exhibited a significant preference to spend time in the chamber containing the novel stranger 2, as compared to time spent in the chamber containing the now-familiar stranger 1 [figure 7.13; two-way ANOVA following significant main effect of side on duration: PME ($F_{2,72}$=185.0, P<0.0001) and DZP ($F_{2,72}$=80.02, P<0.0001)]. A significant preference for social novelty (sniffing duration) was also observed for PME ($F_{1,48}$=64.51, P<0.0001; figure 7.13g) and DZP ($F_{1,48}$=47.10, P<0.0001; figure 7.13h), where mice spent more time sniffing the novel stranger than stranger 1.

In contrast to PME and DZP, PTZ showed a decrease in sociability by significantly decreasing time spent in the chamber containing stranger 1 compared to the empty chamber ($F_{2,72}$=19.0, P<0.0001; figure 7.12c) with post hoc analysis showing significance at 30 mg kg^{-1} (P<0.001). Mice also spent more time sniffing the empty wire cage than stranger 1 ($F_{1,48}$=5.059, P=0.0291; figure 7.12i). A significant preference to spend time in the chamber containing the now-familiar stranger 1, as compared to time spent in the chamber containing the novel stranger 2 was observed for PTZ ($F_{2,72}$=52.25, P<0.0001; figure 7.13c). Mice administered with PTZ also showed an increased preference for sniffing stranger 1 compared to stranger 2 ($F_{1,48}$=9.578, P=0.0033; figure 7.13i).

Differences in number of entries into the two sides were not observed during the test for sociability and social preference for all treated groups (P>0.05).

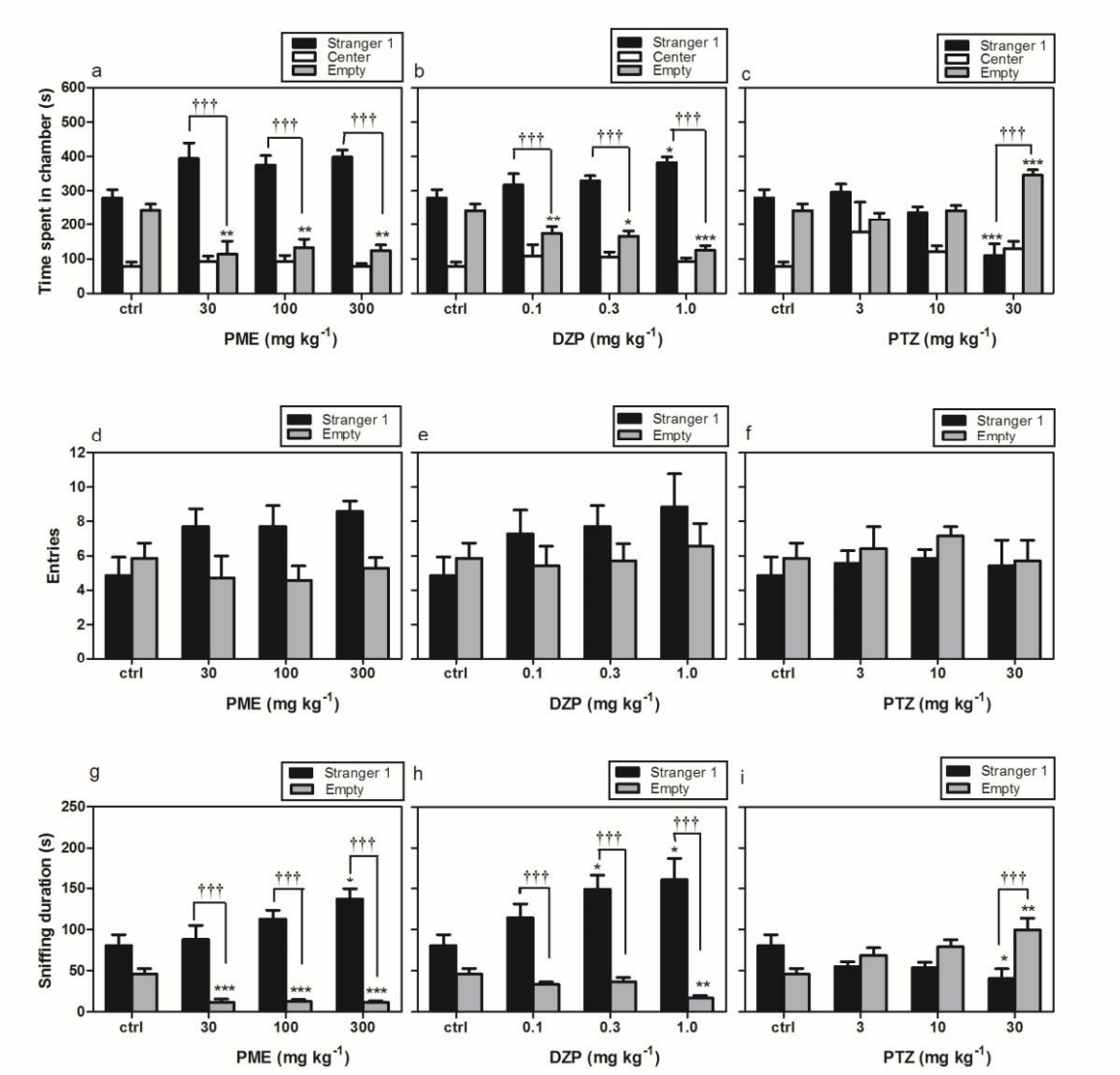


Figure 7.12 Effects of acute PME (30-300 mg kg^{-1}), diazepam (0.1-1.0 mg kg^{-1}) and pentylenetetrazole (330 mg kg^{-1}) treatment on the total time spent in the chambers for PME (a), diazepam (b), PTZ (c); entries into compartments for PME (d), diazepam (e), PTZ (f) and sniffing duration for PME (g), diazepam (h), PTZ (i) in the sociability test. Data are presented as group means±SEM (n=7). Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls' *post hoc* test and significant difference when stranger 1 was compared to empty cage: †††*P*<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

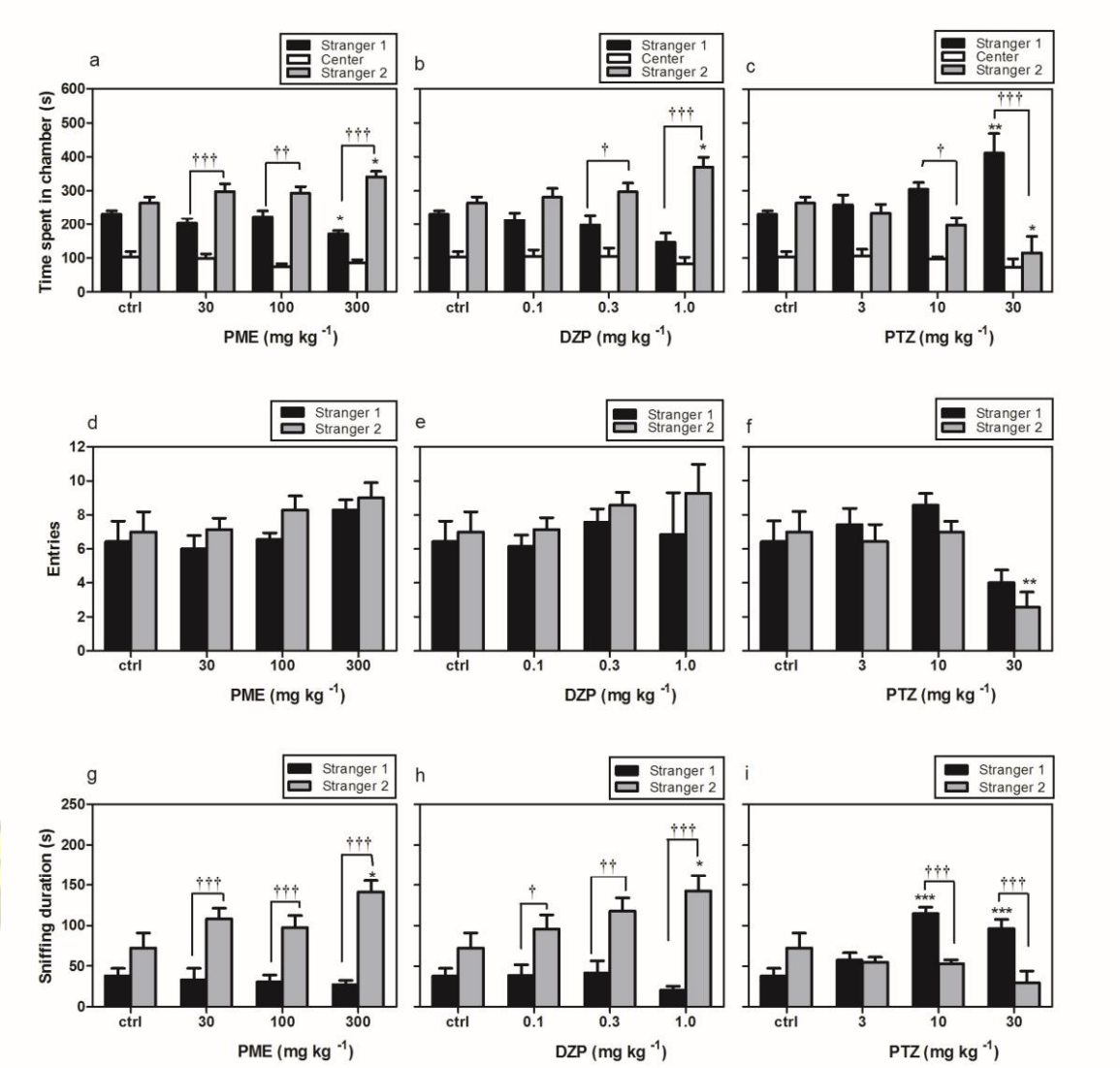


Figure 7.13 Effects of acute PME (30-300 mg kg^{-1}), diazepam (0.1-1.0 mg kg^{-1}) and pentylenetetrazole (330 mg kg^{-1}) treatment on the total time spent in the chambers for PME (a), diazepam (b), PTZ (c); entries into compartments for PME (d), diazepam (e), PTZ (f) and sniffing duration for PME (g), diazepam (h), PTZ (i) in the preference for social novelty test. Data are presented as group mean±SEM (n=7). Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls' *post hoc* test and significant difference when stranger 1 was compared to stranger 2: †*P*<0.05, ††*P*<0.01, †††*P*<0.001 (twoway repeated measures ANOVA followed by Bonferroni's *post hoc* test).

7.3.5 **Stress-Induced Hyperthermia**

ANOVA revealed a significant effect of PME treatment on the magnitude of the SIH response ($F_{3,36}$=2.883, P=0.0491; figure 7.14) with Newman-Keuls' *post hoc* analysis showing a statistical significance at 300 mg kg^{-1} (P<0.05). Moreover, the stronger reduction in T_2 than in T_1 following treatment with PME is reflected in a significant *stress* × *treatment* interaction ($F_{3,72}$=3.561, P=0.0183). Similar to PME, DZP significantly decreased SIH ($F_{3,36}$=4.003, P=0.0149) and a two-way ANOVA showing a significant

stress × *treatment* interaction ($F_{3,72}$=13.18, P<0.0001). Together these data indicate an anxiolytic-like effect of PME and DZP.

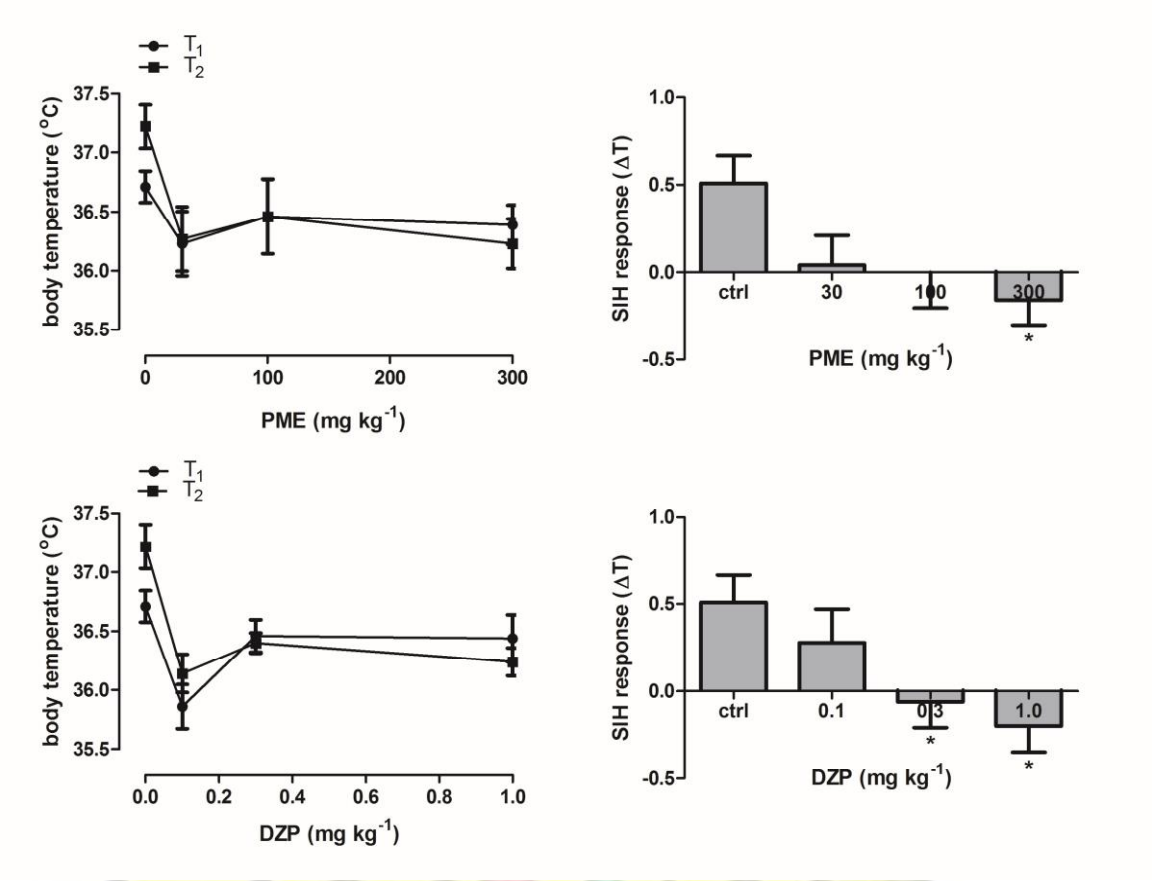


Figure 7.14 Effects of vehicle or various doses of PME and DZP (a standard anxiolytic drug) on SIH (ΔT), basal temperature (T_1), and T_2. Drugs or vehicle were given orally 60 min before the first rectal temperature measurement (T_1). T_2 was measured 10 min later. The difference between T_2 and T_1, ΔT, is indicated at each dose. Data are presented as group mean±SEM (n=10). Significantly different from control: *P<0.05 by Newman-Keuls' *post hoc* test.

7.3.6 **Beam Walk Test**

Figure 7.15 shows the results of the effect of PME and diazepam on motor co-ordination in the mouse beam walk test. One-way ANOVA revealed that pre-treatment of mice with PME (30-300 mg kg^{-1}, *p.o.*) did not significantly affect the time taken by mice to reach the goal box ($F_{3,16}$ = 0.2882, P>0.05) and number of foot slips. Diazepam did not also have significant effect on the time taken to cross the beam at 0.1-1.0 mg kg^{-1} (p>0.05) except 3.0 mg kg^{-1} which caused significant delay in the time to traverse the beam as well as increasing number of foot slips compared to vehicle-treated group (p<0.01).

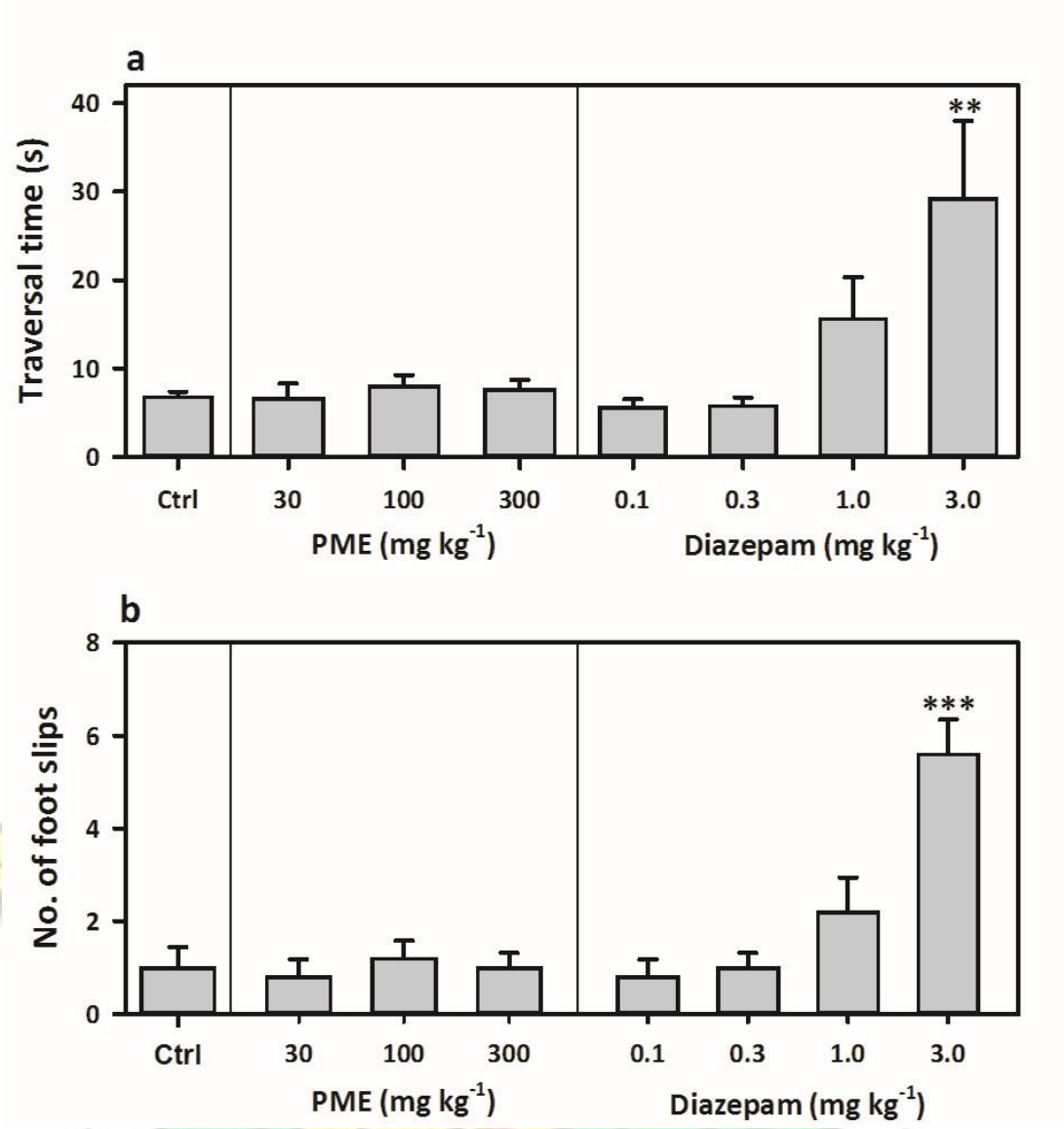


Figure 7.15 Effect of PME and diazepam on motor co-ordination in the mouse beam walk test. Data are expressed as group mean±SEM (n=5). ***P*<0.01, ****P*<0.001 compared to control group (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

7.4 **DISCUSSION**

In this study, administration of hydroethanolic extract of the leaves of *P. microcarpa* possesses anxiolytic-like effect comparable with that of diazepam in pharmacologically validated models of anxiety.

The present study showed that the administration of different doses of PME in mice was able to induce anxiolytic-like effects in the EPM. The elevated plus-maze (EPM) has become the most widely used animal model in contemporary preclinical research on

anxiety. In addition, the EPM affords a good example of a model based on the study of unconditioned responses to less intense threatening situations (Litvin *et al.*, 2008). This test is based on the observation that the natural behaviour of rats or mice is to display an aversion to open spaces; therefore, avoidance of the open arms is interpreted as anxiogenic behaviour (Belzung and Griebel, 2001; Carobrez and Bertoglio, 2005; Galdino *et al.*, 2012). Moreover, the anxiolytic-like effectiveness of a drug can be demonstrated by an increase in exploration of the open arms (time and entries into open arms), while the opposite holds true for drugs with anxiogenic-like effects (Carobrez and Bertoglio, 2005; Lister, 1987; Rodgers and Cole, 1994; Pellow *et al.*, 1985). The number of closed arm entries provides a control measure of motor activity (Bourin *et al.*, 2007). In the present study, PME decreased the avoidance to open arms, increasing the percentage of entries and time in the open arms and decreasing number and time in the closed arms indicating an anxiolytic-like effect. These results were similar to the effects observed after administration of the reference anxiolytic drug diazepam whereas showing effects opposite to that of PTZ (anxiogenic). These data are in agreement with the results of other studies, where diazepam (an anxiolytic) increased the percentage time spent in open arms and open arm entries while PTZ (an anxiogenic) showed opposite in the EPM (Rodgers *et al.*, 1995; 1997).

Ethological approaches to the EPM have evaluated defensive behaviours, especially those associated with risk assessment; for example, stretch-attend posture and head dipping (Rodgers *et al.*, 1997; Carobrez and Bertoglio, 2005). These measures of defence have improved the analysis of the effects observed in this paradigm (Griebel *et al.*, 2000; Wall and Messier, 2001; Roy and Chapillon, 2004). Anxiolytics would decrease the number and time of protected stretch-attend postures or protected head-dips while anxiogenics would increase these parameters (Rodgers *et al.*, 1997; Rodgers and Johnson, 1995; Woode *et al.*, 2011). Both PME and diazepam decreased the number, time and percentage of the protected forms of both stretch-attend postures and head-dipping, indicating reduced state of anxiety/fear. However, PTZ increased the protected forms of the risk assessment behaviours which are consistent with its anxiogenic activity.

As an anxiety model, the light/dark test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behaviour of rodents in response to mild stressors, that is, novel environment and light (Crawley and Goodwin, 1980; Bourin and Hascoët, 2003). A natural conflict situation occurs when an animal is exposed to an

unfamiliar environment or novel objects. This conflict is between the tendency to explore and the initial tendency to avoid the unfamiliar (neophobia). Thus, in the light/dark test, the primary indices of anxiety are spatiotemporal; i.e., the time spent in the bright side and the number of transitions made by animals between the two compartments. Anxiolytics have been shown to significantly increase the number of transits between compartments without an increase in spontaneous locomotion (Crawley, 1981; Bourin and Hascoët, 2003). PME and diazepam showed anxiolytic-like effects by decreasing the latency to enter the light compartment as well as increasing time spent in the light. PTZ also showed results consistent with its anxiogenic activity, increasing the latency to enter the light compartment and decreasing both inter-compartment transitions and the time spent in the light.

The open-field model examines anxiety-related behaviour characterized by the normal aversion of the animal to an open, brightly lit area (Choleris *et al.*, 2001; Mechan *et al.*, 2002). Thus, when initially placed in the open field arena, animals begin investigating the context and spontaneously prefer the periphery of the open field to activity in central parts of the apparatus. This high level of peripheral activity is often accompanied by thigmotaxis (wall-hugging) behaviour, a reaction in which the animal remains close to vertical surfaces (Wilson *et al.*, 1976; Treit and Fundytus, 1988). However, anxiolytics increase preference for the central area, enhancing the crossing number and the time spent in the central area of the apparatus (Prut and Belzung, 2003). In this test, PME without having any effect on total distance increased the preference for the central area and the time spent in the central area of the apparatus indicative of anxiolytic-like effects. Diazepam, also consistently and significantly decreased the number of anxiety-related behaviours that were performed in the corners and along the walls (thigmotaxis) as well as increasing activity in the center. PTZ however decreased the entries and time in the central zone which is consistent with its anxiogenic activity.

Abnormal social behaviours or low levels of social interaction are symptoms of several psychiatric disorders, including autism, anxiety, depression, schizophrenia, and social phobias(Crawley, 2007b). Crawley's three-chamber social approach test consists of sociability test—tendency to initiate social contact and a social novelty preference test—tendency to initiate social contacts with a new individual as compared to someone familiar from past experience (Moy *et al.*, 2004). This test has been successfully employed to study social affiliation and social memory in several inbred and mutant mouse lines as reported

by various studies (Clapcote *et al.*, 2007; Labrie *et al.*, 2008; Kaidanovich-Beilin *et al.*, 2009; Crawley, 2007a). Sociability is defined as the subject mice spending more time in the chamber containing the novel target mouse than in the chamber containing the inanimate novel object. Preference for social novelty is the propensity to spend time with a previously unencountered mouse rather than with a familiar mouse (Moy *et al.*, 2004; Silverman *et al.*, 2010).

PME and DZP treated mice spent more time in the side of the social test box containing the unfamiliar stranger than in the empty side. Mice treated with PME and DZP spent more time sniffing the wire cage containing the stranger than the empty wire cage reflecting social approach behaviour. In the social preference test, mice treated with PME and DZP increased preference (increased time spent in chamber and sniffing) for the novel unfamiliar conspecific placed in the formerly empty side (stranger 2). This was interpreted as the ability of mice to discriminate between the two strangers, and to recognize the one that had not been encountered before (Moy *et al.*, 2004). The increased sociability and preference for social novelty in PME and DZP treated mice is therefore indicative of an increased social interaction. Unlike PME and DZP, PTZ decreased sociability and social novelty preference in mice.

The number of entries between compartments provides an independent measure of general exploratory locomotion (Silverman *et al.*, 2010). Mice treated with test compounds had no effect on number of entries; showing general exploratory activity did not appear to affect sociability and social novelty preference. An increase in social interaction, without a concomitant increase in motor activity, is indicative of an anxiolytic effect, whereas a specific decrease in social interaction indicates an anxiogenic effect (File and Seth, 2003). Therefore from the present study, like DZP (Kita *et al.*, 2004), PME exhibited an anxiolytic-like effect whereas pentylenetetrazole had an anxiogenic action (File and Lister, 1984) in the social interaction test.

Stress-induced hyperthermia (SIH) in singly housed male mice appears to be a robust, reproducible and easy paradigm to study putative anxiolytic-like effects of drugs (Van der Heyden *et al.*, 1997; Olivier *et al.*, 2002). Reduction of SIH (ΔT) by a drug is interpreted as anti-stress or anxiolytic like effect (Zethof *et al.*, 1995; Van der Heyden *et al.*, 1997; Pattij *et al.*, 2001; Olivier *et al.*, 2002). An unchanged stress-induced hyperthermic response indicates absence of any effect on anxiety or stress, whereas an increased SIH

could indicate an anxiogenic-like effect (Zethof *et al.*, 1995; Olivier *et al.*, 2003). Clinically effective anxiolytic compounds such as benzodiazepines (including diazepam, alprazolam, oxazepam, and chlordiazepoxide) and 5-HT_{1A} receptor agonists (such as buspirone and flesinoxan) decrease the SIH, suggesting GABAergic and serotonergic mechanisms underlie SIH (Pattij *et al.*, 2001; Olivier *et al.*, 2002; 2003). However, non-anxiolytic drugs including dopaminergic and noradrenergic compounds do not influence the SIH response (Bouwknecht *et al.*, 2007). From the present study, PME and diazepam significantly antagonized stress-induced hyperthermic response indicative of an anti-stress or anxiolytic like effect. This further confirms the fact that PME has anxiolytic like effect and could be acting via GABAergic and serotonergic mechanisms.

In the beam walk test, motor coordination and balance of mice are assessed by measuring the ability of the mice to traverse a narrow beam to reach an enclosed safety platform or goal box (Brooks and Dunnett, 2009; Drucker-Colín, 2010). In this study, unlike diazepam, PME at the doses used did not impair motor coordination in the beam walk test indicating an anxiolytic-like effect without motor impairment.

7.5 CONCLUSION

Results of the present study indicate that PME possess anxiolytic-like effects without affecting motor impairments.

Chapter 8 ACUTE AND SUBACUTE TOXICITY STUDIES

8.1 INTRODUCTION

Over the last few years, there has been a tremendous rise in the acceptance and public interest in natural therapies both in developing and developed countries. It is estimated that about 75 % of the world population—primarily those of developing countries—rely on traditional remedies (mainly herbs) for the healthcare of its people (Gilani and Atta ur, 2005). In developed countries, herbal therapies are viewed as natural, time-tested and therefore safe compared with what are perceived as synthetic drugs. Also, in developing countries, there may be access to herbal therapies but not to pharmaceuticals, because of cultural and economic factors (Schachter, 2009).

Although some herbal medicines have promising potential and are widely used, many of them remain untested and their use also not monitored. This makes knowledge of their potential adverse effects very limited and identification of the safest and most effective therapies difficult (WHO, 2002). Herbs have been shown to be capable of producing a wide range of undesirable or adverse reactions some of which are capable of causing serious injuries, life-threatening conditions, and even death. It is therefore important to assess the toxicity of herbal medicines.

Although various parts of *Pseudospondias microcarpa* are extensively used in African traditional medicine, no scientific study has been reported on its toxicity. Therefore, the present study determined acute and sub-acute toxicity of *P. microcarpa* in rats.

8.2 METHODS

8.2.1 Acute Toxicity Study

Male Sprague-Dawley rats (120-150 g) were orally treated with the extract (30, 100, 300, 1000 and 3000 mg kg^{-1}) or distilled water (10 ml kg^{-1}) and placed in observation cages. The rats were evaluated for general pharmacological and physiological behaviours as well as mortality at 0, 15, 30, 60, 120, 180 min, up to 24 h after treatment.

8.2.2 Sub-acute toxicity

Sprague-Dawley rats were randomized into six groups with 5 animals each. Five experimental groups were administered PME orally at doses of 30, 100, 300, 1000 and 3000 mg kg^{-1} for 2 weeks. The control group received distilled water orally at the volume

of 10 mL kg^{-1}. During the experimental period, animals were observed daily for general appearance and signs of toxicity.

8.2.2.1 *Preparation of serum and isolation of organs*

At the end of the study, animals were fasted overnight and sacrificed by cervical dislocation. About 1.5 mL of blood was collected into vacuum tubes containing 2.5 µg of ethylenediamine tetra acetic acid (EDTA) as an anticoagulant for haematological assay and 3.5 ml into sample tubes containing a separating gel for biochemical parameters. The blood for the biochemical parameters was centrifuged (4000 rpm at 4 °C for 10 min) to obtain serum and stored at -20 ºC. Organs harvested included liver, kidney, brain, stomach, heart and spleen.

8.2.2.2 *Haematological Assays*

Haematological analysis was performed using the haematological analyser ABX micros ES 60 (HORIBA Medical Diagnostics, France). The parameters examined included white blood cells (WBC), red blood cells (RBC), haematocrit (HCT), haemoglobin (HGB), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), lymphocytes (LMP), platelet distribution width (PDW), red cell (erythrocyte volume) distribution width , relative volume of thrombocytes (PCT), platelets (PLT) and mean platelet (thrombocyte) volume (MPV).

8.2.2.3 *Biochemical Assay*

Biochemical values were measured with a Cobas integra 400 (Hoffmann-La Roche Ltd., Basel, Switzerland), which assessed levels of alkaline phosphatase (ALP), alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), gamma-glutamyl tranferase (GGT), total protein, albumin, globulin, total bilirubin (T-Bil), direct bilirubin (D-Bil), indirect bilirubin (I-Bil), creatinine, urea, triglyceride (TG), cholesterol, high density lipoproteins (HDL), low density lipoproteins (LDL) and very low density lipoproteins (VLDL).

8.2.2.4 *Body and organ weight assessment*

Brain, liver, kidney, stomach, heart and spleen were isolated and weighed. Body weights of the rats were taken on days 0 and 15. Relative organ weight (ROW) was then calculated.

$$\text{ROW} = \frac{\text{absolute organ weight (g)}}{} \times 100$$

body weightof animalon sacrifice day (g)

8.2.2.5 *Histopathological examinations*

Sections of the tissue from liver, kidney, spleen, brain, heart and stomach were used for histopathological examination. Tissues were fixed in 10 % neutral buffered formalin (pH 7.2) and dehydrated through a series of ethanol solutions, embedded in paraffin and routinely processed for histological analysis. Sections of 2 µm thickness were cut and stained with haematoxylin-eosin for examination. The stained tissues were observed through an Olympus microscope (BX-51) and photographed by INFINITY 4 USB Scientific Camera (Lumenera Corporation, Otawa, Canada).

8.2.3 **Statistics**

Data were presented as mean±SEM. The presence of significant differences among means of groups was determined by one-way ANOVA using GraphPad Prism for Windows version 5 (GraphPad Software, San Diego, CA, USA). Significant difference between pairs of groups was calculated using the Newman-Keuls' multiple comparison test.

8.3 **RESULTS**

8.3.1 **Acute toxicity**

Treatment of rats with the extract produced sedation and analgesia at all doses used. No deaths were recorded over the 24 h observation period, indicating an LD_{50} above 3000 mg kg^{-1} (Table 8.1).

Table 8.1 Effects of *Pseudospondias microcarpa* hydroethanolic leaf extract in the primary observation test in rats

Dose (mg kg^{-1})	Mortality D/T	Effects
0	0/5	no change
30	0/5	sedation, analgesia
100	0/5	sedation, analgesia
300	0/5	sedation, analgesia

1000	0/5	sedation, analgesia
3000	0/5	sedation, analgesia

D/T: Dead/Treated

8.3.2 **Subacute toxicity**

No deaths were recorded after 14 days of treatment with the extract. Other signs of toxicity were absent except sedation which was observed throughout the treatment period.

8.3.2.1 ***Effect of extract on body weight and organ weight***

The extract had no significant effect on body weight change although this parameter was decreased at the highest dose (3000 mg kg^{-1}). Treatment with the extract increased weight of the spleen at 100, 300 and 1000 mg kg^{-1} (all at $P<0.05$) when compared to the control group (figure 8.1).

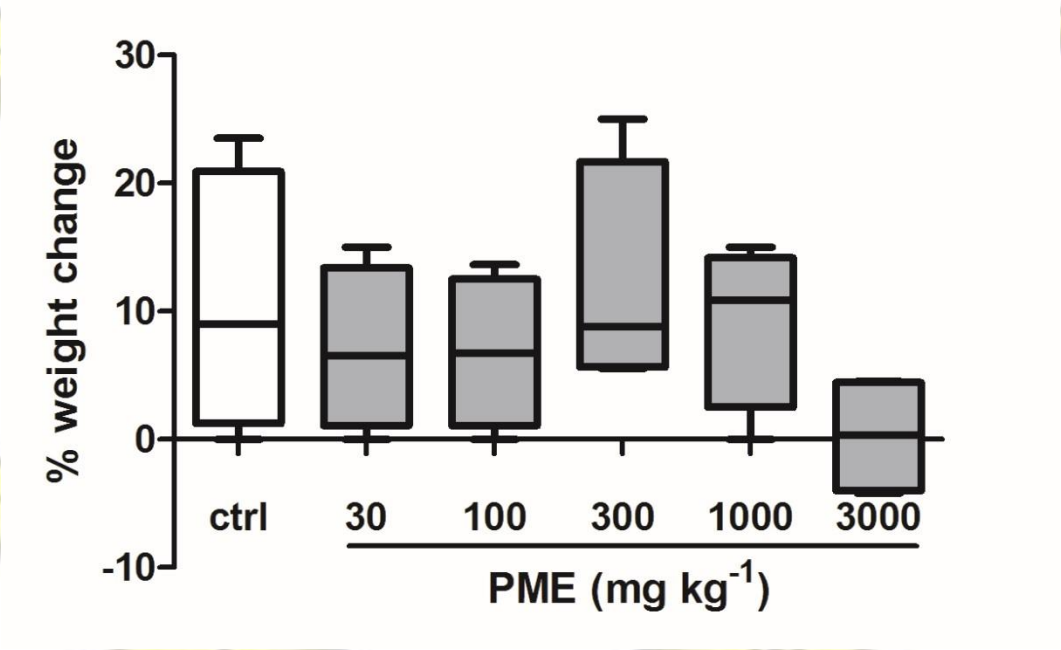


Figure 8.1 Effect of 14-day treatment of PME on the % change in body weights of rats in the sub-acute toxicity test. Data are expressed as mean±SEM (n=5). Treated groups were compared to controls with a one-way ANOVA followed by Newman-Keuls' test. The lower and upper margins of the boxes represent the 25th and 75th percentiles, while the extended arms represent the 10th and 90th percentiles respectively.

Table 8.2 Effects of PME on relative organ weights (ROW) of rats in the sub-acute toxicity test

Organs	PME (mg kg^{-1})

	control	30	100	300	1000	3000
Heart	0.39±0.01	0.36±0.02	0.36±0.01	0.37±0.01	0.37±0.02	0.37±0.01
Liver	3.03±0.26	2.66±0.11	2.90±0.09	3.05±0.18	3.05±0.10	2.75±0.05
Kidney	0.65±0.01	0.62±0.02	0.68±0.01	0.72±0.02	0.70±0.02	0.66±0.02
Spleen	0.48±0.04	0.44±0.02	0.65±0.02*	0.69±0.09*	0.69±0.02*	0.45±0.03
Stomach	1.08±0.05	1.04±0.04	1.13±0.08	1.17±0.07	1.13±0.04	1.07±0.05
Brain	0.93±0.03	0.87±0.02	0.94±0.03	1.01±0.06	1.00±0.06	0.88±0.05

Data are presented as mean±SEM (n=5). *P<0.05 considered statistically significant from control.

8.3.2.2 ***Effect of extract on haematological parameters***

Except for lymphocytes (%) which showed a significant decrease ($F_{5,23}$=3.93, P=0.013), all other parameters remained unaffected by the extract (table 8.3).

Table 8.3 Effect of 14-day treatment of PME on haematological parameters in rats

Parameters	PME (mg kg^{-1})					
	control	30	100	300	1000	3000
WBC (10^3/mm^3)	4.90±1.66	4.63±1.18	5.10±1.32	4.68±0.36	5.40±1.19	6.40±1.28
LYM (%)	90.33±4.02	86.85±1.12	77.0±2.66*	73.3±2.07*	75.7±2.07*	74.8±3.14*
RBC (10^6/mm^3)	7.49±1.07	7.21±0.21	6.41±0.19	6.33±0.46	6.54±0.39	7.28±0.23
HGB (g/dL)	13.08±1.57	12.68±0.33	11.43±0.19	11.20±0.88	12.00±0.90	12.58±0.55
HCT (%)	43.73±5.27	41.83±2.19	37.40±1.43	37.78±2.48	38.43±0.66	40.25±1.67
MCV (µm^3)	59.00±2.04	57.75±1.65	58.25±0.94	60.25±2.06	59.25±3.04	55.25±1.03
MCH (pg)	17.58±0.51	17.58±0.41	17.85±0.29	17.68±0.18	18.25±0.48	17.28±0.44
MCHC (g/dL)	30.33±0.60	30.45±0.91	30.70±0.86	29.60±0.99	31.10±1.98	31.20±0.89
RDW (%)	16.03±0.87	16.25±0.87	16.70±0.68	16.68±0.57	15.93±0.45	13.80±1.15
PLT (10^3/mm^3)	883.3±52.71	963.0±165.7	800.0±127.3	848.5±103.8	875.5±139.5	850.8±89.91
MPV (µm^3)	6.80±0.30	7.43±0.27	6.78±0.39	6.73±0.32	6.95±0.33	7.58±0.25
PCT (%)	0.60±0.04	0.71±0.12	0.54±0.07	0.58±0.09	0.62±0.11	0.64±0.06
PDW (%)	10.85±0.66	10.85±1.77	10.93±0.96	11.63±0.52	10.30±2.52	9.15±2.30

Data are presented as mean±SEM (n=5). *P<0.05 considered statistically significant from control.

8.3.2.3 ***Effect of extract on biochemical parameters***

As shown in table 8.4, alanine transaminase (ALT) and aspartate transferase (AST) levels were decreased although not statistically significant when compared to the control group. Bilirubin levels at the doses of 30 and 100 mg kg^{-1} were also decreased although ANOVA showed no significant difference. ANOVA showed a significant decrease in the levels of triglyceride ($F_{5,23}$=3.086, P=0.034) and VLDL ($F_{5,23}$=3.834, P=0.015) with NewmanKeuls' *post hoc* analysis revealing significance at 100 mg kg^{-1} (both at P<0.05).

Table 8.4 Effect of 14-day treatment of PME on biochemical parameters in rats

Parameters	control	PME (mg kg^{-1})				
		30	100	300	1000	3000
AST (U/L)	244.9±43.44	273.30±9.17	191.70±11.1	250.0±23.06	196.40±8.28	200.90±3.82
ALT (U/L)	122.4±30.54	103.1±17.78	77.70±7.96	106.6±10.85	86.15±6.86	95.0±23.82
ALP (U/L)	171.6±28.38	207.3±38.76	176.4±14.01	207.3±31.67	197.3±35.09	168.7±22.60
GGT (U/L)	0.97±0.33	0.78±0.24	1.45±0.47	1.20±0.33	0.95±0.22	1.43±0.43
Total pr (g/L)	66.98±0.47	67.23±2.63	61.63±2.60	63.08±1.78	62.80±2.25	64.18±1.46
Albumin (g/L)	43.83±1.11	45.33±2.20	41.83±1.73	41.95±1.73	39.88±2.13	38.30±1.13
Globulin (g/L)	23.10±1.14	21.73±1.51	19.80±0.91	21.15±0.79	22.93±3.81	25.88±2.11
Bil.(µmol/L)	1.00±0.39	0.62±0.12	0.48±0.16	1.13±0.44	0.88±0.34	0.78±0.26
D-Bil (µmol/L)	0.33±0.13	0.22±0.05	0.18±0.05	0.45±0.24	0.28±0.11	0.20±0.07
I-Bil (µmol/L)	0.67±0.26	0.40±0.08	0.30±0.11	0.68±0.34	0.60±0.23	0.58±0.19
Urea (mmol/L)	6.85±0.94	7.33±0.47	7.61±0.67	7.74±0.40	8.40±0.87	8.54±0.43
Creat (mmol/L)	47.25±6.20	48.75±6.32	46.25±5.43	53.75±7.56	41.75±5.89	51.25±5.39
Chol (mmol/L)	2.20±0.14	2.36±0.16	1.97±0.13	2.20±0.28	2.20±0.17	1.89±0.07
TG (mmol/L)	0.60±0.03	0.57±0.05	0.4±0.01*	0.56±0.04	0.61±0.06	0.53±0.02
HDL (mmol/L)	1.70±0.02	1.86±0.10	1.62±0.10	1.56±0.10	1.64±0.13	1.57±0.07
LDL (mmol/L)	0.20±0.10	0.23±0.06	0.18±0.04	0.39±0.02	0.29±0.08	0.25±0.15
VLDL(mmol/L)	0.20±0.01	0.26±0.02	0.1±0.01*	0.24±0.02	0.27±0.02	0.23±0.01
Coronary risk	1.30±0.07	1.25±0.05	1.22±0.02	1.42±0.12	1.35±0.06	1.17±0.02

Data are presented as mean±SEM (n=5). *P<0.05 considered statistically significant from control.

8.3.2.4 *Histopathological changes*

Plates 8.1-8.6 show the photomicrographs of sections of the isolated organs of control and PME-treated rats for the 14-day sub-acute toxicity study. Histopathological evaluation of the organs isolated from rats sacrificed at the end of the sub-acute toxicity study revealed no significant extract-related morphological changes compared to the control animals. Sections from the splenic tissue (Plate 8.3) were essentially normal, showing preservation of the lymphoid follicles. A few follicular enlargement as well as minimal dilation of the sinusoids was observed. There was no demonstrable morphological change in the heart sections compared with the control group as normal cardiac myocytes and blood vessels were seen. All sections of the kidneys showed essentially normal glomerulus, tubules and blood vessels. Inflammation and necrosis were also absent. Furthermore, no casts in the tubules as well as other deposits in the kidneys was observed. Brain sections showed well preserved brain tissue. No significant morphological changes like necrosis and red neurons were observed in all the doses of the extract as compared to the control. With regards to the stomach, there was essentially no ulceration and inflammation. In addition, the mucosa glands and muscularis propia showed no abnormalities. At all the doses used, the extract showed no tendency to induce gastritis. As shown in table 8.5, the specific morphological changes assessed in the liver included fatty change, hydropic swelling, inflammation and fibrosis. The extract-induced inflammation was mainly restricted to the portal triad with occasional spill over into the hepatocytes in all the doses. Mild fibrosis (at 3000 mg kg^{-1}) and mild fatty change (at 1000 and 3000 mg kg^{-1}) was observed in some liver sections. In addition, hydropic swelling was observed in all rats treated with PME at 1000 and 3000 mg kg^{-1}. The toxic effect of the extract on the liver is relatively mild and only trace evidence is seen at the high doses (1000 and 3000 mg kg^{-1}).

Table 8.5 Histopathological results of the liver in rats orally treated with PME for 2 weeks

Dose (mg kg^{-1})	Fatty change	Hydropic swelling	Mild inflammation	Mild fibrosis
Saline	0/4	0/4	0/4	0/4
30	0/4	0/4	0/4	0/4
100	0/4	0/4	4/4	0/4

300	0/4	1/4	4/4	0/4
1000	2/4	4/4	4/4	0/4
3000	2/4	4/4	4/4	2/4

Results are expressed as the number of rats with pathological findings per total number of rats sectioned.

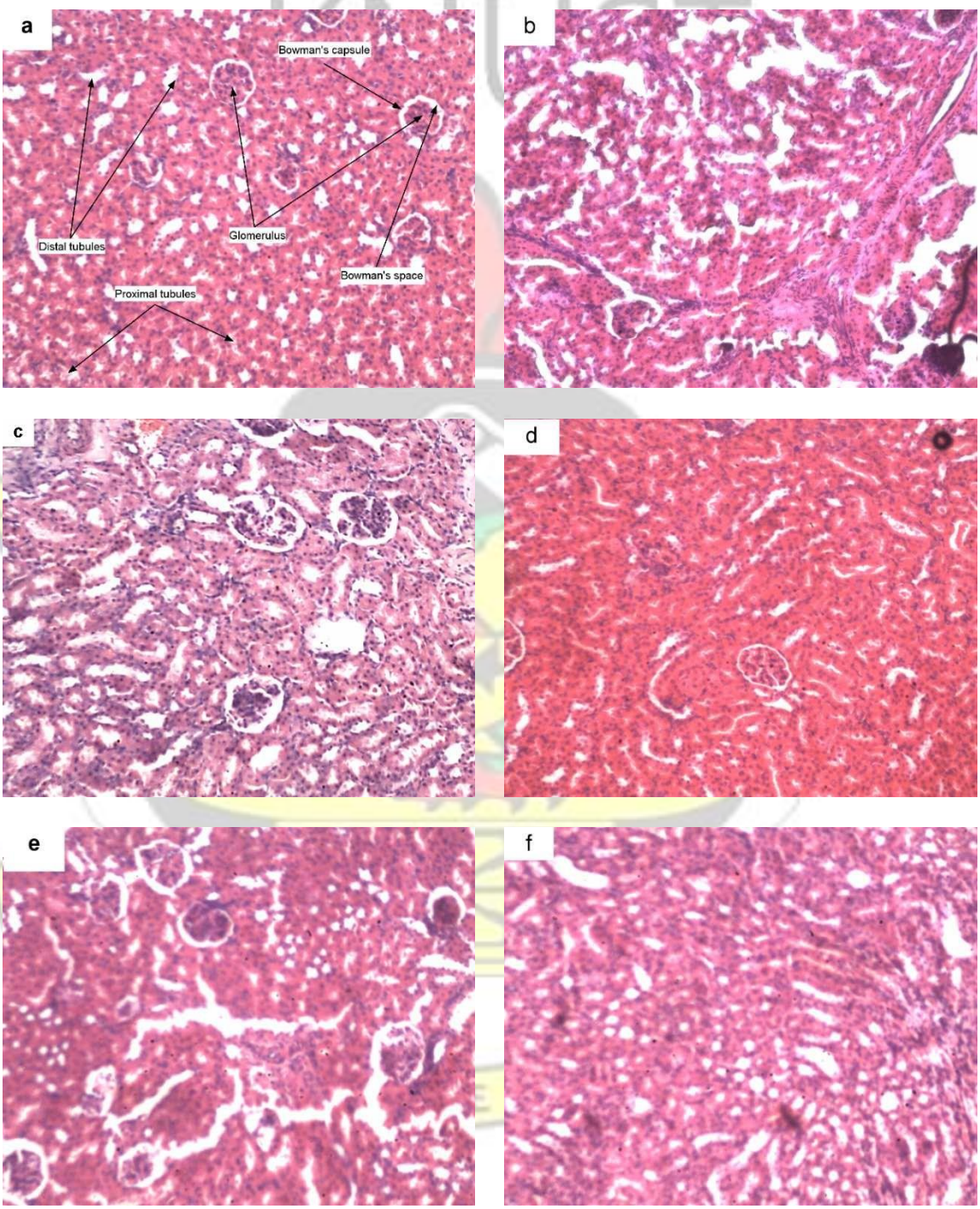


Plate 8.1 Photomicrograph of the sections of the kidney in control rats (a), and rats treated orally with 30 mg kg^{-1} (b), 100 mg kg^{-1} (c), 300 mg kg^{-1} (d), 1000 mg kg^{-1} (e) and 3000 mg kg^{-1} (f) of the extract for 14 days in the sub-acute toxicity study (H & E, ×400).

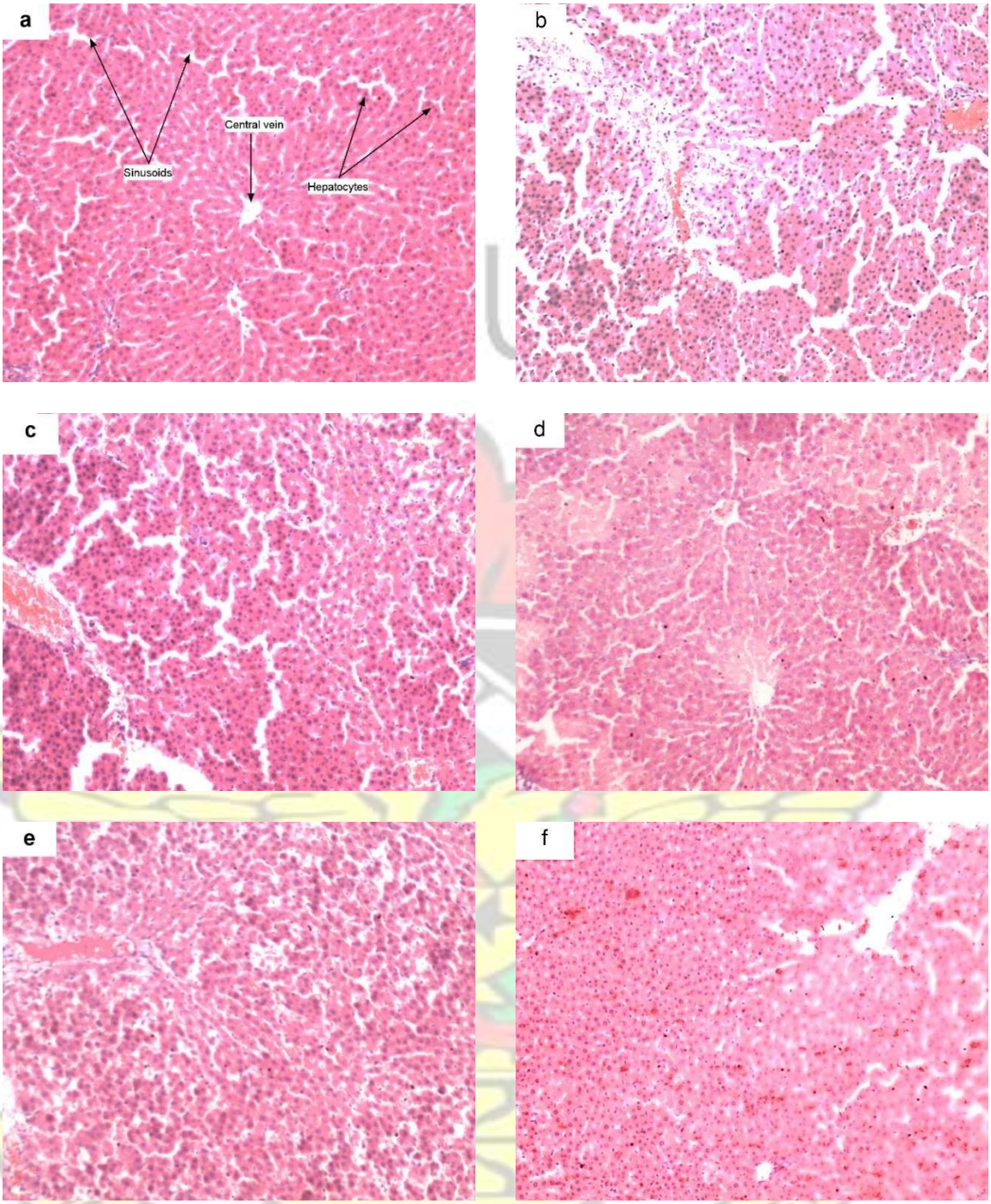


Plate 8.2 Photomicrograph of the sections of the liver in control rats (a), and rats treated orally with 30 mg kg^{-1} (b), 100 mg kg^{-1} (c), 300 mg kg^{-1} (d), 1000 mg kg^{-1} (e) and 3000 mg kg^{-1} (f) of the extract for 14 days in the sub-acute toxicity study (H & E, ×400).

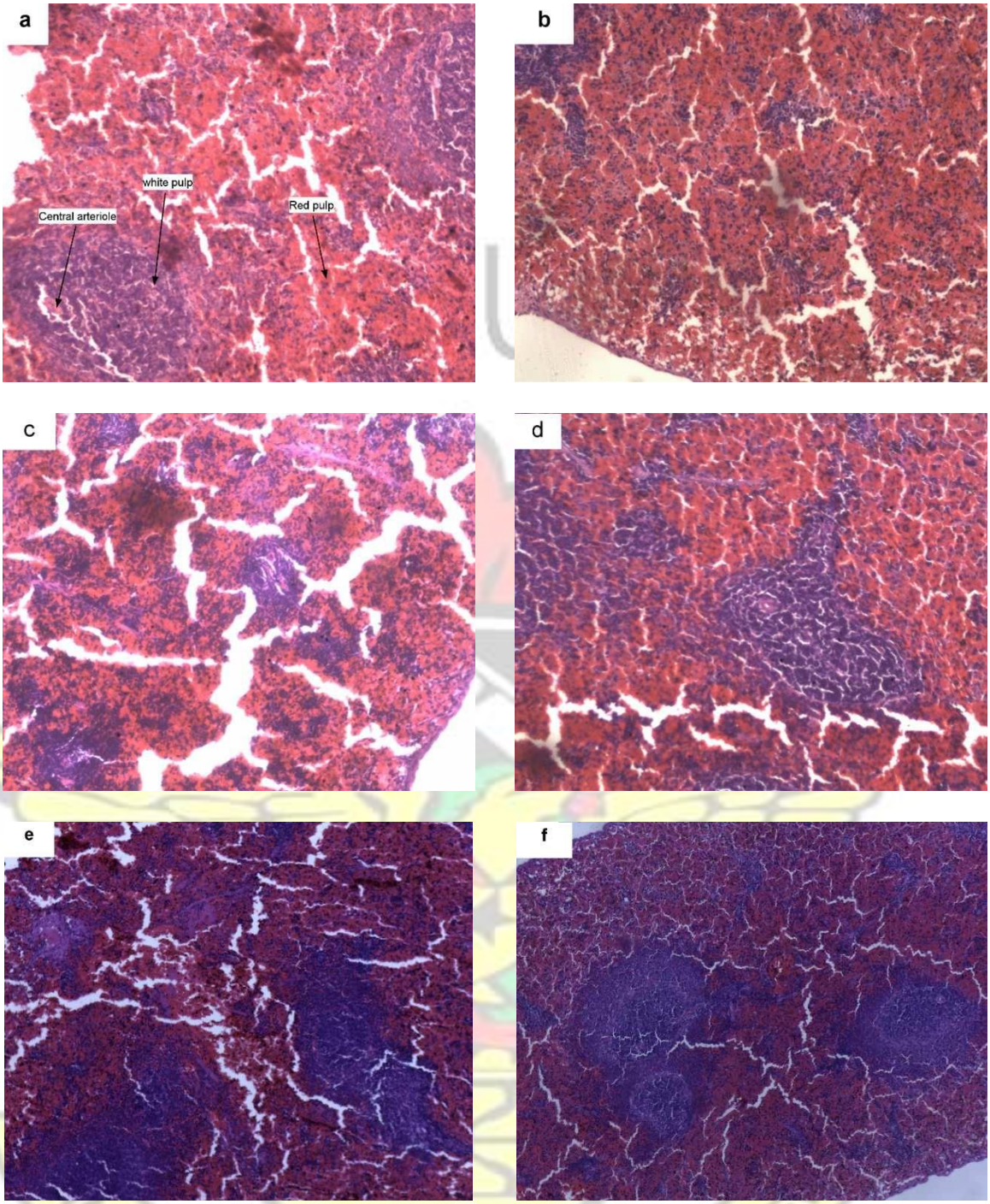


Plate 8.3 Photomicrograph of the sections of the spleen in control rats (a), and rats treated orally with 30 mg kg^{-1} (b), 100 mg kg^{-1} (c), 300 mg kg^{-1} (d), 1000 mg kg^{-1} (e) and 3000 mg kg^{-1} (f) of the extract for 14 days in the sub-acute toxicity study (H & E, ×400).

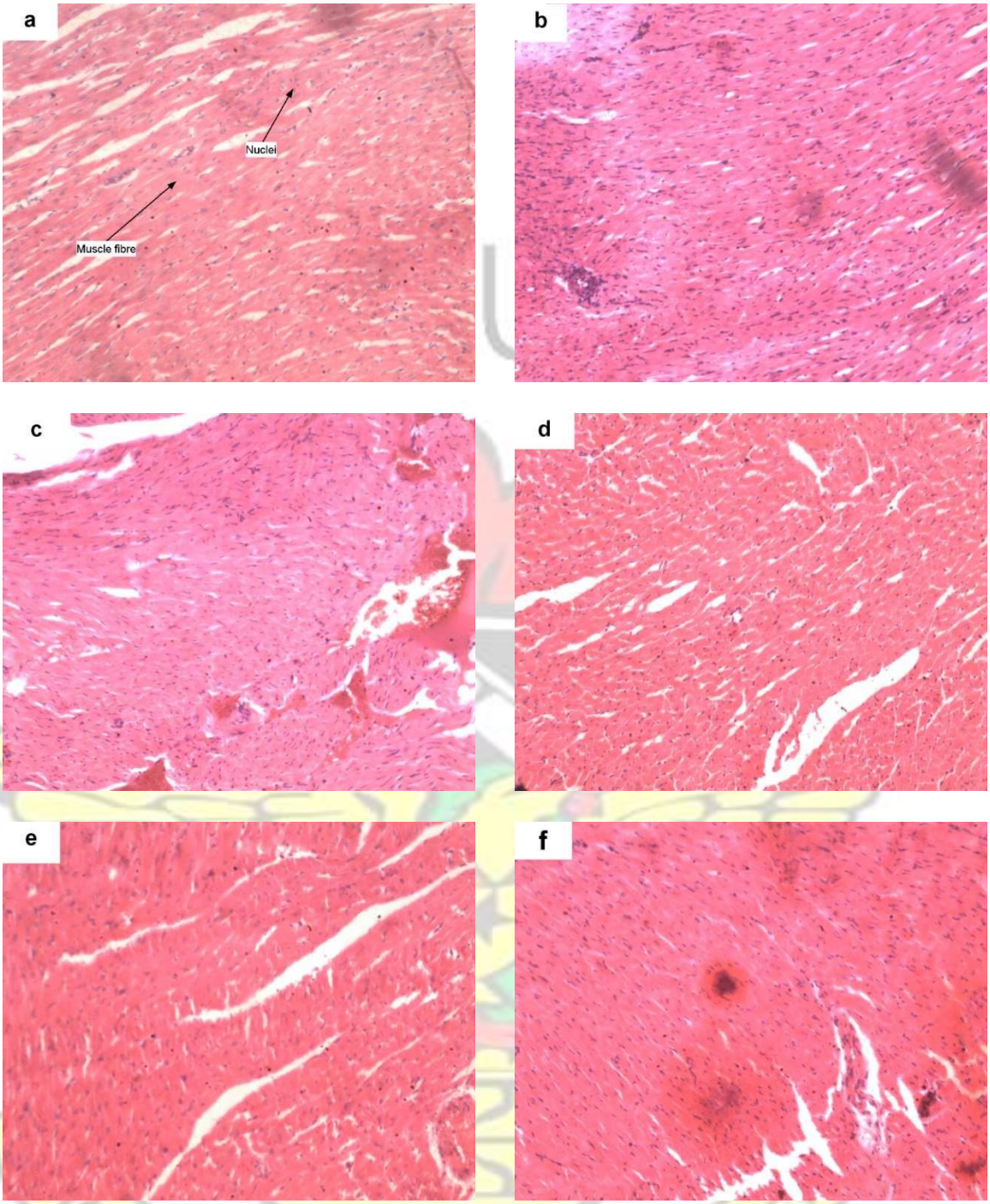


Plate 8.4 Photomicrograph of the sections of the heart in control rats (a), and rats treated orally with 30 mg kg^{-1} (b), 100 mg kg^{-1} (c), 300 mg kg^{-1} (d), 1000 mg kg^{-1} (e) and 3000 mg kg^{-1} (f) of the extract for 14 days in the sub-acute toxicity study (H & E, ×400).

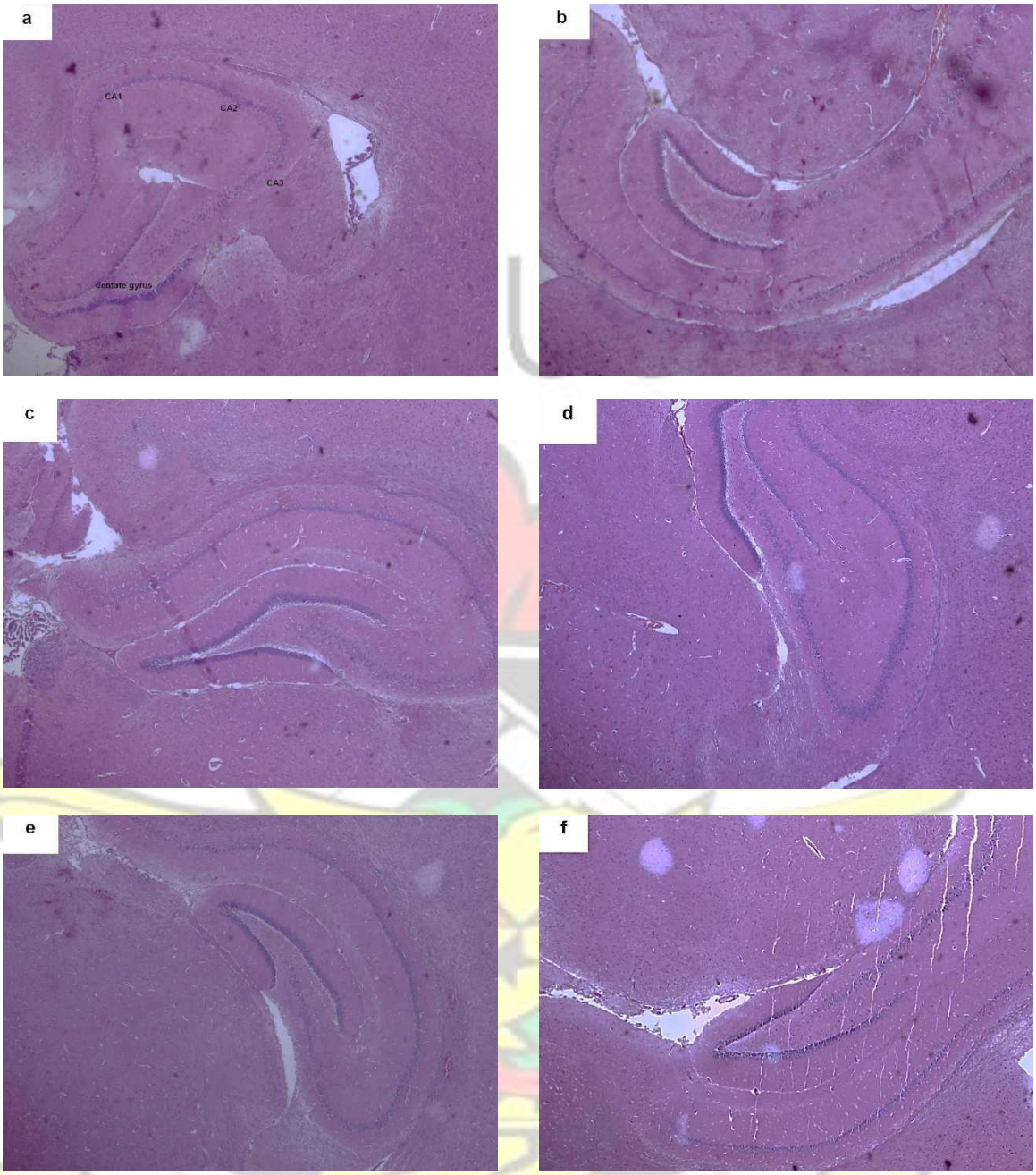


Plate 8.5 Photomicrograph of the sections of the brain in control rats (a), and rats treated orally with 30 mg kg^{-1} (b), 100 mg kg^{-1} (c), 300 mg kg^{-1} (d), 1000 mg kg^{-1} (e) and 3000 mg kg^{-1} (f) of the extract for 14 days in the sub-acute toxicity study (H & E, ×400).

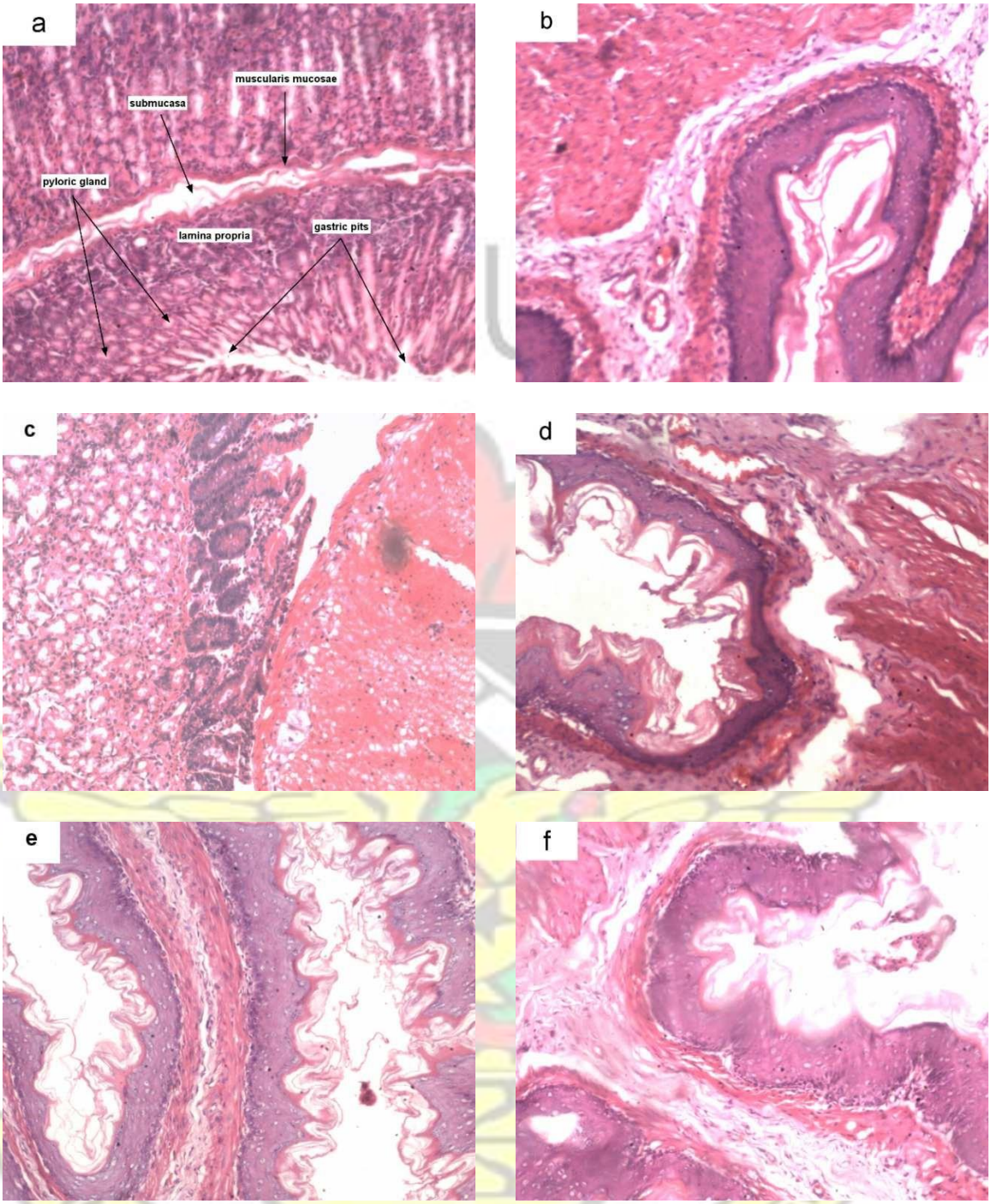


Plate 8.6 Photomicrograph of the sections of the stomach in control rats (a), and rats treated orally with 30 mg kg^{-1} (b), 100 mg kg^{-1} (c), 300 mg kg^{-1} (d), 1000 mg kg^{-1} (e) and 3000 mg kg^{-1} (f) of the extract for 14 days in the sub-acute toxicity study (H & E, ×400).

8.4 DISCUSSION

Medicinal plants are often considered safe for human use due to their natural origin.

However, various reports suggest the potential risks involved with such plants (Jordan *et al.*, 2010). Thus, there is the need to assess the safety of these medicinal plants before use. This study therefore assessed both acute and sub-acute toxicity studies of PME in rats.

In the acute toxicity study, rats treated with PME showed signs of sedation and analgesia, suggesting possible central depressant and analgesic effects. The LD_{50} of the plant extract, given orally, was found to be above 3000 mg kg^{-1}. At the relatively high doses used, the plant extract caused no mortality and appeared to cause no apparent toxicity suggesting that PME is relatively non-toxic.

In toxicological evaluation, it is important to assess sub-acute toxicity profile of test compounds since repeated dosing helps to evaluate morphological and physiological changes in organs (Kouadio *et al.*, 2014). Therefore, a sub-acute toxicity study was performed in rats and similar to observations in the acute toxicity test, treatment of the extract for 14 days caused no mortality. In addition, water and food consumption was normal.

In general, changes in the body weights of animals can be used as an indicator of adverse effects of drugs and chemicals (Adedapo *et al.*, 2005; Arsad *et al.*, 2013). In this study, the change in body weight of animals treated with the extract was not significant when compared to the control group indicating absence of any severe adverse effects. Organs such as the heart, liver, spleen, kidney, brain, stomach and lungs are the primary organs affected by the metabolic reactions induced by toxicants. Therefore, the relative organ weight is an important index of physiological and pathological status in man and animals (Dybing *et al.*, 2002; Jothy *et al.*, 2011; Konate *et al.*, 2012). There was no significant difference in the organ-to-body ratio of the various organs when compared to the control group, except for the spleen which showed an increase. However, gross pathological examination of the extract-treated groups did not reveal any abnormalities, presence of lesions or changes in the colour of the spleen.

Analysis of blood parameters in animal studies is relevant to evaluate the risk of alterations of the haematological system in human toxicity and also to explain blood relating functions of a plant extract or its products (Olson *et al.*, 2000; Afzan *et al.*, 2012; Yakubu and Afolayan, 2009). In this study, no significant difference was found in the majority of haematological parameters between the treated and the control groups except for the observed decrease in lymphocytes (%). Lymphocytes are the main effector cells of the immune system and thus protect the body from infections (Hayes and Kruger, 2014). The

reduction in lymphocytes in the present study may therefore affect the effector cells of the immune system indicating that the body is low on infection resistance. Parameters including MCHC, MCH and MCV relate to individual red blood cells whereas HGB, RBC, PCV and RCDW are linked to the total population of red blood cells. Therefore, the unchanged effect of the extract on these parameters may imply that neither the incorporation of haemoglobin into red blood cells nor the morphology and osmotic fragility of the red blood cells was altered (Adebayo *et al.*, 2005; Ashafa *et al.*, 2011). This excludes the possibility of anaemia or disturbances linked to erythrocytes and indicates non-toxic effects of PME to the haematopoietic system.

Liver and kidney functions tests as well as serum lipid profile are important parameters in determining the safety of functional ingredient or plant extracts (Patel *et al.*, 2008; Syahida *et al.*, 2012). To evaluate kidney function, measurements of urea and creatinine levels in the blood are usually performed (Emeigh Hart, 2005). These two parameters are usually markedly increased to four or five times higher than the normal values in control animals in cases of acute or chronic renal toxicity (Arsad *et al.*, 2013). This study shows that treatment of *Pseudospondias microcarpa* extract in rats for 14 days does not produce possible kidney malfunction since the biomarkers of kidney function (urea and creatinine) were not affected.

The activities of AST and ALT are the most sensitive tests employed in the diagnosis of hepatic diseases (Kim *et al.*, 2005). When liver cell plasma is damaged, various enzymes normally located in the cytosol are released into the blood, thereby causing increased enzyme levels in the serum (Brinda *et al.*, 2013; Shenoy *et al.*, 2001). Thus, increased levels of ALT, AST, ALP, and GGT may be interpreted as a result of liver cell destruction or changes in the membrane permeability (Lim *et al.*, 2000; Kim *et al.*, 2005). Estimation of these enzymes in the serum is therefore a useful quantitative marker of the extent and type of hepatocellular damage. Treatment with the extract had no effect on the levels of these serum enzymes indicating non-toxic effects on liver function.

An important physiologic role of the liver is the removal of toxic endogenous and exogenous substances from the blood. Thus, tests based on excretory functions of the liver are related to bilirubin metabolism (Naik, 2012). Bilirubin is the product of haem following the breakdown of red blood cells by phagocytic cells. It is carried by serum albumin to the liver where most of it is conjugated with glucuronide prior to excretion into the bile. Increased levels in the blood results in jaundice and could be due to increased haemolysis

of red blood cells, primary hepatocellular damage or mechanical biliary duct obstruction (Jansen and Bittar, 2004). Therefore, this metabolite serves as a good indicator to assess the functional capacity of the liver. In this study, serum levels of direct, indirect and total bilirubin after treatment with the extract for 14 days were not elevated indicating that PME did not have any deleterious effects on hepatic metabolism or biliary excretion. However, although not significant, the extract decreased levels of direct, indirect and total bilirubin especially at the low doses used (30 and 100 mg kg^{-1}). The bilirubin-lowering effect as well as decreased ALT levels could suggest possible hepatoprotective effects of the extract.

Cholesterol is an essential constituent of most biological membranes, besides acting as a precursor for the synthesis of bile acids, hormones and vitamins (Repa and Mangelsdorf, 2000; Zarzecki *et al.*, 2014). However, increased levels of cholesterol leads to atherosclerosis, hyperproteinaemia, cirrhosis, haemolytic jaundice, malnutrition and increased risk of cardiovascular diseases (Sodipo *et al.*, 2011; Woo *et al.*, 2009). Low density lipoprotein is a primary carrier of plasma cholesterol and is often referred to as bad cholesterol since increased levels cause atherosclerosis (Toyin *et al.*, 2008). In addition, elevated LDL levels have been reported to be associated with hepatic lesion and damage (Edrington *et al.*, 1995; Brinda *et al.*, 2013). Furthermore, increased levels of serum triglyceride leads to hyperlipidaemia and low levels imply that there is no risk factor related to atherosclerosis (Sodipo *et al.*, 2011). In the present study, decreased levels of triglycerides, LDL and VLDL particularly at the low dose of 100 mg kg^{-1} coupled with normal cholesterol levels further confirm normal liver function and decreased risk of atherosclerosis. The decreased levels of VLDL and LDL cholesterols could also indicate hypolipidermic effects of the extract.

Evaluation of pathological alterations induced in laboratory animals by novel drugs represents the basis of their safety assessment before they can be used in the clinical setting and this is largely based on conventional histopathological techniques (Greaves, 2011; Prabu *et al.*, 2013). Therefore, histological analysis was done to further examine the pathological state of the various organs. Except for the liver which showed mild fatty change, hydropic swelling and mild inflammation at the highest doses (1000 and 3000 mg kg^{-1}), detailed gross and histopathological examination of the various organs showed no significant pathological changes. These changes in liver are mild and may not be considered clinically significant since serum levels of hepatic enzymes (ALT and AST), which are considered markers of liver function, were not significantly elevated. However,

caution should be taken in using this extract beyond 3000 mg kg^{-1}. In addition, chronic toxicity studies should be done in rats and other species to ascertain the safety of the plant extract.

8.5 CONCLUSION

Results of this study shows that oral administration of the ethanolic leaf extract of *Pseudospondias microcarpa* is relatively safe in rats. However, caution should be exercised when extrapolating this results to man.

Chapter 9 GENERAL DISCUSSION AND CONCLUSIONS

9.1 GENERAL DISCUSSION

The present study has demonstrated that the ethanolic leaf extract of *Pseudospondias microcarpa* possesses anticonvulsant, antidepressant, anxiolytic and sedative effects without affecting neuromuscular function. This study has also established that the extract exerts its antidepressant effect through the serotoninergic system, L-arginine-NO-cGMP pathway as well as interacting with NMDA receptors. Moreover, the study also revealed that the extract possesses antidepressant effect in chronic models that mimics human depression and enhances memory and learning.

The two main neurotransmitters in the brain are L-glutamate (excitatory) and gammaamino butyric acid (inhibitory) and an abnormal function of either of these could result in a seizure (Avanzini and Franceschetti, 2003). Impairment of GABA function is widely recognized to provoke seizures, whereas facilitation has an anticonvulsant effect (Löscher, 1999). $GABA_A$ blockers (pentylenetetrazole and picrotoxin) are well-known epileptogenic agents and are commonly used in experimental studies. In the present study, PME blocked seizures induced by PTZ and picrotoxin indicating probable interaction with GABA receptors. In addition, the extract exhibited anticonvulsant effect against INH-induced convulsions, further confirming the GABA enhancing activity of the plant extract. The extract's effect on the GABAergic system was further confirmed using flumazenil as an antagonist. Flumazenil, a specific antagonist of the benzodiazepine site in the $GABA_A$BZD receptor complex, blocked the anticonvulsant effect of the extract. The extract, like diazepam, may therefore be acting via direct activation of benzodiazepine site of the $GABA_A$ receptor complex. Most GABAergic chemoconvulsants induce 'absence-like' and myoclonic seizures (Kubova, 2009). Therefore, PME could have activity against generalized myoclonic and absence seizures. The convulsant action of strychnine is through blockade of chloride channel associated with glycine receptors (Curtis *et al.*, 1971). Seizures induced by strychnine were blocked by the extract at the doses used. Thus, PME also exerts its anticonvulsant effect by enhancing the inhibitory effect of glycine at the glycine receptors. In the 4-AP test, PME increased the latency to seizures and reduced the incidence of mortality. Action of 4-AP occurs through a K^+-channel blockade at the presynaptic neuron level (Brito *et al.*, 2009) as well as enhancing glutamatergic neurotransmission (Tapia *et al.*, 1999). Thus, the anticonvulsant activity of PME against

seizures induced by 4-AP might be due to activation of K^+ channels and/or inhibition of the glutamate signal pathway. In this study, even though not effective in the MES test, PME protected against 6 Hz-induced psychomotor seizures indicating anticonvulsant activity against complex partial seizures. Thus, like levetiracetam (Surges *et al.*, 2008), PME could as well possess a distinct profile of activity from the commonly used antiepileptic drugs, suggesting possible efficacy in pharmacoresistant epilepsies.

It has also been demonstrated that decreased NO levels result in suppression of convulsions, and inhibition of NOS activity shows anticonvulsant property against pentylenetetrazole (PTZ)-induced seizures in rats. In addition, NO is a major stimulator of cGMP generation via soluble guanylate cyclase, which plays a major role in seizure (Snyder and Bredt, 1991), and methylene blue regulates intra-cellular cGMP concentrations by inhibiting soluble guanylate cyclase (Meller and Gebhart, 1993). Furthermore, sildenafil (a PDE5 inhibitor) enhances NO-mediated effects by inhibiting cGMP degradation in target tissues (Boolell *et al.*, 1996; Jackson *et al.*, 1999; Gholipour *et al.*, 2009). In this study, the anticonvulsant effect of PME was potentiated by L-NAME and methylene blue. In contrast, sildenafil or L-arginine inhibited its anticonvulsant effect indicating that PME could probably be acting as a NOS inhibitor and/or decreasing cGMP levels. Potential levels of interaction between GABA or NMDA and the NO system in the regulation of seizure susceptibility have been demonstrated (Paul, 2003; Paul and Subramanian, 2002; Manzoni *et al.*, 1992). Therefore, the influence of NO modulators in the anticonvulsant activity of PME could possibly be through its interaction with GABA and/or NMDA
receptors.

Depression represents one of the most common comorbidities in patients with epilepsy (Kanner and Balabanov, 2002; Harden, 2002). Both clinical and experimental evidence suggest that the imbalances in such neurotransmitters as GABA, glutamate, norepinephrine and serotonin, which are commonly observed in epilepsy patients, may concurrently contribute to the evolvement of depression (Kanner and Balabanov, 2002; Jobe, 2003; Kondziella *et al.*, 2007; Kanner, 2005). Therefore, the antidepressant effect of PME was investigated. The extract produced an antidepressant-like effect in both the FST and TST by decreasing immobility without affecting motor coordination. Thus, the antidepressant effect of the extract in addition to its anticonvulsant activity makes it advantageous in the

management of epileptic conditions comobid with depression. In the TST, PME did not significantly affect pedaling but caused an increase in time spent swinging and curling. Traditional antidepressants that inhibit serotonin and/or NA reuptake decrease immobility and increase swinging behaviour, while opioids, having decreased immobility, increase curling behaviour (Berrocoso *et al.*, 2012). Therefore, the increase in curling behaviour could indicate a possible opioidergic activity of the extract. This opioidergic activity may also explain the analgesic activity observed in the tail immersion test and further supports the traditional use of the plant in the management of pain. In the FST, PME in a similar fashion as fluoxetine increased swimming behaviour; whereas no changes were observed in the climbing behaviour, suggesting that the mechanism of the antidepressant-like activity of PME is related to the modulation of the serotonergic system. In addition, the antidepressant-like effect of PME was abolished by *p*CPA (an inhibitor of serotonin synthesis) and cyproheptadine (a 5-HT receptor antagonist), suggesting that 5-HT in the brain is essential for its action. Furthermore, administration of PME or fluoxetine potentiated 5-HTP-induced HTR in mice. This potentiation of HTR may be due to the PME or fluoxetine-mediated inhibition of the 5-HT reuptake and resulting increase of the content of 5-HT in synapses.This effect demonstrates that the serotonergic mechanism underlie the acute behavioural effects of PME on tests of depressive behaviour. However, PME did not potentiate NA toxicity indicating non-involvement of the noradrenergic system in its antidepressant-like effects.

The NMDA class of glutamate receptors have been implicated in the pathophysiology of major depression and the mechanism of action of antidepressant treatment (Petrie *et al.*, 2000; Hashimoto, 2011; Wlaz *et al.*, 2011; Ghasemi *et al.*, 2010a). In addition, glycine receptor antagonists and partial agonists have favourable safety profile (Beardsley *et al.*, 2002; Hashimoto, 2011) making them potential candidates for new antidepressant drugs (Danysz and Parsons, 1998; Poleszak *et al.*, 2011). D-cycloserine—a partial agonist of glycine$_B$ site of the NMDA receptor complex—potentiated the antidepressant effects of PME whereas D-serine—a full agonist—abolished its effects. This suggests a possible participation of the glycine site of the NMDA receptor complex in the antidepressant-like activity of PME. In addition, the L-arginine-NO-cGMP pathway was found to be involved in the antidepressant effects of the extract. This is evident as a synergistic antidepressantlike effect was observed when PME was administered with N^G nitro-L-arginine-methyl ester (L-NAME), a non-selective nitric oxide synthase inhibitor or

methylene blue, an inhibitor of both NOS and sGC. Moreover, the antidepressant-like effect of PME was reversed by pretreatment with sildenafil (a selective PDE5 inhibitor) or L-arginine (a NOS substrate). Therefore, effect of the extract could possibly be due to inhibition of NOS or decreased levels of cGMP levels.

Although the classical antidepressants are effective in treating most depressive episodes, a significant proportion of depressed patients do not display signs of mood improvement until 2–3 weeks after the start of the treatment (Poleszak *et al.*, 2011). However, the extract has shown to possess rapid and sustained antidepressant effect probably through its interaction with NMDA receptors. Thus, this could be of therapeutic benefit to patients suffering from major depression.

Cognitive impairment is a core endophenotype of major depression (Hasler *et al.*, 2004; Pittenger and Duman, 2007). Patients with major depression also exhibit prominent deficits in explicit memory (Konstantine *et al.*, 1998), a cognitive capacity well established to depend on the function of the hippocampus and the medial temporal lobe (Squire *et al.*, 2004). Lines of evidences have suggested that impaired cognition is an element of depression and that antidepressant therapy may improve the cognitive function (Fann *et al.*, 2001; Nowakowska *et al.*, 2001; Harmer *et al.*, 2002; Meneses, 2002; Yau *et al.*, 2002; Song *et al.*, 2006). The extract enhanced memory and learning in the chronic depression models used, thus indicating its ability to improve cognitive function in episodes of major depression.

Anxiety disorders represent a frequent and clinically important comorbid disorder in epilepsy patients (Vazquez and Devinsky, 2003). GABA, along with serotonin and norepinephrine, is one of several neurotransmitters that appear to be involved in the pathogenesis of anxiety (Stahl, 2003). The $GABA_A$ receptor subtype regulates excitability and rapid changes in fear arousal, such as anxiety, panic, and the acute stress response. Drugs that stimulate $GABA_A$ receptors, such as BDZs and barbiturates, have anxiolytic and antiseizure effects via $GABA_A$-mediated reduction of neuronal excitability (Mula *et al.*, 2007). Results of the present study showed that PME possess anxiolytic-like effects in the EPM, OFT, LDB, SIH, and social interaction tests without affecting motor impairments. This indicates that the GABA and/or serotonergic system could be implicated in the anxiolytic activity of PME.

Enhancement of GABAergic neuronal inhibition underlies the therapeutic action in the treatment of generalized anxiety disorders, panic anxiety, sleep disturbances and epilepsy including status epilepticus (Ma *et al.*, 2009; Seo *et al.*, 2007). In addition, it is well known that benzodiazepines act as anxiolytics (in low doses), anticonvulsants, and also produce sedation and myorelaxant effects at higher doses (Venancio *et al.*, 2011; de Melo *et al.*, 2006). It is not therefore surprising that the extract exhibited sedative effects in addition to its anticonvulsant, and anxiolytic effects. However, unlike benzodiazepines, it has no influence on myorelexant effect or motor coordination. This makes it a better candidate as an anxiolytic, sedative or anticonvulsant.

The phytochemical screening indicated the presence of flavonoids, tannins, saponins, glycosides, terpenoids and alkaloids. As has been reported by several authors, the presence of many biologically active phytochemicals such as flavonoids, triterpenes , alkaloids, steroids, tannins and glycosides in various plant extracts may be responsible for their respective pharmacological properties (Liu *et al.*, 2010; Ibarra *et al.*, 2010; Yokosuka and Mimaki, 2009; Maganha *et al.*, 2010).

The LD_{50} of the plant extract, given orally, was found to be above 3000 mg kg^{-1} in both mice and rats. At the relatively high doses used, the plant extract caused no mortality and appeared to cause no apparent toxicity suggesting that PME is relatively non-toxic.

9.2 CONCLUSIONS

Results obtained in this study indicate that the ethanolic leaf extract of *Pseudospondias microcarpa* possesses anticonvulsant, antidepressant, anxiolytic-like and sedative effects without influencing motor coordination. Moreover, it exhibits rapid and sustained antidepressant effect as well as improving learning and memory.

- The extract exerts its anticonvulsant activity by enhancing GABAergic and glycinergic neurotransmission, activation of potassium channels, inhibition of glutamatergic activity as well as interaction with the nitric oxide pathway.
- Antidepressant-like effects of the extract is dependent on the serotoninergic system, NMDA receptor complex and L-arginine-NO-cGMP pathway.

These results suggest that *Pseudospondias microcarpa* could be of potential interest as an alternative therapeutic tool that could help the conventional pharmacotherapy of epilepsy, depression, anxiety and cognitive deficits.

9.3 RECOMMENDATIONS

- The active component(s) in PME that are responsible for the anticonvulsant and antidepressant effects should be isolated and characterized.
- The exact mechanism(s) underlying the anticonvulsant and anxiolytic effects should be established.
- Effects of the extract in chronic models of epilepsy should also be investigated.
- Signaling pathways (cellular mechanisms) underlying the rapid antidepressant actions of the extract should also be assessed.
- Chronic toxicity studies should be carried out to establish the safety level of the plant.

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APPENDIX

DETAILED OBSERVATIONS IN THE IRWIN'S TEST

Dose (mg/kg)	0							30							100							300						
Time(min)	0-15	15	30	60	120	180	24h	0-15	15	30	60	120	180	24h	0-15	15	30	60	120	180	24h	0-15	15	30	60	120	180	24h
Lethality	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Convulsions	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tremor	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Straub tail	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sedation	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	++	++	+	-	+	++	++	++	++	++	-
Excitation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Abnormal gait	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Abnormal gait (tiptoe)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Jumps	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motor incoordination	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Loss of balance	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fore-paw treading	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Writhes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Piloerection	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stereotypies(sniffing)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stereotypies(chewing)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stereotypies(head movements)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Head twitches	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scratching	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Respiration	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aggressiveness	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fear	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Reactivity to touch	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Muscle tone	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Loss of writhing reflex	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Ptosis	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Exophthalmos	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Loss of grasping	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Akinesia	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-
catalepsy	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Loss of traction	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Loss of corneal reflex	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Analgesia	#	-	-	-	-	-	-	#	+	+	++	++	++	-	#	+	+	++	+++	++	-	#	++	++	++	++	++	-
Defaecation	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Salivation	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Lacrimation	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Urination	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Change in Rectal temperature	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-	#	-	-	-	-	-	-

Dose (mg/kg)	1000							3000						
Time(min)	0-15	15	30	60	120	180	24h	0-15	15	30	60	120	180	24h
Lethality	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Convulsions	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tremor	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Straub	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sedation	-	++	++	++	++	+++	-	+	++	++	+++	+++	+++	-
Excitation	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Abnormal gait	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Abnormal gait(tiptoe)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Jumps	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motor incoordination	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Loss of balance	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fore-paw treading	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Writhes	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Piloerection	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stereotypies(sniffing)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stereotypies(chewing)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stereotypies(head movements)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Head twitches	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scratching	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Respiration	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aggressiveness	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fear	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Reactivity to touch	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Muscle tone	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Loss of writhing reflex	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Ptosis	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Exophthalmos	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Loss of grasping	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Akinesia	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Catalepsy	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Loss of traction	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Loss of corneal reflex	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Analgesia	#	-	++	+++	+++	++	-	#	+	++	+++	+++	+++	-
Defaecation	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Salivation	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Lacrimation	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Urination	#	-	-	-	-	-	-	#	-	-	-	-	-	-
Change in Rectal temperature	#	-	-	-	-	-	-	#	-	-	-	-	-	-

PME was administered at the doses of 30, 100, 300, 1000 and 3000 mg kg^{-1}, *p.o.*; 5 mice per group. Observations were performed at 15, 30, 60, 120, 180 min and 24 h after administration. + = slight increase, ++ = moderate increase, +++ = marked increase and - = no effect. # Parameters not measured.

TABLE OF ED_{50} AND E_{MAX} VALUES FOR VARIOUS TESTS

Test	**Extract**		**Standard drug**	
	ED_{50}	**E_{max}**	**ED_{50}**	**E_{max}**
PTZ seizures				
			Diazepam	
Latency	416.4	80	0.20	100
Frequency	16.27	106	0.18	100
Duration	23.86	88.91	0.12	100
PTX seizures				
			Diazepam	
Latency	196.6	80	0.14	100
Frequency	57.13	80	0.05	100
Duration	15.50	80	0.05	100
STN seizures				
			Diazepam	
Latency	33.09	80	0.16	100
Frequency	6.54	80	0.11	100
Duration	10.31	80	0.10	100
4-AP seizures				

			Carbamazepine	
Latency (clonic)	267.9	85.39	117.0	100
Latency (tonic)	242.5	94.03	79.59	100
FST				
			Fluoxetine	
Immobility	26.08	62.83	3.48	72.77
			Desipramine	
			2.10	69.14
			Fluoxetine	
Swimming	38.15	94.53	4.12	118.6
			Desipramine	
			1.30	43.29
TST				
			Fluoxetine	
Immobility	41.01	80.98	1.43	62.21
			Desipramine	
			2.69	80.10

Repeated open space swim model				
			Fluoxetine	
Immobility	3.14	53.28	1.86	29.02

			Desipramine	
			3.03	33.98
			Fluoxetine	
Total distance	5.85	98.02	15.29	84.36
			Desipramine	
			15.57	100
			Fluoxetine	
TST (immobility)	29.74	78.82	7.99	100
			Desipramine	
			4.45	100
			Fluoxetine	
MWM	4.36	98.81	1.51	100
			Desipramine	
			0.50	100