

**ANTICONVULSANT, ANTIDEPRESSANT AND  
ANXIOLYTIC EFFECTS OF *MALLOTUS  
OPPOSITIFOLIUS* (GEISELER) MÜLL. ARG.  
(EUPHORBIACEAE)**

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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.

# KNUST

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## ABSTRACT

*Mallotus oppositifolius* is used in Ghana for CNS disorders but very little scientific evidence exists to support its use. Thus central effects of 70% v/v hydroalcoholic extract of the leaves of *Mallotus oppositifolius* (MOE) was assessed. Anticonvulsant effects of the extract in acute and chronic seizure models were evaluated. The study also investigated the effect of the extract on animal models of depression and anxiety.

In a preliminary screening of the central effects of the extract, oral dose of MOE induced sedation (1000 – 3000 mg kg<sup>-1</sup>); caused neuromuscular deficits in the rotarod test (300 – 3000 mg kg<sup>-1</sup>); reduced spontaneous locomotor activity in the activity cage; exhibited anticonvulsant effect (30 – 3000 mg kg<sup>-1</sup>) and central analgesic effect in the tail immersion test (100 – 3000 mg kg<sup>-1</sup>). The LD<sub>50</sub> was approximately 6000 mg kg<sup>-1</sup> in mice.

*M. oppositifolius* (10 - 100 mg kg<sup>-1</sup>, *p.o.*) exhibited anticonvulsant effect in the picrotoxin and strychnine induced seizure tests. The extract significantly delayed onset of myoclonic jerks and clonic convulsions; decreased the frequency and duration of clonic convulsions in these models. In the MES test, the extract caused a significant and dose dependent decrease in the duration of tonic limb extensions. In the pilocarpine induced *status epilepticus*, MOE delayed the onset of clonic convulsions and decreased the duration of these seizures. Furthermore, the extract protected mice against mortality induced by 4-aminopyridine and delayed the onset of both clonic and tonic convulsions. Flumazenil, a GABA<sub>A</sub>/benzodiazepine antagonist, reversed the anticonvulsant effect of the extract in the PTZ-induced seizure test suggesting enhancement of GABA<sub>A</sub> neurotransmission is involved in the anticonvulsant effect of the extract. Isobolographic analysis of the combination of diazepam and extract showed a synergistic effect but the mode of action of this effect may not be dependent on enhancement of GABA<sub>A</sub> neurotransmission since flumazenil failed to reverse their anticonvulsant effect.

Oral doses of MOE (10 - 100 mg kg<sup>-1</sup>), fluoxetine (3 - 30 mg kg<sup>-1</sup>) and imipramine (3 - 30 mg kg<sup>-1</sup>) decreased the frequency of immobility and immobility periods of mice in both the FST and TST when compared to control group, indicating significant antidepressant activity. In the open space swim test, a chronic depression model, MOE demonstrated antidepressant-like effect on the first day of treatment and sustained it throughout the period of drug treatment. MOE decreased immobility time while increasing the distanced travelled by the mice. The depression induced in this model induced significant impairment in spatial learning and memory in the Morris water maze—this was reversed by the extract and fluoxetine but not imipramine. Extract, fluoxetine and imipramine treatments did not have significant effects on weight variation. A day after the 14th day of drug treatment, the antidepressant effect was still

significant. A 3-day subcutaneous pretreatment with 200 mg kg<sup>-1</sup> para-chlorophenylalanine (pCPA), reversed the antidepressant effect of MOE and fluoxetine but not imipramine, suggesting that serotonergic enhancement may be involved in the behavioural effect of the extract. This was confirmed by the ability of the extract to potentiate the head twitch responses induced by 5-hydroxytryptophan in mice, a model sensitive to 5-HT<sub>2A</sub> receptor activation. Pretreatment with  $\alpha$ -methyldopa (400 mg kg<sup>-1</sup>) however, failed to reverse the behavioural effect of the extract and fluoxetine treatments in the forced swim test. The same result as above was observed for extract and fluoxetine treatments when mice were pretreated with reserpine (1 mg kg<sup>-1</sup>) or a combination of  $\alpha$ -methyldopa (200 mg kg<sup>-1</sup>) and reserpine (1 mg kg<sup>-1</sup>). This suggests that the antidepressant effect of the extract may not be dependent on central noradrenergic mechanisms. Administration of D-serine (600 mg kg<sup>-1</sup>), a full agonist on the glycine site of the NMDA receptors, reversed the antidepressant effect of the extract, fluoxetine and desipramine in both the TST and FST. D-cycloserine (2.5 mg kg<sup>-1</sup>), a partial agonist potentiated this behavioural effect in both extract and fluoxetine treated mice but not desipramine in both the TST and FST. This suggests possible involvement of glycine/NMDA receptor or pathway antagonism in the antidepressant effect of the extract. MOE slightly increased curling score in the tail suspension test and this was significantly potentiated by D-cycloserine, suggestive of possible opioidergic activity.

MOE (10 - 100 mg kg<sup>-1</sup>, *p.o.*) showed anxiolytic effect in the three anxiety models used namely; elevated plus maze, light-dark box and open field tests. *M. oppositifolius* treatment significantly increased the percentage of centre entries and the percentage time spent in the centre of the open field. *M. oppositifolius* also increased the time spent in the lit area and the latency to leave the lit area in light/dark box. In the EPM, it significantly increased open arm activities by increasing percentage open arm entries and duration. MOE also decreased risk assessment behaviours such as the head dips, stretch-attend postures and rearing.

Acute and subacute toxicity in rats did show deaths after 14 day treatment with the extract (30 – 3000 mg kg<sup>-1</sup>). Extract treatment did not affect weight of rats or the relative organ weights. Haematological or serum biochemical parameters were not affected except increases in serum bilirubin (300 and 3000 mg kg<sup>-1</sup>), urea and creatinine (30 and 100 mg kg<sup>-1</sup>). Histopathological examination did not reveal toxic effect on the stomach, heart, liver and spleen. There were however some morphological changes of the kidney at 30 mg kg<sup>-1</sup>.

These results suggest that the extract has anticonvulsant effect possibly through enhancement of GABAergic, glycinergic and potassium channel activation or increased potassium conductance. Possible inhibition of muscarinic and glutamatergic transmission may also be

involved. The antidepressant-like effects of the extract may be due to the interplay of serotonergic, glycine/NMDA and opioidergic pathways. The extract also demonstrated anxiolytic-like effects possibly by the involvement of GABAergic and serotonergic mechanisms.

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# TABLE OF CONTENTS

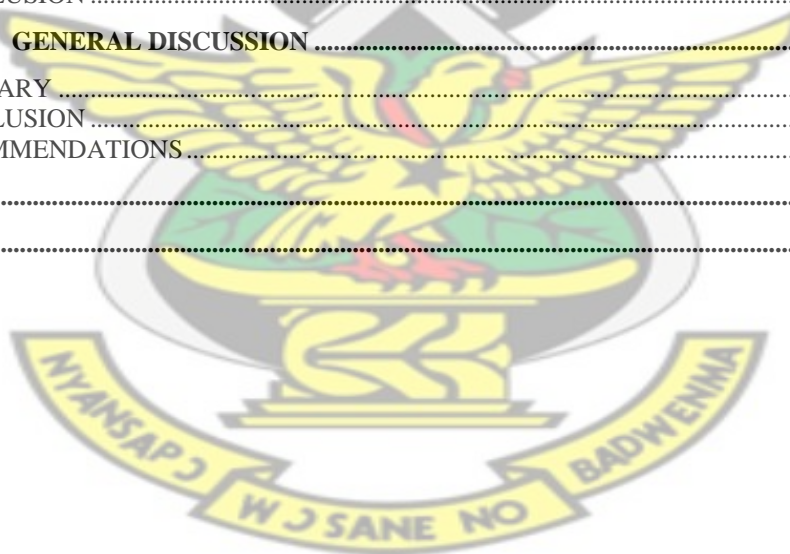
DECLARATION.....	II
ABSTRACT .....	III
ACKNOWLEDGEMENT.....	VII
TABLE OF CONTENTS.....	VIII
LIST OF TABLES .....	XII
LIST OF FIGURES .....	XIII
LIST OF PLATES.....	XV
ABBREVIATIONS .....	XVI
<b>CHAPTER 1 INTRODUCTION.....</b>	<b>1</b>
1.1 GENERAL INTRODUCTION.....	1
1.2 THE PLANT <i>MALLOTUS OPPOSITIFOLIUS</i> .....	2
1.2.1 Name .....	2
1.2.2 Description.....	2
1.2.3 Ecological and geographical distribution .....	4
1.2.4 Traditional uses .....	4
1.2.5 Previous works on <i>M. oppositifolius</i> .....	4
1.2.5.1 Chemical constituents .....	4
1.2.5.2 Antimicrobial activity .....	5
1.2.5.3 Effect on root-knot nematode infection .....	5
1.2.5.4 Antifungal Property .....	5
1.3 EPILEPSY, THERAPY AND DRUG SCREENING .....	5
1.3.1 Background.....	5
1.3.2 Pathophysiology of epilepsy.....	7
1.3.3 Classification of epilepsy.....	8
1.3.4 History of antiepileptic drugs.....	9
1.3.5 Mechanism of action of antiepileptic drugs.....	9
1.3.5.1 Modulation of ion channels .....	9
1.3.5.2 Enhancement of inhibitory neurotransmission .....	11
1.3.5.3 Attenuation of excitatory neurotransmission .....	12
1.3.6 Dietary treatment of epilepsy.....	13
1.3.7 Experimental Models for Anticonvulsant screening.....	14
1.3.7.1 Acute models.....	14
1.3.7.2 Chronic models .....	15
1.4 DEPRESSION.....	15
1.4.1 Background.....	15
1.4.2 Theories of Depression.....	16
1.4.2.1 Monoamine Hypothesis .....	16
1.4.2.2 Neurotrophic Hypothesis .....	18
1.4.2.3 Neuroendocrine Factors in the Pathophysiology of Depression .....	18
1.4.3 Antidepressant Drugs.....	19
1.4.3.1 Monoamine Oxidase Inhibitors .....	19
1.4.3.2 Tricyclic Antidepressants.....	20
1.4.3.3 Selective Serotonin Reuptake Inhibitors (SSRIs).....	22
1.4.3.4 Atypical Antidepressants .....	23
1.4.4 Animal models of depression.....	23
1.4.4.1 Forced Swimming Test .....	23
1.4.4.2 Tail Suspension Test .....	23
1.5 ANXIETY.....	24
1.5.1 Background.....	24

1.5.2	<i>Animal models used for screening anxiolytics</i> .....	25
1.5.2.1	<i>The elevated plus-maze test</i> .....	25
1.5.2.2	<i>Light Dark Exploration test</i> .....	26
1.6	<b>JUSTIFICATION</b> .....	26
1.7	<b>PURPOSE AND OBJECTIVES OF STUDY</b> .....	27
<b>CHAPTER 2 PLANT COLLECTION, EXTRACTION AND PHYTOCHEMICAL ANALYSIS</b> .....		<b>28</b>
2.1	<b>PLANT COLLECTION AND EXTRACTION</b> .....	28
2.1.1	<i>Plant collection</i> .....	28
2.1.2	<i>Extraction</i> .....	28
2.2	<b>PHYTOCHEMICAL TESTS</b> .....	28
2.2.1	<i>Test for alkaloids</i> .....	28
2.2.2	<i>Test for tannins</i> .....	28
2.2.3	<i>Test for saponins</i> .....	29
2.2.4	<i>Test for reducing sugars</i> .....	29
2.2.5	<i>Test for sterols</i> .....	29
2.2.6	<i>Test for terpenoids</i> .....	29
2.3	<b>RESULTS</b> .....	29
2.4	<b>DISCUSSION</b> .....	30
2.5	<b>CONCLUSION</b> .....	30
<b>CHAPTER 3 PRELIMINARY CNS SCREENING</b> .....		<b>31</b>
3.1	<b>INTRODUCTION</b> .....	31
3.2	<b>MATERIALS AND METHODS</b> .....	31
3.2.1	<i>Animals</i> .....	31
3.2.2	<i>Drugs and chemicals</i> .....	31
3.2.3	<i>Irwin test</i> .....	32
3.2.4	<i>Activity meter test</i> .....	32
3.2.5	<i>Rotarod test</i> .....	32
3.2.6	<i>Convulsive threshold test (PTZ seizure test)</i> .....	32
3.2.7	<i>Pentobarbitone interaction test</i> .....	33
3.2.8	<i>Tail immersion test</i> .....	33
3.2.9	<i>Analysis of Data</i> .....	34
3.3	<b>RESULTS</b> .....	34
3.3.1	<i>Irwin test</i> .....	34
3.3.2	<i>Activity meter test</i> .....	35
3.3.3	<i>Rotarod</i> .....	35
3.3.4	<i>PTZ seizure test</i> .....	36
3.3.5	<i>Pentobarbital interaction test</i> .....	37
3.3.6	<i>Tail immersion test</i> .....	40
3.4	<b>DISCUSSION</b> .....	41
3.5	<b>CONCLUSION</b> .....	43
<b>CHAPTER 4 ANTICONVULSANT EFFECT AND ISOBOLOGRAPHIC ANALYSIS OF DRUG INTERACTION</b> .....		<b>44</b>
4.1	<b>INTRODUCTION</b> .....	44
4.2	<b>MATERIALS AND METHODS</b> .....	45
4.2.1	<i>Animals</i> .....	45
4.2.2	<i>Drugs and chemicals</i> .....	45
4.2.3	<i>Pentylenetetrazole-induced seizure test</i> .....	45
4.2.4	<i>Picrotoxin-induced seizure test</i> .....	45
4.2.5	<i>Maximal electroshock seizure test</i> .....	45
4.2.6	<i>Strychnine induced seizure test</i> .....	46
4.2.7	<i>Pilocarpine-induced status epilepticus</i> .....	46
4.2.8	<i>Possible Mechanisms</i> .....	46



4.2.8.1	Involvement of GABAergic mechanisms .....	46
4.2.8.2	Involvement of potassium channels.....	47
4.2.9	Isobolographic analysis of drug interaction between extract and diazepam .....	47
4.2.10	Rotarod test.....	48
4.2.11	Statistical analysis .....	48
4.3	RESULTS .....	49
4.3.1	PTZ-induced seizures .....	49
4.3.2	Picrotoxin-induced seizures .....	51
4.3.3	Maximal electroshock seizure (MES) test.....	53
4.3.4	Strychnine-induced convulsions.....	54
4.3.5	Pilocarpine-induced status epilepticus .....	56
4.3.6	Involvement of GABAergic mechanisms.....	57
4.3.7	Involvement of potassium channels: The 4-aminopyridine induced seizure test .....	58
4.3.8	Isobolographic analysis of extract and diazepam .....	60
4.3.9	Rotarod .....	62
4.4	DISCUSSION.....	63
4.5	CONCLUSION .....	66
<b>CHAPTER 5</b>	<b>ANTIDEPRESSANT EFFECT .....</b>	<b>67</b>
5.1	INTRODUCTION.....	67
5.2	MATERIALS AND METHODS .....	68
5.2.1	Animals .....	68
5.2.2	Chemicals.....	68
5.2.3	Acute antidepressant model.....	68
5.2.3.1	Forced Swimming test .....	68
5.2.3.2	Tail suspension test .....	69
5.2.4	Chronic depression.....	69
5.2.4.1	Open space swim test .....	69
5.2.4.2	Tail suspension test .....	69
5.2.4.3	Spatial learning and memory task- Morris water maze.....	70
5.2.4.4	Weight variation .....	70
5.2.5	Possible Mechanisms.....	70
5.2.5.1	Involvement of noradrenergic systems.....	70
5.2.5.2	Involvement of serotonergic systems .....	71
5.2.5.3	Involvement of glycine/NMDA neurotransmission .....	71
5.2.6	Statistics .....	72
5.3	RESULTS .....	72
5.3.1	Forced swimming and Tail suspension tests.....	72
5.3.2	The open space swim test .....	76
5.3.3	Spatial memory and learning in the Morris water maze task. ....	79
5.3.4	Weight variation .....	81
5.3.5	Tail suspension test (TST). ....	82
5.3.6	Involvement of noradrenergic mechanisms .....	83
5.3.7	Involvement of serotonergic mechanism.....	84
5.3.8	Involvement of glycine/NMDA receptor complex.....	88
5.4	DISCUSSION.....	91
5.5	CONCLUSION .....	96
<b>CHAPTER 6</b>	<b>ANXIOLYTIC EFFECT .....</b>	<b>97</b>
6.1	INTRODUCTION.....	97
6.2	MATERIALS AND METHODS .....	97
6.2.1	Animals .....	97
6.2.2	Chemicals.....	97
6.2.3	Elevated Plus-Maze test .....	97
6.2.4	Light/Dark Box .....	98
6.2.5	Open-field test.....	98

6.3	RESULTS .....	99
6.3.1	<i>Effect of MOE and DZP on mice in the elevated plus-maze .....</i>	99
6.3.2	<i>Effect of MOE and DZP on mice in the light-dark test.....</i>	102
6.3.3	<i>Effect of MOE and DZP on mice in the open field test.....</i>	103
6.4	DISCUSSION.....	105
6.5	CONCLUSION .....	107
<b>CHAPTER 7</b>	<b>ACUTE AND SUBACUTE TOXICITY STUDIES.....</b>	<b>108</b>
7.1	INTRODUCTION.....	108
7.2	METHOD .....	108
7.2.1	<i>Acute toxicity.....</i>	108
7.2.2	<i>Subacute toxicity.....</i>	109
7.2.2.1	Preparation of serum and isolation of organs .....	109
7.2.2.2	Effect of extract on haematological parameters .....	109
7.2.2.3	Effect of extract on serum biochemical parameters .....	109
7.2.2.4	Effect of extract on body and organ weights .....	110
7.2.2.5	Histological examination .....	110
7.2.3	<i>Statistics .....</i>	110
7.3	RESULTS .....	111
7.3.1	<i>Acute toxicity.....</i>	111
7.3.2	<i>Subacute toxicity.....</i>	111
7.3.2.1	Effect of extract on body weight and organ weight .....	111
7.3.2.2	Effect of extract on haematological parameters .....	113
7.3.2.3	Effect of extract on serum biochemical parameters .....	114
7.3.2.4	Histopathological changes .....	117
7.4	DISCUSSION.....	124
7.5	CONCLUSION .....	126
<b>CHAPTER 8</b>	<b>GENERAL DISCUSSION .....</b>	<b>127</b>
8.1	SUMMARY .....	127
8.2	CONCLUSION .....	131
8.3	RECOMMENDATIONS .....	132
	<b>REFERENCES .....</b>	<b>133</b>
	<b>APPENDIX.....</b>	<b>170</b>



## LIST OF TABLES

Table 2.1 Phytochemical constituents of the hydroalcoholic extract from the leaves of <i>M. oppositifolius</i> .....	29
Table 3.1 Observations in the acute toxicity test after oral administration of <i>M. oppositifolius</i> in mice.....	34
Table 4.1 ED <sub>50</sub> and E <sub>max</sub> Values of the Latency to Convulsions.....	62
Table 4.2 ED <sub>50</sub> and E <sub>max</sub> Values of the frequency of Convulsions .....	62
Table 4.3 ED <sub>50</sub> and E <sub>max</sub> Values of the Duration of Convulsions.....	62
Table 4.4 showing the latency for mice to fall off the rotarod .....	63
Table 5.1 ED <sub>50</sub> and E <sub>max</sub> values of drugs used in the forced swim (FST) and tail suspension tests (TST).....	74
Table 5.2 ED <sub>50</sub> and E <sub>max</sub> values of drugs used in the open space swim test. ....	79
Table 5.3 ED <sub>50</sub> and E <sub>max</sub> values of drugs used in the Morris water maze test. ....	81
Table 6.1 Effect of Drug treatment on the frequency of some ethological parameters.....	101
Table 6.2 Effect of Drug treatment on the duration of some ethological parameters .....	102
Table 7.1 Observations in the acute toxicity test after oral administration of <i>M. oppositifolius</i> in rats.....	111
Table 7.2 Haematological values of control and rats treated with <i>M. oppositifolius</i> for 14 days.....	115
Table 7.3 Clinical biochemistry values of control and rats treated with <i>M. oppositifolius</i> for 14 days. ....	116



## LIST OF FIGURES

Figure 1.1 Plant showing the leaves of <i>M. oppositifolius</i> .....	3
Figure 1.2 Sites of action of antiepileptics in GABAergic synapse.....	12
Figure 3.1 Effect of MOE, diazepam and caffeine on spontaneous activity in mice..	35
Figure 3.2 Effect of MOE, diazepam and d-tubocurarine on motor coordination in mice.....	36
Figure 3.3 Effect of MOE and diazepam on convulsions in the pentylenetetrazole seizure test in mice.....	37
Figure 3.4 Effect of MOE and diazepam and caffeine in the pentobarbitone sleeping test..	38
Figure 3.5 Effect of MOE, diazepam and caffeine on sleep after pre-treating with phenobarbitone in the pentobarbitone sleeping test.....	39
Figure 3.6 Effect of phenobarbitone on sleep after pre-treatment with extract in the pentobarbitone sleeping test.....	40
Figure 3.7 Effect of MOE and morphine on the time course curve of the tail immersion test and the area under the curve in mice..	41
Figure 4.1 Effect of MOE and diazepam on convulsions in the pentylenetetrazole induced seizure test in mice. ....	50
Figure 4.2 Dose–response curves of MOE and diazepam in the PTZ test in mice.....	51
Figure 4.3 Effect of MOE and diazepam on convulsions in the picrotoxin induced seizure test in mice.....	52
Figure 4.4 Dose–response curves of MOE and diazepam in the picrotoxin test in mice.....	53
Figure 4.5 Effect of MOE and carbamazepine on the duration of tonic limb extensions maximal electroshock seizure test in mice.....	54
Figure 4.6 Dose–response curves of MOE and carbamazepine in the MES test .....	54
Figure 4.7 Effect of MOE and diazepam on the convulsions in strychnine induced seizure test in mice.....	55
Figure 4.8 Dose–response curves of MOE and diazepam in the strychnine seizure test.....	56
Figure 4.9 Effect of MOE and diazepam on convulsions in the pilocarpine <i>status epilepticus</i> in mice.....	57
Figure 4.10 Dose–response curves for the MOE and diazepam in the pilocarpine <i>status epilepticus</i> in mice.....	57
Figure 4.11 Effect of MOE and diazepam on the effect of flumazenil pretreatment on the clonic convulsions in the pentylenetetrazole induced seizure test in mice.....	58
Figure 4.12 Effect of MOE and valproate on convulsions in the 4-AP seizure test.....	59
Figure 4.13 A Kaplan-Meier estimates of the percentage survival of mice for extract and sodium valproate.....	59
Figure 4.14 Dose–response curves MOE and sodium valproate in the 4-AP seizure test..	60
Figure 4.15 Effect of fractions of extract and diazepam combinations and their antagonism with flumazenil on convulsions in the PTZ test..	61
Figure 4.16 Dose response curve for fractions of extract and diazepam combinations and isobologram in the PTZ seizure test. ....	61
Figure 5.1 Effects of extract, fluoxetine and imipramine on mobility and immobility in FST..	73
Figure 5.2 Dose–response curves of the extract, fluoxetine and imipramine in the forced swimming test in mice.....	74
Figure 5.3 Effect of the extract, fluoxetine and imipramine treatment on mobility and immobility in the TST.....	75



Figure 5.4 Dose–response curves of the extract, fluoxetine and imipramine showing % decrease in frequency and % decrease in immobility in the tail suspension test in mice.....	76
Figure 5.5 Effects of extract, fluoxetine and imipramine treatment on the duration of immobility in the open space swim test.....	77
Figure 5.6 Effects of extract, fluoxetine and imipramine treatment on the distance travelled in the open space swim test.....	78
Figure 5.7 Dose–response curves showing the effect of extract, fluoxetine and imipramine on % decrease in immobility time and % increase in distance travelled in the open space swim test in mice.....	79
Figure 5.8 Effects of MOE, fluoxetine and imipramine treatment on the spatial learning and memory in the Morris water maze test.....	80
Figure 5.9 Dose–response curves showing the effect of MOE, fluoxetine and imipramine on % decrease in the escape latency in the Morris water maze test in mice.....	81
Figure 5.10 Effect of extract, fluoxetine and imipramine treatment on weight changes.....	82
Figure 5.11 Effect of the extract, fluoxetine and imipramine treatment on duration of mobility and immobility in the tail suspension test.....	83
Figure 5.12 Effects of reserpine and $\alpha$ -methyl dopa on duration of immobility of extract, fluoxetine and imipramine treatment in the FST.....	84
Figure 5.13 Effects of pCPA pretreatment on the mean immobility counts; swimming counts and climbing counts of oral doses of extract, fluoxetine and imipramine .....	86
Figure 5.14 Effect of MOE and fluoxetine on the time course curve of head twitch response test and their corresponding AUCs.....	87
Figure 5.15 Dose–response curves of % increase in head twitches by oral dose of the extract and fluoxetine in response to 5-hydroxytryptophan administration in mice.....	88
Figure 5.16 Effects of D-serine or D-cycloserine pretreatment on mean immobility count, pedalling count and curling count of extract in the tail suspension test.....	89
Figure 5.17 Effects of D-serine or D-cycloserine pretreatment on mean immobility count, swimming count and climbing count of extract, fluoxetine and desipramine treatment in the forced swimming test. ....	90
Figure 6.1 Effect of the extract and diazepam treatment on the open and closed arm entries of the elevated plus maze.....	100
Figure 6.2 Effect of the extract and diazepam treatment on the time spent in the open and closed arm of the elevated plus maze.....	101
Figure 6.3 Effect of the extract and diazepam treatment on the latency to enter dark compartment and the frequency of transitions in the light dark test.....	103
Figure 6.4 Effect of the extract and diazepam treatment on the frequency of entries into the corner, peripheral or central compartments in the open field test. ....	104
Figure 6.5 Effect of the extract and diazepam treatment on the time spent in the corner, peripheral or central compartments in the open field test. ....	105
Figure 7.1 Effect of oral administration of <i>Mallotus oppositifolius</i> extract, on the % change in body weights of rats in the sub-acute toxicity test. ....	112
Figure 7.2 Effect of oral administration of <i>Mallotus oppositifolius</i> extract, on the relative organ weights (ROW) of rats in the sub-acute toxicity test.....	113
Figure 8.1 A simplified representation of the proposed mechanisms of action of the hydroalcoholic extract of the leaves of <i>Mallotus oppositifolius</i> .. ....	131



## LIST OF PLATES

Plate 7.1 Photomicrograph of the sections of the liver in control rats and rats treated orally with the extract for 14 days in the sub-acute toxicity study.....	118
Plate 7.2 Photomicrograph of the sections of the spleen in control rats and rats treated orally with the extract for 14 days in the sub-acute toxicity study.....	119
Plate 7.3 Photomicrograph of the sections of the heart in control rats and rats treated orally with of the extract for 14 days in the sub-acute toxicity study .....	120
Plate 7.4 Photomicrograph of the sections of the stomach in control rats and rats treated orally with the extract for 14 days in the sub-acute toxicity study .....	121
Plate 7.5 Photomicrograph of the sections of the kidney in control rats and rats treated orally with the extract for 14 days in the sub-acute toxicity study .....	122
Plate 7.6 Photomicrograph of the sections of the brain showing regions of the hippocampus in control rats and rats treated orally with the extract for 14 days in the sub-acute toxicity study.....	123



## ABBREVIATIONS

AEDs	Antiepileptic drugs
AMPA	Alpha-3-Hydroxy-5-Methoxy-4-Isoxazole Propionic Acid
CBZ	Carbamazepine
CNS	Central nervous system
DCS	Dextro-cycloserine
DS	Dextro-serine
DSP	Desipramine
DZP	Diazepam
EPM	Elevated Plus Maze
FLX	Fluoxetine
FST	Forced Swimming Test
GABA	Gamma Amino Butyric Acid
HLTE	Hind limb tonic extension
ICR	Imprinting Control Region
IMI	Imipramine
IPSCs	Inhibitory Post-Synaptic Currents
IGluR	Ionotropic glutamate receptors
LDB	Light-Dark Box
mGluR	Metabotropic glutamate receptors
MES	Maximal Electroshock Test
MIC	Minimum inhibitory concentration
MOE	<i>Mallotus oppositifolius</i> extract
NMDA	N-methyl- D-aspartate
<i>p.o.</i>	<i>Per os</i>
PTX	Picrotoxin
PTZ	Pentylenetetrazole
SSRI	Selective serotonin reuptake inhibitor
TST	Tail suspension test
Z <sub>add</sub>	Theoretical ED <sub>50</sub>

## Chapter 1

### INTRODUCTION

#### 1.1 GENERAL INTRODUCTION

Virtually all medicines centuries before the advent of modern medicine, synthetic chemistry and the pharmaceutical industry, came from plants (Agosta, 1997). These medicinal plants continue to serve as important repositories for the discovery of novel bioactive compounds which can be utilized as lead molecules for the development of new drugs (Cragg *et al.*, 1997). Aspirin, atropine, scopolamine, taxol, theophylline, tubocurarine, vincristine and vinblastine are a few examples of medicines derived from plant sources. At least 25% of drugs in the modern pharmacopoeia are derived from plants and many others, which are synthetic analogues or built on prototype compounds isolated from plants (Cox and Balick, 1994; Jones, 1996).

The use of traditional medicine and medicinal herbs is currently enjoying a renaissance in popularity in the West as well, and in many parts of the world it is the source of primary health care. People often resort to traditional and other forms of complementary and alternative medicines for chronic conditions which do not respond well to conventional or modern drug treatments. Among these are neurological disorders such as anxiety, depression, epilepsy and pain (Spinella, 2001).

Epilepsy is one of the major neurological disorders affecting approximately 2% of the population (Pitkanen and Lukasiuk, 2009). Considerable progress in the pharmacotherapy of epilepsy has been made over the last few decades, including the introduction of new antiepileptic drugs such as retigabine, felbamate, lamotrigine, etc. (Bazil and Pedley, 1998; McCabe, 2000). However, current drug therapy of epilepsy is fraught with side-effects, teratogenic effects, long term toxicity and about a third of patients do not respond to these pharmacotherapies (Raza *et al.*, 2001; Loscher, 2002). Furthermore, there is currently no drug available which prevents the development of epilepsy (Temkin, 2001; Temkin *et al.*, 2001). Epilepsy in particular is a condition where traditional healers are very critical in providing treatment in the rural settings (Stafford *et al.*, 2008). For example, a sample in Nigeria revealed that 52% of epilepsy patients use some form of traditional medicine (Danesi and Adetunji, 1994). Considering the great reliance on traditional medicinal plants for treatment of diseases and the potential for drug discovery, it is justifiable 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.

The plant *Mallotus oppositifolius* (family Euphorbiaceae) is a shrub that is commonly found in drier types of forest and secondary growth throughout the West African region. Locally it is known as 'sroti' in Ewe or 'sratadua' in Akan (Burkill, 1985). Economically, *M. oppositifolius* twig is used as chewing sticks for cleaning the teeth; the stem is used as yam stakes. In Nigeria the leaves are taken by the Hausas in cold infusion to expel tapeworm, while the decoction is a vermifuge in Ivory Coast. In Ghana, the crushed leaves are applied to inflammation of the eye during an attack of small pox. Leaf-sap is an eye-instillation in Ubangi for eye-troubles and in Congo (Brazzaville) it is administered in attacks of epilepsy. The leaves are also used for treating convulsion (Burkill, 1985).

This work seeks to investigate the anticonvulsant effect of the hydroalcoholic leaf extract of the plant in classical animal models of epilepsy namely, maximal electroshock seizure (MES) test, pentylenetetrazole (PTZ), picrotoxin (PTX), strychnine, 4-aminopyridine induced seizure tests and pilocarpine induced *status epilepticus*.

A relationship between epilepsy and psychiatric disorders like depression and anxiety has been recognized since antiquity. Statement found in Hippocrates' writings: "melancholics ordinarily become epileptics, and epileptics melancholics: what determines the preference is the direction the malady takes; if it bears upon the body, epilepsy, if upon the intelligence, melancholy" corroborates this fact. Moreover antiepileptic drugs like carbamazepine have been used in managing refractory depression (Rogawski and Löscher, 2004). This is probably because there is some overlap in the neurobiology of these conditions. Thus the effect of the extract on animal models of depression, forced swimming test, tail suspension test and the open space swim test was also investigated. The elevated plus maze, light-dark box and open field were employed to assess the anxiolytic effects of the extract. Acute and subacute toxicity studies were also carried.

## 1.2 THE PLANT *MALLOTUS OPPOSITIFOLIUS*

### 1.2.1 Name

Botanical name: *Mallotus oppositifolius*

Family: Euphorbiaceae

Local name (s): Sroti (Ewe); Osratadua (Twi)

### 1.2.2 Description

*Mallotus oppositifolius* is a dioecious shrub or a small tree that grows up to 6 to 13 m tall. The young shoots are densely stellate-hairy; older twigs almost glabrous, often purplish brown. Leaves are opposite and simple; petiole is long and short in each pair; 2.5- 11cm long when long and 0.5- 2 cm long when short, slightly thickened at the both ends; stipules are long, soon



falling; blade is broadly ovate to oblong-ovate, 3-18-21 cm  $\times$  2-13 cm, unequal in size in each pair; base shallowly cordate to rounded or truncate, margins almost entire or more or less deeply toothed or lobed, 3 veined from the base, sparingly stellate-hairy to almost glabrous, sparingly gland dotted, also with simple hair beneath. Inflorescence a terminal or axillary raceme; male inflorescence up to 10- 15 cm long, female one up to 10- 18 cm long, bracts 0.5- 1.5 mm long, triangular, each 1-5 flowered. Flowers are unisexual, fragrant, petals absent, male flowers with jointed pedicel 3-7 mm long, sepals 3-4, elliptical about 2mm long, strongly reflexed, pale yellow-green, disk absent, stamens numerous, filament about 2 mm long, free, greenish white, female flowers with pedicels 2-3 mm long, extending to 2-5 cm in fruit, calyx lobes 3-5-6 ovate to lanceolate about 2 mm long, united at the base, recurved, densely short-hairy and covered with yellow glands, 3-lobed, styles 3 about 1.5 mm long, free, plerose. Fruit a deeply 3-lobed capsule 5-7 mm  $\times$  7-9 mm, short-hairy and gland-dotted 3 seeded. Seeds are almost globose, 3.5-4 mm  $\times$  about 3 mm, smooth, shiny, grayish and olive-brown.



Figure 1.0 Plant showing the leaves of *M. oppositifolius*



### 1.2.3 Ecological and geographical distribution

*Mallotus oppositifolius* is widely distributed and occurs from Senegal to Ethiopia, Angola, Mozambique and Madagascar. It is also widely distributed in Ghana and Nigeria.

### 1.2.4 Traditional uses

In West Africa most plant parts, but especially the leaves, are commonly used for medicinal purposes. A leaf or stem bark infusions are taken as anthelmintic to expel tapeworms and to treat diarrhoea. The crushed or chewed fresh leaves, sometimes mixed with butter, are put on cuts and sores as a haemostatic and antibacterial; on skin eruptions and rashes for fast healing. They are also applied to burns for analgesic effect (Adekunle and Ikumapayi, 2006). A steam bath with the leaves is taken to treat headache, epilepsy or mental illness. Leaf sap is used as nasal drops or eye drops and the heads are massaged with the pulped leaves to treat headache. The crushed leaves or leaf sap are applied to aching teeth and inflamed eyes. The ground leaves in salted water are applied to extract poisons in snakebites and the extract is also drunk for this purpose (Farombi *et al.*, 2001). Crushed leaves or a leaf infusion are applied to treat urinary infections, venereal diseases, malaria, leprosy, chickenpox and female sterility. A leaf and fruit infusion is taken to treat dysentery and diarrhoea, or the leaves are added to food. A leaf and root decoction is drunk to treat anaemia and general fatigue. A root and leaf paste is applied to treat convulsions, stomach-ache and chest pains. An infusion of the roots together with the seeds of *Aframomum melegueta* K. Schum is taken as an enema to treat lumbago. In eastern Africa a root decoction is taken as an aphrodisiac. The root decoction and leaf sap are taken to treat pneumonia, vomiting and chest pain. *Mallotus oppositifolius* is commonly browsed by cattle. The wood is also used as firewood and to make tool handles or yam stakes; thinner stems or the bark are sometimes used as binding material and the twigs are commonly used as chew sticks. Refer to (Burkill, 1985).

### 1.2.5 Previous works on *M. oppositifolius*

#### 1.2.5.1 Chemical constituents

Phytochemical screening of *M. oppositifolius* revealed the presence of secondary metabolites such as alkaloids, phenols, flavonoids, anthraquinones and cardenolides (Farombi *et al.*, 2001). Five hydrolysable tannins and cytotoxic phloroglucinol have been reported from the bark of *M. japonicus*, another mallotus species. Anthocyanins, butacyanin have also been found in the leaves. Heavy metal analysis of the leaves of *M. oppositifolius* showed that the plant did not contain heavy metals such as cadmium, zinc, lead, chromium, and nickel. The presence of

copper, iron and manganese was less than 0.95% individually (Adekunle and Ikumapayi, 2006). Rottlerin, a protein kinase C inhibitor, has also been found in its bark and leaves (Oliver, 1960).

#### **1.2.5.2 Antimicrobial activity**

*M. oppositifolius* leaf extract exhibited significant antimicrobial activity against *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus*. *M. oppositifolius* significantly enhanced antibacterial activity of amoxicillin against *Staph. aureus* and *B. subtilis* (Gbedema *et al.*, 2010).

#### **1.2.5.3 Effect on root-knot nematode infection**

The undiluted leaf extracts of *M. oppositifolius* inhibited egg hatching (100%) and caused mortality in larva even after 12 h of exposure time. The juvenile mortality increased with increase in exposure time (Okeniyi, 2010).

#### **1.2.5.4 Antifungal Property**

*M. oppositifolius* crude extracts (ethanolic and aqueous) had significant antifungal activity on *Aspergillus flavus*, *Candida albicans*, *Microsporum audouinii*, *Penicillium sp.*, *Trichoderma mentagrophytes* and *Trichoderma sp.* The ethanolic extracts of *M. oppositifolius* used did not inhibit the growth of *T. cutaneum*. (Adekunle and Ikumapayi, 2006).

### **1.3 EPILEPSY, THERAPY AND DRUG SCREENING**

#### **1.3.1 Background**

The word “epilepsy” comes from the Greek word *epilepsia* which means to be taken, seized or attacked. Epilepsy is not a single entity but an assortment of different seizure types and syndromes originating from several mechanisms that have in common the sudden, excessive, and synchronous discharge of cerebral neurons (McNamara and Puranam, 1996; Fisher *et al.*, 2005). Seizures are finite episodes of brain dysfunction resulting from abnormal discharge of cerebral neurons. This abnormal electrical activity may result in a variety of events, including loss of consciousness, abnormal movements, atypical or odd behaviour, or distorted perceptions that are of limited duration but recur if untreated. The site of origin of the abnormal neuronal firing determines the symptoms that are produced. For example, if the motor cortex is involved, the patient may experience abnormal movements or a generalized convulsion. Seizures originating in the parietal or occipital lobe may include visual, auditory, or olfactory hallucinations (Stafstrom, 2005).

Epilepsy is one of the most prevalent neurological disorders. It is estimated that over 50 million people suffer from epilepsy, 85% of whom live in developing countries (Sander and Shorvon, 1996; Stafford *et al.*, 2008). It is the second most common neurological disorder after stroke and it is estimated that approximately 2% of the population worldwide is affected by some form of epilepsy (Pitkanen and Lukasiuk, 2009). In most African communities epilepsy carries a social stigma and sufferers are often shunned or discriminated against with respect to education, employment and marriage (Baskind and Birbeck, 2005; Stafford *et al.*, 2008). This makes early treatment difficult.

Drug or vagal nerve stimulator therapy is the most widely effective mode for the treatment of patients with epilepsy, although surgery is also an option. However, for most patients, pharmacotherapy is still considered the mainstay of treatment (Simoens, 2010). Drug therapy of epilepsy with currently available antiepileptic drugs (AEDs) is often plagued with significant adverse effects such as, foetal abnormalities e.g. cleft palate, cleft lip, congenital heart disease; slowed growth rate and mental deficiency, rash, blood dyscrasias, vitamin K and folate deficiencies, loss of libido, hormonal dysfunction, and bone marrow hypoplasia. Furthermore, a significant proportion of patients (up to 40%) do not respond to these agents and this proportion is quite high in the developing countries (Regesta and Tanganelli, 1999; Kwan and Brodie, 2001). Moreover, all the currently available AEDs have potential for adverse effects on cognition and behaviour (Samren *et al.*, 1997; Duncan, 2002). The practice of polypharmacy in the therapy of epilepsy increases the risk of side effects and drug interactions (Shorvon and Reynolds, 1979; Mula and Trimble, 2009). Due to lack of facilities for proper diagnosis, treatment and monitoring of serum levels of AEDs all problems with the current AED therapy of epilepsy seem more prevalent in underdeveloped countries (Tran *et al.*, 2007; Carpio and Hauser, 2009). Another critical issue associated with currently available AEDs is recent clinical and experimental data that strongly suggest that AED therapy does not alter the course or natural history of epilepsy and though AEDs suppress the seizures, they may not affect the underlying disorder (Taylor *et al.*, 1995; Shinnar and Berg, 1996; Loscher, 2002). Only a very few AEDs have been shown to be antiepileptogenic including valproate and phenobarbitone (Silver *et al.*, 1991; Duncan, 2002) and levetiracetam (Loscher *et al.*, 1998; Duncan, 2002) but these are not well substantiated. Clearly there is a pressing need for alternative pharmacotherapies of epilepsy which are not only anticonvulsant but also antiepileptogenics and will not infringe on the patient's quality of life. Natural products and plants for that matter, used in traditional medicine can serve as an invaluable source for novel antiepileptic compounds (Meldrum, 1997; Stafford *et al.*, 2008). They may provide better alternatives to the currently prescribed medications.



### 1.3.2 Pathophysiology of epilepsy

The susceptibility for generating an epileptic seizure varies between individuals because of differences in threshold for the development of epileptic seizures from one person to the other. Any brain can elicit a seizure if provoked sufficiently. Only if seizures become recurrent and are not provoked by systemic disease, is the individual diagnosed with epilepsy. Seizure phenomenology varies from patient to patient and more than one seizure type can occur within the same patient (McNamara and Puranam, 1996; Fisher *et al.*, 2005). Irrespective of the fact that epilepsy can be caused by a variety of intracranial structural, cellular or molecular conditions and manifests itself in different ways, the epileptic seizure always reflect abnormal hypersynchronous electrical activity of neurons, caused by an imbalance between excitation and inhibition in the brain. The neuronal membrane potential is regulated by an accurate balance between excitatory postsynaptic potential (EPSP) and inhibitory postsynaptic potential (IPSP). If this balance is compromised, an epileptic seizure can be generated (Dudek and Staley, 2007). More than 100 neurotransmitters or neuromodulators have been shown to play a role in the process of neuronal excitation. L-glutamate, the major excitatory amino acid plays a major role in the spread of seizure activity by acting at more than half of the neuronal synapses in the brain. There is also an increased release of glutamate in the brain associated with seizure activity. Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter of the CNS. Under normal conditions the excitatory post-synaptic potentials are followed immediately by GABAergic inhibition. Neuronal hypersynchronization occurs if excitatory mechanisms dominate; either initiated by increased excitation or decreased inhibition. As the abnormal neuronal hypersynchronous activity continues, more and more neurons are activated (high frequency depolarization or repolarization), generating the epileptic seizure that can be registered in the electroencephalogram. The existence of excitatory connections between pyramidal neurons generating epileptic bursts through a positive-feedback mechanism in epileptogenic areas and the fact that neurons in some epilepsy prone regions, (e.g. the structures of the limbic system in the temporal lobe, especially the hippocampal CA3 region), possess the capability to generate 'intrinsic bursts', dependent on voltage dependent calcium currents or persistent sodium currents has been identified as key features of epileptogenic circuits (Dudek and Staley, 2007). Sex hormones also influence the regulation of GABAergic transmission in the CNS (Verrotti *et al.*, 2009). Animal models have shown that the infusion of oestrogens lowers the threshold for experimentally provoked seizures, and that this effect of oestrogen is intensified if a cortical lesion is already present. Progesterone has been shown to possess an inhibitory effect on spontaneous and experimentally provoked seizures. Progesterone probably works through a direct activation of the GABA complex to enhance the effect of GABA.

Additionally, progesterone possesses the capability of inhibiting glutamatergic activity. The convulsant or anticonvulsant effects of oestrogens and progesterone respectively, are demonstrated in women with catamenial epilepsy (seizure clustering around the time of menses) (Morris and Vanderkolk, 2005). There is an increase in seizure frequency, immediately before the menstrual period, which correlates with a decrease in progesterone level and an additional increase in seizure frequency immediately before the time of ovulation, correlating with a high oestrogen level with no simultaneous increase in progesterone. At the end of ovulation, simultaneously with an increase in the progesterone level, the seizure frequency decreases (Verrotti *et al.*, 2009).

### 1.3.3 Classification of epilepsy

The clinical classification of epilepsy recognizes two categories, namely; partial seizures and generalized seizures, although there are some overlaps and many varieties of each (Bienvenu *et al.*, 2002). In partial seizures the discharge begins locally and often remains localized. These may produce relatively simple symptoms such as involuntary muscle contractions, abnormal sensory experiences or autonomic discharge without loss of consciousness or they may cause more complex effects on consciousness, mood and behaviour, often termed psychomotor epilepsy. Generalized seizures involve the whole brain, including the reticular system, thus producing abnormal electrical activity throughout both hemispheres. Immediate loss of consciousness is characteristic of generalized seizures (Bienvenu *et al.*, 2002; Engel, 2006). The main categories are generalized tonic-clonic seizures (*grand mal*) and absences seizures (*petit mal*). A generalized tonic-clonic seizure consists of an initial strong contraction of the whole musculature, causing a rigid extensor spasm. Respiration stops and defecation, micturition and salivation often occur. This tonic phase lasts for about 1 minute and is followed by a series of violent, synchronous jerks, which gradually dies out in 2-4 minutes. Most types of epilepsy are characterized by more than one type of seizure (Engel, 2006). Patients with focal (or partial) epilepsy may have simple partial, complex partial and secondarily generalized tonic-clonic seizures (e.g. partial seizures with secondary generalization). Patients with generalized epilepsy may have one or more of the following seizure types: absence, myoclonic, and tonic, clonic, tonic-clonic and atonic. Thus, no seizure type is specific for a single type of epilepsy. Seizures are symptoms, and patients should be treated for a type of epilepsy, not for a type of seizure (Benbadis and Tatum, 2001).



### 1.3.4 History of antiepileptic drugs

From 1857 to 1958, potassium bromide, phenobarbital (PB), and a variety of drugs that were derived mainly by modification of the barbiturate structure, including phenytoin (PHT), primidone (PRM), trimethadione, and ethosuximide (ESM) became the first generation antiepileptics entering the market (Krall *et al.*, 1978; Shorvon, 2009). The second generation AEDs, including carbamazepine (CBZ), valproate (VPA), and the benzodiazepines, introduced between 1960 and 1975, differed chemically from the barbiturates (Shorvon, 2009). The era of the third-generation AEDs started in the 1980s with “rational” developments such as progabide, vigabatrin (VGB), and tiagabine (TGB), that were designed to selectively target a mechanism that was thought to be critical for the occurrence of epileptic seizures (Loscher and Schmidt, 1994). Other examples include retigabine (a potassium channel activator), eslicarbazepine and lacosamide. The new, third-generation AEDs have undoubtedly expanded the therapeutic options, in particular for those in need of a change in medical regimen (Kwan and Brodie, 2000; Brodie *et al.*, 2007; Marson *et al.*, 2007).

### 1.3.5 Mechanism of action of antiepileptic drugs

Antiepileptic effects are produced by drugs that modulate ion channels, enhance inhibition mediated by GABA<sub>A</sub> receptors and/or glycinergic systems, the regionally specific transmitter systems (including monoamines such as catecholamines, serotonin and histamine, and neuropeptides such as opioid peptides, galanin and neuropeptide Y) and the inhibitory neuromodulator adenosine (Errington *et al.*, 2005; Surges *et al.*, 2008). In addition, blockade of glutamate receptors (including those of the NMDA, AMPA, kainate and group I metabotropic (mGluR1 and mGluR5 types) can also protect against seizures in animal models. In principle, it might be possible to prevent seizures by targeting any one or a combination of these systems (Rostock *et al.*, 1996; Rogawski, 2006). Consideration will be given to three of the major mechanisms of action of antiepileptic drugs: modulation of ion channels, enhancement of GABA inhibitory neurotransmission, and attenuation of glutamate mediated excitatory transmission (Kwan *et al.*, 2001).

#### 1.3.5.1 Modulation of ion channels

The intrinsic excitability of the nervous system is ultimately controlled by voltage-gated ion channels which regulate the flow of cations across surface and internal cell membranes. Voltage-gated sodium channel is arguably the most important ion channel responsible for depolarization of cell membranes. Modulation of the gating of brain sodium channels is believed to account, at least in part, for the ability of several AEDs to protect against generalized tonic-clonic and partial seizures (Deckers *et al.*, 2003). Phenytoin and

carbamazepine are prototype sodium channel blockers and this mechanism is shared by the newer drugs lamotrigine, felbamate, topiramate, oxcarbazepine and zonisamide (Schauf, 1987; Schmutz *et al.*, 1994; Taglialatela *et al.*, 1996; Albus and Williamson, 1998; Taverna *et al.*, 1999; Ambrosio *et al.*, 2001; Remy *et al.*, 2003). By binding mainly to the inactivated state of the sodium channel these drugs produce a voltage- and frequency-dependent reduction in channel conductance, resulting in a limitation of repetitive neuronal firing with little or no effect on the generation of single action potentials (Kwan *et al.*, 2001). Their actions translate indirectly into reduced transmitter output at synapses (Leach *et al.*, 1986). AEDs might also act by blocking the persistent sodium current (current that flows due to the overlap between the voltage ranges for sodium channel activation and inactivation) (Maurice *et al.*, 2001; Taddese and Bean, 2002). It has been reported that phenytoin (Chao and Alzheimer, 1995; Segal and Douglas, 1997; Lampl *et al.*, 1998; Niespodziany *et al.*, 2004), valproate (Taverna *et al.*, 1998) and topiramate (Taverna *et al.*, 1999) inhibit the persistent sodium current at concentrations lower than those that block fast sodium current. The selective reduction of late, persistent sodium channel openings might contribute to the ability of these drugs to protect against seizures with minimal interference in normal function (Rogawski and Loscher, 2004).

Voltage-gated calcium channels are also involved in depolarization. They are often recruited in response to initial sodium-dependent action potential generation. The N-, P- and Q-type calcium channels have been implicated in the control of neurotransmitter release at the synapse, whereas the T-type channel, expressed predominantly in the thalamocortical relay neurons, is believed to play a role in the distinctive rhythmic discharges of generalized absence seizures (Kwan *et al.*, 2001). The efficacy of ethosuximide against generalized absence seizures is believed to be mediated by blockade of the T-type calcium channel. Evidence suggests that valproate may have similar effects (Deckers *et al.*, 2003). Lamotrigine has also been reported to limit neurotransmitter release by blockade of the N- and P- subtypes of voltage-sensitive calcium channel while gabapentin binds to the  $\alpha 2\delta$ -subunit of the L-type channel (Kwan *et al.*, 2001).

Potassium channels also play a major role in the control of resting membrane potential, responsiveness to synaptic inputs, spike frequency adaptation and neurotransmitter release. They are therefore potential targets for antiepileptic drugs. Genetic, molecular, physiological and pharmacological evidence supports a role of some K<sup>+</sup> channels in the control of neuronal excitability and epileptogenesis (Wickenden, 2002). Retigabine functions through its ability to activate potassium currents (Rostock *et al.*, 1996; Rundfeldt, 1997).

### 1.3.5.2 Enhancement of inhibitory neurotransmission

Potentialiation of inhibitory neurotransmission mediated by GABA is a key mechanism of AED action. Compared to excitatory synapses, neurons that use GABA as their neurotransmitter represent only a small fraction of neurons in key regions implicated in epileptic activity, such as the neocortex, hippocampus and amygdala (Galarreta and Hestrin, 1998). These inhibitory connections are still capable of restraining the natural tendency of excitatory neurons from undergoing the transition into synchronized epileptiform discharges. Following synaptic release, GABA acts through GABA<sub>A</sub>, GABA<sub>B</sub> and GABA<sub>c</sub> receptors to bring about neuronal hyperpolarization leading to synaptic inhibition. Potassium bromide, the first effective epilepsy treatment, augments GABA<sub>A</sub> receptor-mediated inhibition by enhancing the sensitivity of GABA<sub>A</sub> receptors to GABA and increasing GABA<sub>A</sub> receptor currents (Gallagher *et al.*, 1978; Akaike *et al.*, 1989; Brunig *et al.*, 1999). Bromide salts continue to be widely used for treating epileptic dogs and cats in veterinary medicine although clinically it has been replaced by less toxic agents. Drugs that act by interacting with GABA<sub>A</sub> receptors or by modifying the activity of enzymes and transporters typically have a broad spectrum of antiepileptic activity in human seizure disorders, although, with the exception of benzodiazepine receptor agonists, they are generally ineffective in absence seizures. The actions of valproate and gabapentin (by increasing GABA synthesis and turnover) overlap with drugs that interact with GABA systems. The antiepileptic activity of benzodiazepine-like agents occurs through positive allosteric modulation of GABA<sub>A</sub> receptors containing the  $\alpha 2$  subunit,  $\beta 1$  subunit (necessary for seizures protection) and effects on tonic GABA<sub>A</sub> receptor currents, which originate from GABA acting on extrasynaptic receptors (Rudolph *et al.*, 1999; Crestani *et al.*, 2000). Benzodiazepines have an important clinical role in the acute treatment of status *epilepticus* though sedation, muscle relaxation but development of tolerance and dependence limit their chronic use. Benzodiazepines, such as clonazepam have anti-absence activity by inhibiting the 3-Hz spike-and-wave activity believed to underlie the generalized absence seizures, in thalamocortical circuits) (Sohal *et al.*, 2003). Thalamic reticular neurons exert an inhibitory influence on thalamocortical relay neurons that is necessary for de-inactivation of the T-type calcium currents that underlie bursting. Benzodiazepines reduce the inhibitory output of the reticular neurons by effects on benzodiazepine-sensitive  $\alpha 3$ -containing GABA<sub>A</sub> receptors and therefore prevent absence seizure activity.

Barbiturates such as phenobarbital also act as positive allosteric modulators of GABA<sub>A</sub> receptors to shift the relative proportion of openings to favour the longest-lived open state associated with prolonged bursting (brief openings in rapid succession), thereby increasing the overall probability that the channel is open (Macdonald and Olsen, 1994). In addition,



barbiturates act on other ion channel systems, including calcium and sodium channels, and this probably contributes to their therapeutic activity and might also be a factor in their side effects (French-Mullen *et al.*, 1993). Felbamate (Rho *et al.*, 1997) and topiramate (White *et al.*, 1997; Gordey *et al.*, 2000) might also act, in part, through positive modulation of GABA<sub>A</sub> receptors. The concentration of GABA in the brain is controlled by two pyridoxal-5'-phosphate-dependent enzymes, glutamate decarboxylase (GAD) and GABA transaminase (GABAT). The AED vigabatrin ( $\gamma$ -vinyl GABA) elevates brain GABA by acting as an irreversible suicide inhibitor of GABAT (De Biase *et al.*, 1991). The AED tiagabine is a potent and selective competitive inhibitor of GABA transporter 1 (GAT1) that binds with high affinity for the transporter and prevents GABA uptake without itself being transported (Suzdak and Jansen, 1995). By slowing the reuptake of synaptically released GABA, tiagabine prolongs inhibitory postsynaptic potentials (Thompson and Gahwiler, 1992; Jackson *et al.*, 1999).

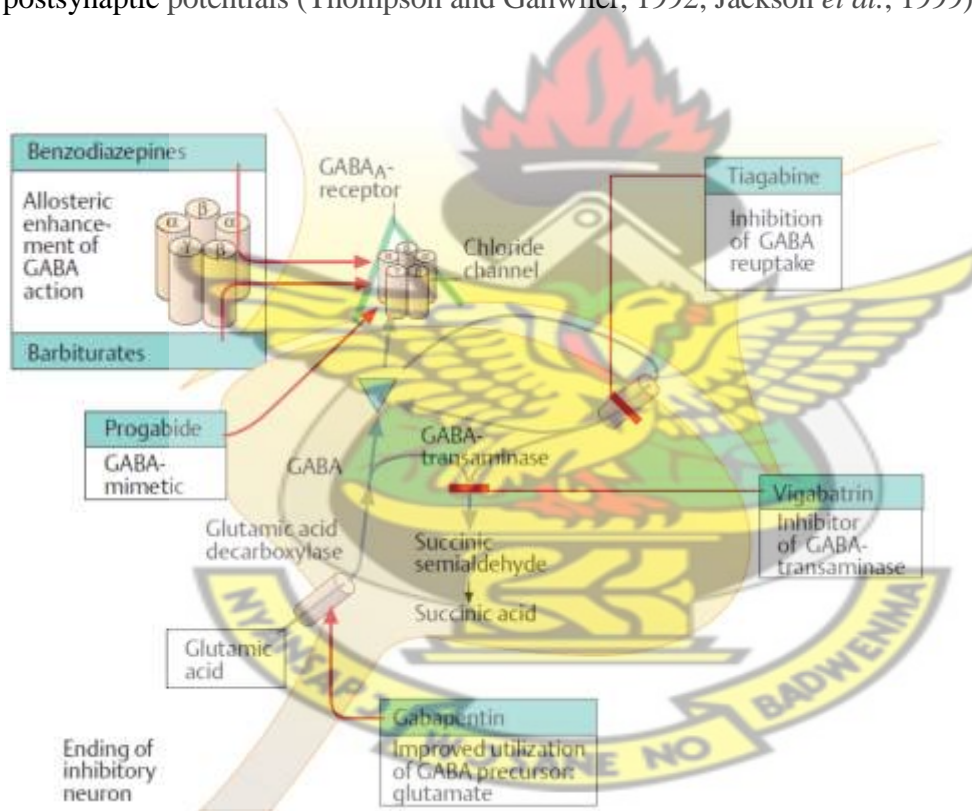


Figure 1.2 Sites of action of antiepileptics in GABAergic synapse

### 1.3.5.3 Attenuation of excitatory neurotransmission

Glutamate is the principal excitatory neurotransmitter in the mammalian brain. Following synaptic release, it exerts its effects on both ionotropic (AMPA, kainate and NMDA) and metabotropic receptor types. The ionotropic glutamate receptors form ligand-gated ion

channels permeable to sodium and depending on subtype and subunit composition, calcium ions. The AMPA and kainite subtypes are implicated in fast excitatory neurotransmission, whereas the NMDA receptor, quiescent at resting membrane potential, is recruited during periods of prolonged depolarization (Kwan *et al.*, 2001). NMDA receptors consist of NR1 subunits combined with one or more NR2 (A–D) subunits, which form channels that are permeable to sodium and calcium ions. The activity of NMDA receptors is regulated via a strychnine-insensitive glycine-binding site and other modulatory sites, such as polyamines,  $Zn^{2+}$  and  $H^+$ . At resting membrane potentials, the pore of this receptor is blocked by magnesium ions, which are removed after membrane depolarization. The role of NMDA receptors in experimental epileptogenesis, neuroplasticity, seizures and excitotoxicity has been firmly established (Delorenzo *et al.*, 2005; Lason *et al.*, 2011). Antagonists of NMDA receptors, such as dizocilpine or ketamine, inhibit seizures induced by pentylenetetrazole, pilocarpine, maximal electroshock or sensory stimulation. Furthermore, they delay the development of amygdala kindling but have a weaker effect on fully developed seizures in this model. Unfortunately, both competitive and non-competitive NMDA receptor antagonists show serious undesired effects, such as psychomotor, memory and cognitive disturbances, and psychotomimetic-like effects in experimental animals and in initial clinical studies. However allosteric modulators of NMDA receptors, especially modulators that interact with the strychnine-insensitive glycine-binding site and the polyamine-binding site, are more promising as potential AEDs. The partial agonist of the glycine binding site, D-cycloserine, exerts anticonvulsant activity most likely via the desensitization of NMDA receptors. This compound also augments the seizure suppressing effects of some AEDs and, in low doses, has a positive influence on memory processes. Beneficial effects in experimental models of seizures have been observed after the concomitant administration of glycine- and polyamine-binding site antagonists. In 2008 lacosamide (LCM), an antagonist of the glycine binding site on NMDA receptors, was registered as an AED. Blockade of the NMDA subtype of glutamate receptor and AMPA receptors have also been reported to contribute to the antiepileptic effects of felbamate and topiramate respectively (Deckers *et al.*, 2003).

#### 1.3.6 Dietary treatment of epilepsy

A growing body of evidence demonstrates that dietary therapies for epilepsy are highly effective, with approximately 30–60% of children overall having at least a 50% reduction in seizures after 6 months of treatment (Henderson *et al.*, 2006; Neal *et al.*, 2008; Freeman, 2009; Nabbout *et al.*, 2010). Ketogenic diets may be also effective for adult *status epilepticus* and



adult epilepsy (Wusthoff *et al.*, 2010; Smith *et al.*, 2011) and as a first-line treatment of seizures associated with glucose transporter 1 deficiency (De Vivo *et al.*, 2002).

All dietary therapies used to treat epilepsy share the common characteristic of restricting carbohydrate intake to shift the predominant caloric source of the diet to fat (Huffman and Kossoff, 2006). Body tissues are thereby forced to catabolize fats as their primary source of energy, and the catabolism of fats results in ketones, hence the origin of the common descriptor for these therapies, “the ketogenic diet.” The precise mechanisms by which the ketogenic diet yields its anticonvulsant effect are not known, but appear unique from the mechanisms of action for other anticonvulsants (Bough and Rho, 2007).

### 1.3.7 Experimental Models for Anticonvulsant screening

#### 1.3.7.1 Acute models

The maximal electroshock (MES) test and the pentylenetetrazole (PTZ) seizure are often classified as acute animal models (Loscher, 2002). The first important neuropharmacological step in detecting the potential value of candidate anticonvulsant compounds is the classical maximal electroshock (MES) test in mice, introduced by Putnam and Merritt (Putnam and Merritt, 1937). The MES is the most widely used animal model in AED discovery, because seizure induction is simple and the predictive value for detecting clinically effective AEDs is high (Loscher, 2002). A powerful detection system is ensured when the MES is combined with the pentylenetetrazole (PTZ) seizure test. These are the two primary bioassays employed in the in vivo screening of new anticonvulsant compounds (Krall *et al.*, 1978; Loscher *et al.*, 1988; Raza *et al.*, 2001). AEDs such as phenytoin, carbamazepine, valproic acid, that inhibit the hind limb tonic extension phase (HLTE) of the electroshock seizure in MES are effective in the therapy of generalized tonic-clonic and partial seizures, while AEDs that inhibit seizures induced by pentylenetetrazole (PTZ) in PTZ test e.g. ethosuximide and phenobarbitone are effective in the treatment of generalized myoclonic and absence seizures (White, 1997; Raza *et al.*, 2001). The subcutaneous administration of bicuculline (BIC), picrotoxin (PTX) and strychnine (STR) are also valuable tests to induce seizures and evaluate the effectiveness and mechanisms of anticonvulsant compounds (Porter *et al.*, 1984; Raza *et al.*, 2001). Clinically efficacious drugs have been discovered by these acute models including ethosuximide, trimethadone and valproate. These show similar anticonvulsant effects in different genetic models of absence epilepsy such as Genetic Absence Epilepsy Rats from Strasbourg (GAERS) or lethargic mice (Loscher, 2002).

### 1.3.7.2 Chronic models

Acute models might not detect all compounds with antiepileptic activity when used alone. The MES test preselects drugs with certain mechanisms, but misses others (Meldrum, 1997). Although the MES test is often considered a mechanism-independent model (Kupferberg, 2001), it is particularly sensitive to drugs blocking sodium channels (Meldrum, 1997). This means that several clinically efficacious AEDs which act by other mechanisms (such as levetiracetam, vigabatrin and tiagabin) and were initially not screened or detected by using MES test would have been missed using this model as the only drug discovery model (Loscher and Schmidt, 1994). Also, the PTZ test might not be able to detect all antiepileptic drugs against non-convulsive seizures (Loscher, 2002). This is due to the fact that lamotrigine, which is very efficacious against non-convulsive seizures in patients, is ineffective in the PTZ test, while vigabatrin and tiagabin, which are quite effective in the PTZ test, are ineffective in patients and even aggravate non-convulsive seizures (Loscher, 2002). In this case chronic seizure models including kindling, genetic models such as GAERS or lethargic mice have been used (Loscher, 2002). Also, even after the primary screening of anticonvulsants, advanced experiments on primate models and 'Kindling in rodents' which may follow include monkey models of absence (petit mal) seizures, aluminium hydroxide induced partial or secondary generalised (grand mal) seizures in monkeys, experimental temporal lobe epilepsy in monkeys and amygdala kindled seizures in rats. Also the plicarpine induced status epilepticus which is a model of temporal lobe epilepsy is also widely used.

## 1.4 DEPRESSION

### 1.4.1 Background

Epileptic patients often present with other psychiatric comorbidities, one of the commonest being depression. There is a general consensus that epileptic patients have a four to fivefold higher incidence of depression and up to a fivefold higher incidence of suicide than the general population (Hauser and Kurland, 1975; Standage and Fenton, 1975; Kogeorgos *et al.*, 1982).

Depression is a chronic recurring illness that affects 120 million people worldwide. Major depressive disorder (depression) is characterized by the core symptoms of depressed mood and a loss of interest and/or pleasure. Other symptoms that may be manifested include significant weight changes (loss or gain), sleep disturbances (insomnia or hypersomnia), fatigue or loss of energy, diminished ability to think or concentrate, feelings of worthlessness or guilt, recurrent thoughts of death or suicide, and psychomotor agitation or retardation (Khawam *et al.*, 2006). In industrialized societies, approximately 5% of the population experienced a major depressive

episode (MDE, defined as the occurrence of a core symptom and 4 or more other symptoms daily or almost daily for  $\geq 2$  weeks) in the past year (Andrews *et al.*, 2002). It is likely that many brain regions mediate the diverse symptoms of depression. Knowledge of the function of these brain regions under normal conditions suggests the aspects of depression to which they may contribute. Neocortex and hippocampus may mediate cognitive aspects of depression, such as memory impairments and feelings of worthlessness, hopelessness, guilt, doom, and suicidality. The striatum (particularly the ventral striatum or nucleus accumbens) and amygdala, and related brain areas, are important in emotional memory, and could mediate the anhedonia (decreased drive and reward for pleasurable activities), anxiety, and reduced motivation that predominates in many patients (Schlaepfer *et al.*, 2008). Given the prominence of so-called neurovegetative symptoms of depression, including too much or too little sleep, appetite, and energy, as well as a loss of interest in sex and other pleasurable activities, a role of the hypothalamus has also been speculated. These various brain regions operate in a series of highly interacting parallel circuits, which perhaps begins to formulate a neural circuitry involved in depression (Nestler *et al.*, 2002).

Over the past four to five decades, a number of antidepressants have been approved for use. The newer ones seem to offer better patient tolerability and safety than the older ones. Yet there are almost 40% of patients whose condition are refractory to current treatment. Current medications still have slow onset of action (Khawam *et al.*, 2006). Though there are evidences to suggest that rapid onset antidepressants are available none have been approved yet (Lucas *et al.*, 2007; Duman and Voleti, 2012). These reasons underscore the need for alternative medicines that will cater for the needs of those with refractory depression and also provide rapid onset of action.

#### 1.4.2 Theories of Depression

There has been a marked shift in the last decade in the understanding of the pathophysiology of major depression. In addition to the older idea that a deficit in function or amount of monoamines (the monoamine hypothesis) is central to the biology of depression, there is evidence that neurotrophic and endocrine factors also play a major role.

##### 1.4.2.1 Monoamine Hypothesis

The monoamine hypothesis of depression suggests that depression is related to a deficiency in the amount or function of cortical and limbic serotonin (5-HT), norepinephrine (NE), and dopamine (DA) (Hirschfeld, 2000). Evidence to support the monoamine hypothesis comes from several sources. Reserpine treatment, which depletes monoamines, is associated with



depression in a subset of patients. Similarly, depressed patients who respond to serotonergic antidepressants such as fluoxetine often rapidly suffer relapse when given diets free of tryptophan, a precursor of serotonin synthesis. Patients who respond to noradrenergic antidepressants such as desipramine are less likely to relapse on a tryptophan-free diet (Hirschfeld, 2000; Yamada and Higuchi, 2002). However, depleting catecholamines in depressed patients who have previously responded to noradrenergic agents likewise tends to be associated with relapse. Administration of an inhibitor of norepinephrine synthesis is also associated with a rapid return of depressive symptoms in patients who respond to noradrenergic but not necessarily in patients who had responded to serotonergic antidepressants (Yamada and Higuchi, 2002).

Another line of evidence supporting the monoamine hypothesis comes from genetic studies (Yamada and Higuchi, 2002). A functional polymorphism exists for the promoter region of the serotonin transporter gene, which regulates how much of the transporter protein is available. Subjects who are homozygous for the *s* (short) allele may be more vulnerable to developing major depression and suicidal behavior in response to stress. In addition, homozygotes for the *s* allele may also be less likely to respond to and tolerate serotonergic antidepressants. Conversely, subjects with the *l* (long) allele tend to be more resistant to stress and may be more likely to respond to serotonergic antidepressants (Drevets *et al.*, 1999; Yamada and Higuchi, 2002). Studies of depressed patients have sometimes shown an alteration in monoamine function. For example, some studies have found evidence of alteration in serotonin receptor numbers (5-HT<sub>1A</sub> and 5-HT<sub>2C</sub>) or norepinephrine ( $\alpha_2$ ) receptors in depressed and suicidal patients, but these findings have not been consistent (Drevets *et al.*, 1999). Perhaps the most convincing line of evidence supporting the monoamine hypothesis is the fact that all available antidepressants appear to have significant effects on the monoamine system. All classes of antidepressants appear to enhance the synaptic availability of 5-HT, norepinephrine, or dopamine (Elhwuegi, 2004).

The monoamine hypothesis is at best incomplete (Boerkamp and Wijnberger, 2011). Many studies have not found an alteration in function or levels of monoamines in depressed patients. In addition, some candidate antidepressant agents under study do not act directly on the monoamine system (Boerkamp and Wijnberger, 2011). These include glutamate antagonists, melatonin agonists, and glucocorticoid-specific agents. Thus, monoamine function appears to be an important but not exclusive factor in the pathophysiology of depression.



#### **1.4.2.2 Neurotrophic Hypothesis**

There is substantial evidence that nerve growth factors such as brain-derived neurotrophic factor (BDNF) are critical in the regulation of neural plasticity, resilience, and neurogenesis (Groves, 2007; Hasler, 2010). The evidence suggests that depression is associated with the loss of neurotrophic support and that effective antidepressant therapies increase neurogenesis and synaptic connectivity in cortical areas such as the hippocampus. BDNF is thought to exert its influence on neuronal survival and growth effects by activating the tyrosine kinase receptor B in both neurons and glia (Groves, 2007; Hasler, 2010). Animal and human studies indicate that depression, stress and pain are associated with a drop in BDNF levels and that this loss of neurotrophic support contributes to atrophic structural changes in the hippocampus and perhaps other areas such as the medial frontal cortex and anterior cingulate which play important roles in depression. Moreover, all known classes of antidepressants are associated with an increase in BDNF levels in animal models with chronic administration. This increase in BDNF levels is consistently associated with increased neurogenesis in the hippocampus in these animal models (Duman and Li, 2012; Voleti and Duman, 2012). Other interventions thought to be effective in the treatment of major depression, including electroconvulsive therapy, also appear to robustly stimulate BDNF levels and hippocampus neurogenesis in animal models. Human studies seem to support the animal data on the role of neurotrophic factors in stress states. Thus, the neurotrophic hypothesis continues to be intensely investigated and has yielded new insights and potential targets in the treatment of major depressive disorder (MDD) (Duman and Li, 2012; Voleti and Duman, 2012).

#### **1.4.2.3 Neuroendocrine Factors in the Pathophysiology of Depression**

Depression is known to be associated with a number of hormonal abnormalities (Cleare *et al.*, 1995; Cleare *et al.*, 1995). Among the most replicated of these findings are abnormalities in the hypothalamic- pituitary-adrenal (HPA) axis in patients with MDD (Gotlib *et al.*, 2008). Moreover, MDD is associated with elevated cortisol levels, nonsuppression of adrenocorticotrophic hormone (ACTH) release in the dexamethasone suppression test, and chronically elevated levels of corticotropin-releasing hormone (Gotlib *et al.*, 2008; Pariante and Lightman, 2008). The significance of these HPA abnormalities is unclear, but they are thought to indicate a dysregulation of the stress hormone axis. More severe types of depression, such as psychotic depression, tend to be associated with HPA abnormalities more commonly than milder forms of major depression. It is well known that both exogenous glucocorticoids and endogenous elevation of cortisol are associated with mood symptoms and cognitive deficits similar to those seen in MDD (Markopoulou *et al.*, 2009). Thyroid dysregulation has also been

reported in depressed patients (Engum *et al.*, 2002). Up to 25% of depressed patients are reported to have abnormal thyroid function. These include a blunting of response of thyrotropin to thyrotropin-releasing hormone, and elevations in circulating thyroxine during depressed states. Clinical hypothyroidism often presents with depressive symptoms, which resolve with thyroid hormone supplementation (Cole *et al.*, 2002; Engum *et al.*, 2002). Thyroid hormones are also commonly used in conjunction with standard antidepressants to augment therapeutic effects of the latter (Engum *et al.*, 2002). Finally, sex steroids are also implicated in the pathophysiology of depression (Kessler, 2003; Almeida *et al.*, 2004). Estrogen deficiency states, which occur in the postpartum and postmenopausal periods, are thought to play a role in the aetiology of depression in some women. Likewise, severe testosterone deficiency in men is sometimes associated with depressive symptoms (Kessler, 2003). Hormone replacement therapy in hypogonadal men and women may be associated with an improvement in mood and depressive symptoms (Almeida *et al.*, 2004).

### 1.4.3 Antidepressant Drugs

#### 1.4.3.1 Monoamine Oxidase Inhibitors

In 1952 iproniazid, a hydrazine derivative was found to inhibit monoamine oxidase (MAO). It was later used as the first antidepressant to treat depressed patients but owing to hepatotoxicity its use was discontinued (Nelson *et al.*, 1978; Timbrell, 1979). Two other hydrazine-derivative inhibitors of MAO, phenelzine (the structural analog of phenethylamine, an endogenous amine) and isocarboxazid subsequently were introduced into clinical practice. Tranylcypromine, structurally related to amphetamine, was the first MAO inhibitor unrelated to hydrazine to be discovered. The development of reversible, selective MAO inhibitors with potentially broad applications (e.g. selegiline for Parkinson's disease) was stimulated by the understanding that the early MAO inhibitors result in irreversible and nonselective blockade of both MAO-A and MAO-B, which were responsible for the metabolic breakdown of dopamine, norepinephrine, and serotonin in neuronal tissues. The nonselective MAO inhibitors in clinical use are reactive hydrazines (phenelzine and isocarboxazid) or amphetamine derivatives (tranylcypromine). Selegiline, a propargylamine, is relatively specific for MAO-B (Cesura and Pletscher, 1992). Following their oxidation to reactive intermediates by MAO, each of these "suicide" substrates interacts irreversibly to inactivate the flavin prosthetic group of the MAO enzyme (Krishnan, 2007). Cyclization of the side chain of amphetamine resulted in tranylcypromine. After formation of a reactive imine intermediate by MAO, inhibition of MAO by this cyclopropylamine derivative may involve the reaction of a sulfhydryl group in the active site of MAO. Due to the irreversible inactivation of MAO, these compounds produce long-acting

inhibition that may persist for up to 2 weeks after drug discontinuation. Short-acting, reversible inhibitors of MAO-A (RIMAs) with antidepressant activity are also available. These include a piperidylbenzofuran (Brofaromine), a morpholinobenzamide (Moclobemide) and an oxazolidinone (Toloxatone) (Delini-Stula *et al.*, 1988).

#### **1.4.3.2 Tricyclic Antidepressants**

Tricyclic antidepressants are closely related in structure to the phenothiazines and were initially synthesized in 1949 as potential antipsychotic drugs (Gallanosa *et al.*, 1981). Imipramine was found to be of no use in schizophrenia but effective in depression, so related compounds, such as clomipramine, were synthesised. In addition to their effects on amine uptake, most TCAs affect one or more types of neurotransmitter receptor, including muscarinic acetylcholine receptors, histamine receptors and 5-HT receptors. The antimuscarinic effects of TCAs do not contribute to their antidepressant effects but are responsible for their various side effects. Older tricyclic antidepressants with a tertiary-amine side chain (including amitriptyline, doxepin, and imipramine) block neuronal uptake of both serotonin and norepinephrine, whereas clomipramine is relatively selective against serotonin (Gallanosa *et al.*, 1981).

The actions of imipramine-like tricyclic antidepressants also include a range of complex, secondary adaptations to their initial and sustained actions as inhibitors of norepinephrine neuronal transport (reuptake) and variable blockade of serotonin transport (Beasley *et al.*, 1992). Tricyclic-type antidepressants with secondary-amine side chains or the N-demethylated (nor) metabolites of agents with tertiary-amine moieties (e.g., amoxapine, desipramine, maprotiline, norclomipramine, nordoxepin, and nortriptyline) are relatively selective inhibitors of norepinephrine transport. Most tertiary-amine tricyclic antidepressants also inhibit the reuptake of serotonin. It is likely that relatively selective inhibitors of norepinephrine reuptake, including atomoxetine and reboxetine, share many of the actions of older inhibitors of norepinephrine transport (Kratochvil *et al.*, 2003) such as desipramine (Delgado and Michaels, 1999). Among the tricyclic antidepressants, trimipramine is exceptional in that it lacks prominent inhibitory effects at monoamine transport, and its clinical actions remain unexplained. The tricyclic and other norepinephrine-active antidepressants do not block dopamine transport, thereby differing from CNS stimulants, including cocaine, methylphenidate, and amphetamines. Nevertheless, they may facilitate effects of dopamine indirectly by inhibiting the nonspecific transport of dopamine into noradrenergic terminals in cerebral cortex. Tricyclic antidepressants also can desensitize D2 dopamine autoreceptors, through uncertain mechanisms and with uncertain behavioral contributions. In addition to their transport-inhibiting effects, tricyclic antidepressants variably interact with adrenergic receptors.



The presence or absence of such receptor interactions appears to be critical for responses to increased availability of extracellular norepinephrine in or near synapses. Most tricyclic antidepressants have at least moderate and selective affinity for  $\alpha_1$  adrenergic receptors, much less for  $\alpha_2$ , and virtually none for  $\beta$ -receptors. The  $\alpha_2$  receptors include presynaptic autoreceptors that limit the neurophysiological activity of noradrenergic neurons ascending from the locus coeruleus in brainstem to supply mid- and forebrain projections. The same noradrenergic neurons provide descending projections to the spinal cord preganglionic cholinergic efferents to the peripheral autonomic ganglia. Autoreceptor mechanisms also reduce the synthesis of norepinephrine through the rate-limiting enzyme tyrosine hydroxylase, presumably through  $\alpha_2$  adrenergic receptor attenuation of cyclic AMP-mediated phosphorylation-activation of the enzyme. Activation of these receptors inhibits transmitter release by incompletely defined molecular and cellular actions that likely include suppression of voltage-gated  $\text{Ca}^{2+}$  currents and activation of G protein-coupled, receptor-operated  $\text{K}^+$  currents. The  $\alpha_2$  receptor-mediated, presynaptic, negative-feedback mechanisms are rapidly activated after administration of tricyclic antidepressants. By limiting synaptic availability of norepinephrine, such mechanisms normally tend to maintain functional homeostasis. However, with repeated drug exposure,  $\alpha_2$ -receptor responses eventually are diminished. This loss may result from desensitization secondary to increased exposure to the endogenous agonist ligand norepinephrine, or alternatively from prolonged occupation of the norepinephrine transporter itself via an allosteric effect, as suggested for inhibitors of serotonin transporters on serotonergic neurons (Chaput *et al.*, 1991). Over a period of days to weeks, this adaptation allows the presynaptic production and release of norepinephrine to return to, or even exceed, baseline levels (Charney *et al.*, 1987). However, long-term treatment eventually can reduce the expression of tyrosine hydroxylase as well as the norepinephrine transporter (NET) protein (Nestler *et al.*, 1990). Other adaptive changes have been observed in response to long-term treatment with tricyclic antidepressants. These include altered sensitivity of muscarinic acetylcholine receptors as well as decreases in gamma-aminobutyric acid ( $\text{GABA}_B$ ) receptors and possibly N-methyl-D-aspartate (NMDA) glutamate receptors (Kitamura *et al.*, 1992). In addition, cyclic AMP production is increased and the activities of protein kinases altered in some cells, including those acting on cytoskeletal and other structural proteins that may alter neuronal growth and sprouting (Perez *et al.*, 1991). Transcription and neurotrophic factors also are affected, including the cyclic AMP-response-element binding protein (CREB) and brain-derived neurotrophic factor (BDNF) (Duman *et al.*, 1997). Additional changes may be indirect effects of antidepressant treatment or may reflect recovery from depressive illness. These include normalization of glucocorticoid release and glucocorticoid receptor sensitivity, as well



as shifts in the production of prostaglandins and cytokines and in lymphocyte functions (Kitamura *et al.*, 1989).

#### **1.4.3.3 Selective Serotonin Reuptake Inhibitors (SSRIs)**

SSRIs block neuronal transport of serotonin both immediately and chronically, leading to complex secondary responses. Citalopram and fluoxetine are racemates; sertraline and paroxetine are separate enantiomers. Escitalopram is the (S)-enantiomer of citalopram. Fluoxetine and its major metabolite norfluoxetine are highly active against serotonin transport and also may have antimigraine effects not found with the (R)-enantiomer of fluoxetine. The (R)-enantiomer of fluoxetine also is active against serotonin transport and is shorter acting than the (S)-enantiomer, but its clinical development was halted due to adverse electrocardiographic effects. (R)-Norfluoxetine is virtually inactive (Wong *et al.*, 1993). Structure-activity relationships are not well established for the SSRIs. However, it is known that the para-location of the CF<sub>3</sub> substituent of fluoxetine is critical for serotonin transporter potency. Its removal and substitution at the ortho-position with a methoxy group yields nisooxetine, a highly selective norepinephrine-uptake inhibitor.

Increased synaptic availability of serotonin stimulates a large number of postsynaptic 5-HT receptor types (Azmitia *et al.*, 1995). Stimulation of 5-HT<sub>3</sub> receptors is suspected to contribute to common adverse effects characteristic of this class of drugs, including gastrointestinal effects (nausea and vomiting) and delayed or impaired orgasm. Stimulation of 5-HT<sub>2C</sub> receptors may contribute to agitation or restlessness. An important parallel in responses of serotonin and norepinephrine neurons is that negative feedback mechanisms rapidly emerge to restore homeostasis (Azmitia *et al.*, 1995). In the serotonin system, 5-HT<sub>1</sub>-subtype autoreceptors (types 1A and 7 at raphe cell bodies and dendrites, type 1D at terminals) suppress serotonin neurons in the raphe nuclei of the brainstem, inhibiting both tryptophan hydroxylase (probably through reduced phosphorylation-activation) and neuronal release of serotonin. Repeated treatment leads to gradual down-regulation and desensitization of autoreceptor mechanisms over several weeks (particularly of 5-HT<sub>1D</sub> receptors at nerve terminals), with a return or increase of presynaptic activity, production, and release of serotonin (Blier *et al.*, 1990). Additional secondary changes include gradual down-regulation of postsynaptic 5-HT<sub>2A</sub> receptors that may contribute to antidepressant effects directly, as well as by influencing the function of noradrenergic and other neurons via serotonergic heteroreceptors.

Many other postsynaptic 5-HT receptors presumably remain available to mediate increased serotonergic transmission and contribute to the mood-elevating and anxiolytic effects of this class of drugs. As in responses to norepinephrine-transport inhibitors, complex late adaptations

occur upon repeated treatment with serotonin reuptake inhibitors. These may include indirect enhancement of norepinephrine output by reduction of tonic inhibitory effects of 5-HT<sub>2A</sub> heteroceptors. Similar nuclear and cellular adaptations occur as with the tricyclic antidepressants (Azmitia *et al.*, 1995; Hyman and Nestler, 1996).

#### **1.4.3.4 Atypical Antidepressants**

Atypical antidepressants represent a heterogeneous group comprising agents that interfere only weakly or not at all with monoamine reuptake (trazodone, nefazodone, bupropion, mirtazapine), preferentially block reuptake of norepinephrine (reboxetine), or act as dual inhibitors of 5-HT and norepinephrine reuptake (venlafaxine, milnacipran, duloxetine) (Horst and Preskorn, 1998). Venlafaxine appears to be as effective as tricyclic antidepressants in severe depression. Mirtazapine and mianserin are structural analogs of serotonin with potent antagonistic effects at several postsynaptic serotonin receptor types (including 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub>, and 5-HT<sub>3</sub> receptors) and can produce gradual down-regulation of 5-HT<sub>2A</sub> receptors (Golden *et al.*, 1998). Mirtazapine also limits the effectiveness of inhibitory  $\alpha_2$  adrenergic heteroceptors on serotonergic neurons as well as inhibitory  $\alpha_2$  autoreceptors and 5-HT<sub>2A</sub> heteroceptors on noradrenergic neurons. These effects may enhance release of amines and contribute to the antidepressant effects of these drugs. Mirtazapine also is a potent histamine H<sub>1</sub>-receptor antagonist and is relatively sedating (Arko and Florjanc, 2001).

### **1.4.4 Animal models of depression**

#### **1.4.4.1 Forced Swimming Test**

The forced swimming test, originally described by Porsolt *et al.*, (1977) is the most widely used laboratory test for assessing the potential clinical antidepressant activity of drugs (Cryan *et al.*, 2002; 2005). This is largely due to its ease of use, reliability across laboratories, ability to detect a broad spectrum of antidepressants, and its capacity to meet the high through-put demands of the pharmaceutical industry (Borsini and Meli, 1988; Rupniak, 2003). The development of immobility when rodent is placed in an inescapable cylinder of water is thought to reflect the cessation of persistent escape-directed behaviour that serves various adaptive functions in response to stress (Detke *et al.*, 1997; Cryan and Mombereau, 2004).

#### **1.4.4.2 Tail Suspension Test**

The TST is based on the observation that rodents (almost always mice although gerbils and rats have been used (Chermat *et al.*, 1986; Varty *et al.*, 2003), after initial escape-oriented movements, develop an immobile posture when placed in an inescapable stressful situation. The stressful situation involves the haemodynamic stress of being hung in an uncontrollable

fashion by their tail (Thierry *et al.*, 1986). If antidepressant treatments are given prior to the test, the subjects will actively persist engaging in escape-directed behaviours for longer periods of time than after vehicle treatment. The test is usually quite short, and the amount of time they spend immobile is recorded either manually or through an automated device (Steru *et al.*, 1985). Acute antidepressant treatments decrease these immobility scores. An obvious advantage of this test is its ability to detect a broad spectrum of antidepressants irrespective of their underlying mechanism; it is inexpensive, methodologically unsophisticated and easily amenable to automation.

## 1.5 ANXIETY

### 1.5.1 Background

Anxiety is both a normal emotion and a psychiatric disorder. Anxiety is a feeling of apprehension or fear, combined with symptoms of sympathetic activity. It is a normal response to stress and only becomes a clinical problem if it becomes severe or persistent, and interferes with everyday performance. It has a lifetime prevalence of over 5% of the population (Sinclair *et al.*, 2007). A number of pharmacological theories exist which suggest that anxiety is caused by either amine or excitatory amino acid function so anxiolytics have therefore, been developed to target specific brain neurotransmitter systems. The GABA<sub>A</sub> receptor is the brain's main inhibitory receptor and so regulates the activity of many types of neurons, including dopaminergic, noradrenergic and serotonergic. There is considerable evidence that a down-regulation of GABA<sub>A</sub> function may underlie some forms of anxiety, some of which comes from imaging studies (Malizia *et al.*, 1998).

Most anticonvulsant drugs act via GABA and glutamate neurotransmission and so can serve as sources for novel anxiolytics. Benzodiazepines act on GABA<sub>A</sub>-BZ receptor and are effective predominantly in panic disorder (PD), general anxiety disorder (GAD) and social phobia (Malizia *et al.*, 1998). Gabapentin and pregabalin are effective in certain anxiety disorders. Pregabalin, which works via voltage-gated Ca<sup>2+</sup> channels, causing decreased release of several neurotransmitters, has shown short-term efficacy GAD (Sinclair *et al.*, 2007). The anticonvulsant properties of lamotrigine are mediated via NMDA glutamate receptor antagonism. Efficacy has been shown in post-traumatic stress disorder (PTSD) (Sinclair *et al.*, 2007). Tiagabine, a GABA<sub>A</sub> reuptake inhibitor, has had mixed results in clinical trials but has shown efficacy in GAD as well as in PD.



### 1.5.2 Animal models used for screening anxiolytics

Animal tests of anxiety are used to screen for novel compounds for anxiolytic or anxiogenic activity, to investigate the neurobiology of anxiety, and to assess the impact of other occurrences such as exposure to predator odours or early rearing experiences. These models involve exposure of animals to stimuli (exteroceptive or interoceptive) that appear capable of causing anxiety in humans. Despite their apparent diversity, animal anxiety models may be grouped into two general categories involving either conditioned (e.g. Geller-Seifter conflict, potentiated startle) or unconditioned (social interaction and light/dark exploration tests) responses (Rodgers and Dalvi, 1997). An ideal model of anxiety should have predictive, face and construct validities. A model that has predictive validity should display reduced anxiety when treated with anxiolytics, while in a model with face validity, the response of an animal to a threatening stimulus should be comparable to the response known for humans, and the mechanisms underlying anxiety should be exhibited by a model with construct validity (McKinney and Bunney, 1969). Naturally, one or more models usually combine to achieve these parameters. Conditioning models require considerable training of subjects, food or water deprivation and/or the use of electric shock as an aversive stimulus. However, some of these procedures, such as conditioned defensive burying, take advantage of the natural tendency of rodents to make faster stimulus-response associations when faced with ecologically relevant (versus arbitrary) environmental challenges (Treit, 1990). The study of unconditioned responses to various forms of external threat represents a logical extension of this refinement of laboratory methods, providing a high degree of ecological validity for the research and allowing for a very much more complete behavioural characterization of the effects of experimental manipulations. Hence models involving unconditioned behavior were adopted in this study as discussed below:

#### 1.5.2.1 The elevated plus-maze test

The elevated plus-maze (EPM) is the most popular of all currently available animal models of anxiety, and affords an excellent example of a model based on the study of unconditioned or spontaneous behaviour (File, 1992; Rodgers and Dalvi, 1997; Carobrez and Bertoglio, 2005). It was initially described by Pellow and co-workers (Pellow *et al.*, 1985) as a simple method for assessing anxiety responses by rodents. It is made of four arms (two open and two closed) that are arranged to form a plus shape. The assessment of anxiety behaviour of rodents is done by using the ratio of time spent on the open arms to the time spent on the closed arms. In the test, mice or rats are placed at the junction of the four arms of the maze, facing an open arm, and entries/duration in each arm are recorded by a video-tracking system and observer



simultaneously for 5 minutes. Other ethological parameters (i.e. rears, head dips, and stretch-attend postures) reflect anti-anxiety behaviour (Walf and Frye, 2007).

#### **1.5.2.2 Light Dark Exploration test**

The light/dark test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behaviour of the animals, applying mild stressors, i.e. novel environment and light (Bourin and Hascoet, 2003). The test apparatus consists of a small dark secure compartment (one-third) and a large illuminated aversive compartment (two-thirds). A natural conflict situation occurs when an animal is exposed to an unfamiliar environment or novel objects. The conflict is between the tendency to explore and the initial tendency to avoid the unfamiliar (neophobia). The exploratory activity reflects the combined result of these tendencies in novel situations. Thus, in the light/dark test, drug-induced increases in behaviours in the white part of a two-compartment box, in which a large white compartment is illuminated and a small black compartment is darkened, suggest anxiolytic activity. An increase in transitions without an increase in spontaneous locomotion is considered as anxiolytic activity. Transitions have been reported to be an index of activity exploration because of habituation over time and the time spent in each compartment to be a reflection of aversion. Classic anxiolytics (benzodiazepines) as well as the newer anxiolytic-like compounds (e.g. serotonergic drugs or drugs acting on neuropeptides receptors) can be detected using this paradigm. It has the advantages of being quick and easy to use, without requiring the prior training of animals (Bourin and Hascoet, 2003).

### **1.6 JUSTIFICATION**

The current pharmacotherapy for CNS disorders like epilepsy, depression and anxiety is far from optimum (Sun and Alkon, 2002; Misra *et al.*, 2003). Refractoriness and side effects are rife (Sills, 2006). In most African countries, herbal remedies are used for primary healthcare needs (Abu-Irmaileh and Afifi, 2003). These medicinal plants have proven to be important sources of bioactive molecules—one of the reasons why WHO is advocating for the incorporation of traditional medicine in the primary healthcare of developing nations (Calixto, 2000; Abu-Irmaileh and Afifi, 2003). Aside their effectiveness, they are generally considered to be safer compared to their orthodox counterparts because of the many compounds that can act antagonistically to reduce side effects and also synergistically to enhance efficacy. *Mallotus oppositifolius* is used traditionally for epilepsy, pain and treating infections in Ghana, Nigeria and Congo but there exists no scientific evidence supporting their efficacy and safety (Burkill, 1985). The study was therefore conducted to evaluate scientifically the anticonvulsant effect

and antidepressant of the leaves of plant and to assess its possible mechanism(s) of action. This information will go a long way to help in our search for better and alternative drugs for most of these psychiatric ailments that have remained elusive.

## 1.7 PURPOSE AND OBJECTIVES OF STUDY

The primary aim of this study was to investigate the anticonvulsant, antidepressant and anxiolytic activity of *Mallotus oppositifolius* and other neurobehavioural effects that may be associated with antiepileptic drugs. The specific objectives of the thesis were to:

- Conduct phytochemical tests on the extract to confirm the presence of secondary metabolites
- Screen the general CNS activity of the extract
- Evaluate the anticonvulsant activity of the hydroalcoholic leaf extract in pentylenetetrazole, picrotoxin, maximal electroshock, strychnine, 4-aminopyridine - induced seizures and pilocarpine induced *status epilepticus*.
- Examine the antidepressant effect of the extract using classical acute models of depression including the tail suspension and the forced swimming tests and the chronic model, open space swim test.
- Examine the anxiolytic effect of the extract using classical models of anxiety including the elevated plus-maze, light/dark exploration tests and open field test.
- Investigate the effect of the extract on motor coordination and the rotarod test.

## *Chapter 2*

# **PLANT COLLECTION, EXTRACTION AND PHYTOCHEMICAL ANALYSIS**

## **2.1 PLANT COLLECTION AND EXTRACTION**

### **2.1.1 Plant collection**

Leaves of the plant *Mallotus oppositifolius* were collected from the campus of the Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana (6°41'6.4"N, 1°33'42.8"W), in September, 2009. The leaves were authenticated at the Department of Herbal Medicine of the Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Kumasi and a voucher specimen (KNUST/FP/035/09) has been deposited at the herbarium of the Department of Herbal Medicine.

### **2.1.2 Extraction**

The leaves were air-dried indoors for a week and pulverized with a hammer-mill. The powder was extracted by cold maceration using 70% (v/v) ethanol in water over a period of 72 h. The resulting extract was concentrated under moderate temperature (60°C) and pressure to a syrupy mass in a rotary evaporator. The syrupy mass was then dried to a dark brown solid mass on water bath and kept in a dessicator till it was ready to be used. The final yield was 9.5% (w/w). This is subsequently referred to as *Mallotus oppositifolius* extract (MOE) or extract.

## **2.2 PHYTOCHEMICAL TESTS**

The freshly prepared hydroalcoholic extract was analyzed for phytochemical constituents as described by Trease and Evans (1989) for the detection of alkaloids, saponins, reducing sugars, sterols, tannins and terpenoids. These are described as follows:

### **2.2.1 Test for alkaloids**

A sample of the extract (0.5 g) was boiled with 10 ml dilute hydrochloric acid for 5 min. The supernatant liquid was filtered and 3 drops of Dragendorff's reagent was added to 1 ml of the filtrate. The mixture was shaken and the appearance of an orange-red spot precipitate indicates the presence of alkaloids.

### **2.2.2 Test for tannins**

An amount of the extract (0.5 g) was boiled with 25 ml of water for 5 min, cooled and filtered. The volume of the filtrate was adjusted to 25 ml with water. A small quantity of water (10 ml)

and 2 drops of 1 % ferric chloride was added to 1 ml of the filtrate and observed for the appearance of a blue-black or green precipitate.

### 2.2.3 Test for saponins

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

### 2.2.4 Test for reducing sugars

A portion of the extract (0.2 g) was boiled in 5 ml of water. The mixture was cooled and filtered. An equal quantity (5 ml) of Fehling's A and B solutions were added to the filtrate, heated and observed for a red-brown precipitate.

### 2.2.5 Test for sterols

An amount of acetic anhydride (2ml) was added to 0.5 g of the extract with 2 ml of H<sub>2</sub>SO<sub>4</sub>. A colour change from violet to blue or green in the samples indicates the presence of steroids.

### 2.2.6 Test for terpenoids

An amount of 0.5 g of MOE was extracted with 2 ml of chloroform in a test tube followed by addition of 1ml of concentrated sulphuric acid. The reddish-brown coloration at interface shows the presence of terpenoids (Jana and Shekhawat, 2011).

## 2.3 RESULTS

The phytochemical screening revealed the presence of alkaloids, tannins, saponins, reducing sugars and sterols. Terpenoids were absent (Table 2.1).

Table 2.1 Phytochemical constituents of the hydroalcoholic extract from the leaves of *M. oppositifolius*.

Constituent	Results
Saponins	+
Tannins	+
Alkaloids	+
Triterpenes	-
Reducing sugars	+
Sterols	+

—: Not detected, +: Present



## 2.4 DISCUSSION

Phytochemicals like alkaloids, flavonoids, coumarins, glycosides, gums, polysaccharides, phenols, tannins, terpenes and terpenoids are chemical compounds formed during the plants normal metabolic processes (Briskin, 2000; Palombo, 2006). Most of these secondary metabolites are potent bioactive compounds found in medicinal plant parts and have been reported to be responsible for their pharmacological effects (Briskin, 2000; Eastwood, 2001; Rogerio *et al.*, 2010). The hydroalcoholic extract of the leaves of *M. oppositifolius* contained alkaloids, saponins, sterols, tannins and glycosides, an observation consistent with works done by other investigators (Farombi *et al.*, 2001; Adekunle and Ikumapayi, 2006).

Alkaloids, the largest single class of secondary plant substances are ranked among the most efficient and therapeutically significant plant substances (Otani *et al.*, 2005; Makkar *et al.*, 2007). Pure, isolated plant alkaloids and their synthetic derivatives are used as basic medicinal agents for their CNS and analgesic e.g. morphine and codeine (*Papaver somniferum*); anti-inflammatory e.g. colchicine; anticancer e.g. vincristine and vinblastine (*Vinca roseus*); antispasmodic and bactericidal effects (Staerk *et al.*, 2002; Norn *et al.*, 2005; Yang *et al.*, 2006). Tannins are a group of polymeric/phenolic substances capable of tanning leather or precipitating gelatin from a solution; a property known as astringency (Okuda, 2005). Many physiological activities such as stimulation of phagocytic cells, host mediated antitumour activity and wide range of anti-infective action have been assigned to tannins (Okuda *et al.*, 1992; Souza *et al.*, 2007; Buzzini *et al.*, 2008; Kolečkar *et al.*, 2008). Saponins are a group of naturally occurring plant glycosides, characterized by their strong foam-forming properties in aqueous solution (Vincken *et al.*, 2007; Quante *et al.*, 2010). They possess significant anti-cancer, antiinflammatory, antifungal, moluscidal, spermicidal, antidepressant effects, among others (George, 1999; Jung *et al.*, 2005; Dang *et al.*, 2009; Quante *et al.*, 2010). Sterols are cholesterol-like compounds found in plant. Plant sterols are best known for their ability to lower cholesterol level (Pollak, 1985; Katan *et al.*, 2003). They are used to boost the immune system (Bouic *et al.*, 1996; Breytenbach *et al.*, 2001). Moreover they have antidiabetic, anticancer and antimicrobial effects (Donald *et al.*, 1997; Muti *et al.*, 2003).

The pharmacological and therapeutic effects of the plant may be due to the presence of one or more of these secondary metabolites.

## 2.5 CONCLUSION

The hydroalcoholic extract of the leaves of *M. oppositifolius* contain alkaloids, sterols, reducing sugars, saponins and tannins.

## *Chapter 3*

### **PRELIMINARY CNS SCREENING**

#### **3.1 INTRODUCTION**

In the drug discovery process, it is important to assess and monitor the efficacy and safety of drugs especially in the preclinical phase (Kinter and Valentin, 2002). In 2000, the International Conference on Harmonization (ICH), determined guidelines for safety pharmacology. The Conference recommended the monitoring of the functions and effects of test drugs on the cardiovascular, respiratory and central nervous system.

The core CNS battery procedures are applied at the initial stages of the drug discovery process to help eliminate substances with a potential for CNS risk. These protocols include measuring general behavioural signs induced by test substance (Irwin test), effects on spontaneous locomotion (activity meter test), effects on neuromuscular coordination (Rotarod test), effects on the convulsive threshold (PTZ seizure test), interaction with hypnotics (Pentobarbital interaction test) and effects on the pain threshold (tail immersion test) (Porsolt *et al.*, 2002).

*Mallotus oppositifolius* is a common shrub that is used traditionally for treating convulsion, epilepsy, treatment of parasitic, eye and kidney infections, as diuretic, pain killers, and treatment of paralysis, spasm, headache and swellings. Despite its usefulness there is no available scientific data on its CNS effects. Thus these tests were carried out on the leaves to assess the neuropharmacological effects of the leaves of the plant.

#### **3.2 MATERIALS AND METHODS**

##### **3.2.1 Animals**

Male ICR mice were obtained from the Noguchi Memorial Institute for Medical Research, Accra, Ghana and kept at the animal facility of the Department of Pharmacology, KNUST, Kumasi, Ghana. The animals were housed in groups of five in stainless steel cages (34 x 47 x 18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (GAFCO, Tema), given water *ad libitum* and maintained under laboratory conditions. All animals used in these studies were treated according to the Guide for the Care and Use of Laboratory Animals (NRC, 1996) and were approved by the College Ethics Committee.

##### **3.2.2 Drugs and chemicals**

Diazepam (DZP), pentylenetetrazole (PTZ) caffeine (CFN), pentobarbitone (PBT), and phenobarbitone (PHE) were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA. Morphine hydrochloride (MOR) was obtained from Phyto-Riker, Accra, Ghana.

### 3.2.3 Irwin test

Assessment of behaviour and physiological function was carried out similar to the primary observation procedure originally described by Irwin (1968). ICR mice (20- 25 g) were randomly divided into seven groups (n=7) and kept in the experimental environment for 7 days to acclimatize. Animals were fasted overnight, but had access to water *ad libitum*, and then treated orally with *M. oppositifolius* extract in doses of 30 - 6000 mg kg<sup>-1</sup> of body weight. Mice in the control group received 10 ml kg<sup>-1</sup>, *p.o.* of saline (0.9% w/v). The mice were observed at 0, 15, 30, 60, 120 and 180 min, up to 24 hours after treatment for general changes in behaviour and physiological function as well as mortality. The animals were assessed for behaviours related to neurotoxicity, central nervous system (CNS) stimulation and depression. Effects on autonomic functions were also noted, as was the lethality of the test agent. Refer to Appendix.

### 3.2.4 Activity meter test

This test is employed to measure spontaneous locomotor activity with an activity cage (Ugo Basille model 7401, Milan, Italy). ICR mice weighing (20- 25 g), n=7, were given the extract (30- 3000 mg kg<sup>-1</sup>), caffeine (16 mg kg<sup>-1</sup>) or diazepam (8 mg kg<sup>-1</sup>) by the oral route. After 60 minutes, the animals were placed in the activity cage individually and their activities were scored every 5 minutes for 30 minutes.

### 3.2.5 Rotarod test

The effect of the extract on motor coordination was determined using the rotarod (UgoBasile, model 7600, Comerio, Varese, Italy). ICR mice were divided into 8 groups of 7 animals each after an initial screening to obtain a baseline. This screening involved placing each mouse on the rotarod prior to treatment, any mouse that fell off before the cut off time of 3 min was excluded from the experiment. The extract (30 - 3000 mg kg<sup>-1</sup>, *p.o.*) were administered to groups A, B, C, D, E while the reference drugs D-tubocurarine (0.1 mg kg<sup>-1</sup>, *i.p.*) and diazepam (8 mg kg<sup>-1</sup>, *p.o.*) were administered to groups F and G respectively. Group H received distilled water (10 ml kg<sup>-1</sup>, *i.p.*) and served as the control group. The animals were placed on the rotarod bar prior to treatment and at 0.5, 1, 1.5 and 2 h after treatment. The time in seconds for the mouse to fall off within the cut off time of 180 s was noted.

### 3.2.6 Convulsive threshold test (PTZ seizure test)

ICR mice weighing 20-25 g, were divided into 7 groups (n=7). Group 1 was given distilled water (10 ml kg<sup>-1</sup>); groups 2- 6 received MOE (30- 3000 mg kg<sup>-1</sup>, *p.o.*) and group 7, received diazepam (8 mg kg<sup>-1</sup>, *p.o.*). Pentylenetetrazole (100 mg kg<sup>-1</sup>, *s.c.*) was administered to the mice 30 min after distilled water or 1 h after diazepam or MOE. The mice were then placed in a



plastic observation cage and observed for 60 minutes for latency to clonic and tonic convulsions, frequency and duration of clonic and tonic convulsions for 1 h. Clonic convulsions consist of a prolonged and rapid succession of involuntary muscle contractions, while tonic convulsions are characterized by predominant abduction and extension of the limbs and rigid stretching of the body.

### 3.2.7 Pentobarbitone interaction test

The effect of MOE on pentobarbitone-induced sleeping time was studied in mice as described previously (de-Paris *et al.*, 2000). Fifty-six ICR mice were randomly divided into eight groups (n=7). The control group received distilled water (10 ml kg<sup>-1</sup>). The remaining 7 groups received either MOE (30 - 3000 mg kg<sup>-1</sup>, *p.o.*), diazepam (8 mg kg<sup>-1</sup>, *p.o.*) or caffeine (16 mg kg<sup>-1</sup>, *p.o.*). Sodium pentobarbitone (50 mg kg<sup>-1</sup>, *i.p.*) was administered 1 hour after diazepam, caffeine or MOE. The control group received only the pentobarbitone. In a separate experiment, the effect of hepatic enzyme induction on pentobarbitone sleeping time was assessed. Briefly eight groups of mice (n=7) were pretreated with phenobarbitone (25 mg kg<sup>-1</sup>, *i.p.*) for two days and on the third day, the first five groups were given extract (30- 3000 mg kg<sup>-1</sup>, *p.o.*) and pentobarbitone (50 mg kg<sup>-1</sup>, *i.p.*), then the rest of the groups took either, diazepam and pentobarbitone; caffeine (16 mg kg<sup>-1</sup>, *p.o.*) and pentobarbitone (50 mg kg<sup>-1</sup>, *i.p.*) or pentobarbitone alone (50 mg kg<sup>-1</sup>, *i.p.*). In another experiment, the effect of extract (30- 3000 mg kg<sup>-1</sup>) on hepatic enzyme induction or inhibition was investigated. Five groups of mice (n=7) were pretreated with extract for 2 consecutive days and on the third day, they received pentobarbitone (50 mg kg<sup>-1</sup>, *i.p.*). Two parameters were recorded: time elapsed since the administration 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).

### 3.2.8 Tail immersion test

Tail-immersion test was carried out as described (Sewell and Spencer, 1976; Luttinger, 1985) with modifications. The tail of the mouse was immersed in a water bath containing water at a temperature of 48 ± 0.5 °C. The mouse reacts by withdrawing the tail (Bohn *et al.*, 2000). The reaction time (time taken for mice to withdraw tail) was recorded with a stop watch and a cut-off time of 10 s imposed on this measure. Mice were randomly divided into one of the following study groups (seven per group): control, morphine (10 mg kg<sup>-1</sup>, *i.p.*) and MOE (30 - 3000 mg kg<sup>-1</sup>, *p.o.*). The reaction time (T) for the study groups was taken at 0.5, 1, 2 h intervals after a latency period of 30 min (*i.p.*) or 1 h (*p.o.*) following the administration of the morphine



and extract. The percentage maximal possible effect (% MPE) was calculated from the reaction times by the formula:

$$\% \text{ MPE} = \frac{[\text{Post} - \text{drug latency}] - [\text{Pre} - \text{drug latency}]}{[\text{Cut} - \text{off latency}] - [\text{Pre} - \text{drug latency}]} \times 100$$

where 10 s is the cut-off latency time.

### 3.2.9 Analysis of Data

All data are presented as mean  $\pm$  SEM. Data were analyzed using one-way analysis of variance (ANOVA) with drug treatment as a between-subjects factor. Whenever ANOVA was significant, further comparisons between vehicle- and drug-treated groups were performed using the Newman Keuls' *post hoc* test. GraphPad Prism for Windows Version 5 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses.  $P < 0.05$  was considered significant.

## 3.3 RESULTS

### 3.3.1 Irwin test

Convulsions were observed in two mice in the highest dose (6000 mg kg<sup>-1</sup>) after 15 min. During the 24 h period, no other toxic signs were observed except for sedation and loss of muscle coordination from the dose of 300 - 6000 mg kg<sup>-1</sup>. Two mice died on the first day and after 24 hours, there was one more death in the highest dose (Table 3.1).

Table 3.1 Observations in the acute toxicity test after oral administration of *M. oppositifolius* in mice.

Dose	Mortality		Toxicity
	D/T	Latency (h)	
0	0/7	-	-
30	0/7	-	-
100	0/7	-	-
300	0/7	-	Sedation, motor incoordination
1000	0/7	-	Sedation, motor incoordination
3000	0/7	-	Sedation, motor incoordination
6000	3/7	24	Convulsion, sedation, motor incoordination, death

The hydroalcoholic extract of *M. oppositifolius*, was administered orally; each dose was administered to groups of 7 mice. D/T: dead/treated mice; -: no toxic symptoms were seen during the observation period; latency: time to death (in hours) after the dose.

### 3.3.2 Activity meter test

MOE affected spontaneous activity only at 3000 mg kg<sup>-1</sup> ( $F_{7,47}=55.78$ ,  $P<0.0001$ ) (Figure 3.1). Diazepam, the reference CNS depressant (8 mg kg<sup>-1</sup>, *p.o.*), significantly decreased spontaneous activity in contrast to caffeine, (16 mg kg<sup>-1</sup>, *p.o.*), which is the reference CNS stimulant.

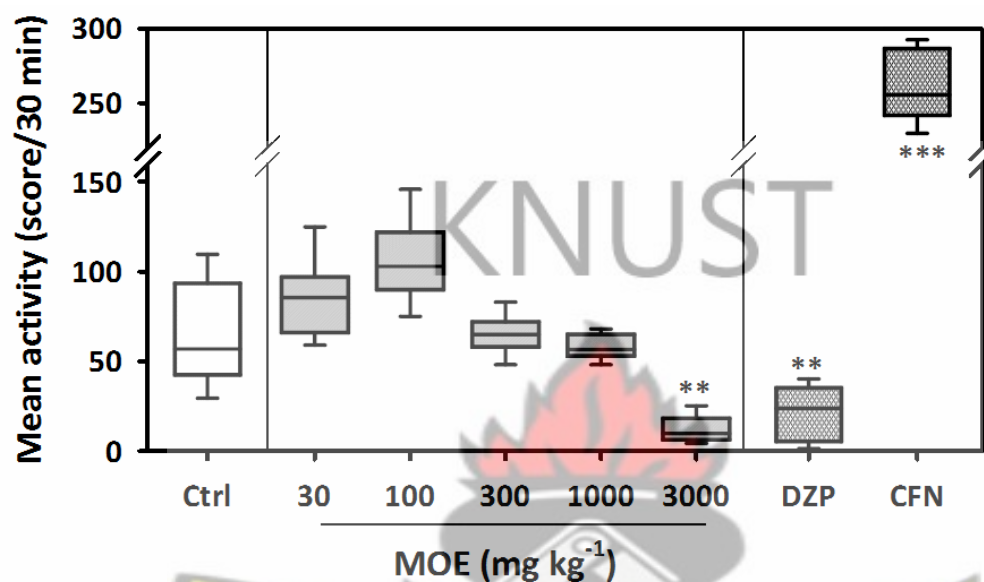


Figure 3.1 Effect of MOE (30 - 3000 mg kg<sup>-1</sup>, *p.o.*), diazepam (8 mg kg<sup>-1</sup>, *p.o.*) and caffeine (16 mg kg<sup>-1</sup>, *p.o.*), on spontaneous activity in mice. Data was presented as mean  $\pm$  S.E.M. (n=7); \*\*\* $P<0.001$ ; \*\* $P<0.01$ ; compared to vehicle-treated group (One-way ANOVA followed by Newman Keuls' test).

### 3.3.3 Rotarod

From the time course curve, MOE caused a slight decrease in the time spent on the rotarod at lower doses but significantly decreased it at higher doses (Figure 3.2a). From the area under the curve (AUC) calculated from the time course curves, MOE caused significant motor impairment from 1000 to 3000 mg kg<sup>-1</sup> ( $F_{7,48}=37.27$ ,  $P<0.0001$ ) (Figure 3.2a). Both diazepam (8 mg kg<sup>-1</sup>) and D-tubocurarine (0.5 mg kg<sup>-1</sup>) also caused significant motor impairment (Figure 3.2a and b).

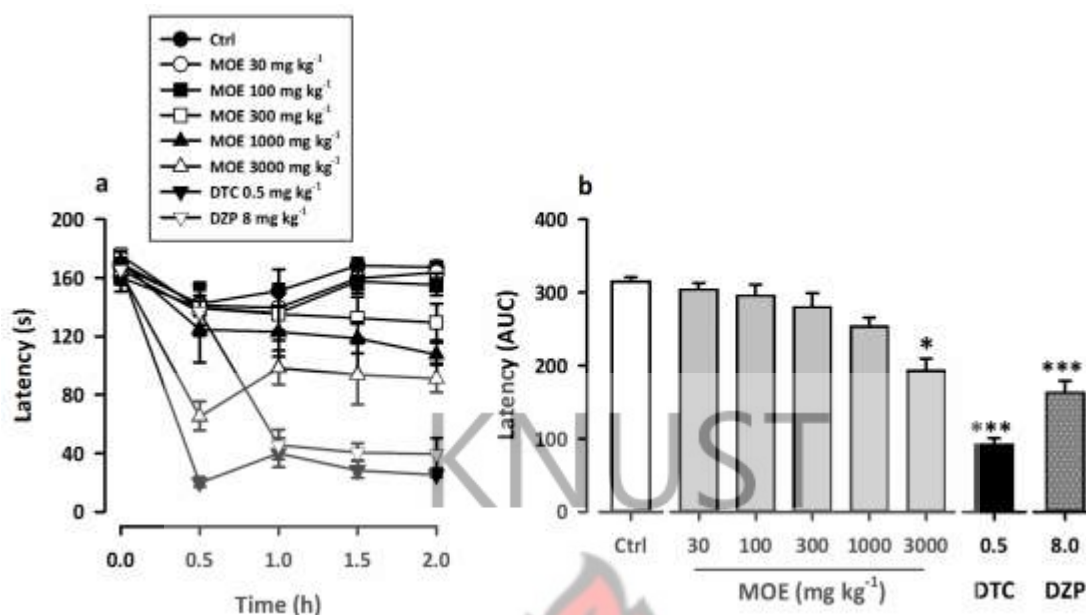


Figure 3.2 Effect of MOE (30 - 3000 mg kg<sup>-1</sup>, *p.o.*), diazepam (8 mg kg<sup>-1</sup>, *p.o.*) and D-tubocurarine (0.5 mg kg<sup>-1</sup>, *i.p.*) on (a) the time course curve of the rotarod test and (b) the area under the curve (AUC), in mice. Data was presented as mean  $\pm$  S.E.M. (n=7); \*\*\* $P$ <0.001; \* $P$ <0.05; compared to vehicle-treated group (One-way ANOVA followed by Newman Keuls' test).

### 3.3.4 PTZ seizure test

MOE (30 - 3000 mg kg<sup>-1</sup>), administered orally, significantly delayed both onset of clonic ( $F_{8,54}=651.2$ ,  $P$ <0.0001) (Figure 3.3a) and tonic convulsions ( $F_{8,54}=625.1$ ,  $P$ <0.0001) (Figure 3.3c) in mice. The frequency of both clonic ( $F_{8,54}=33.28$ ,  $P$ <0.0001) (Figure 3.3a) and tonic convulsions ( $F_{8,54}=26.70$ ,  $P$ <0.0001) (Figure 3.3c) was reduced. Furthermore, duration of clonic convulsions ( $F_{8,54}=26.74$ ,  $P$ <0.0001) (Figure 3.3b) and tonic convulsions ( $F_{8,54}=52.27$ ,  $P$ <0.0001) (Figure 3.3d) were also reduced significantly by the extract. Diazepam (0.3 - 3 mg kg<sup>-1</sup>), the reference drug also demonstrated similar effects as the extract (Figure 3.3a-d).

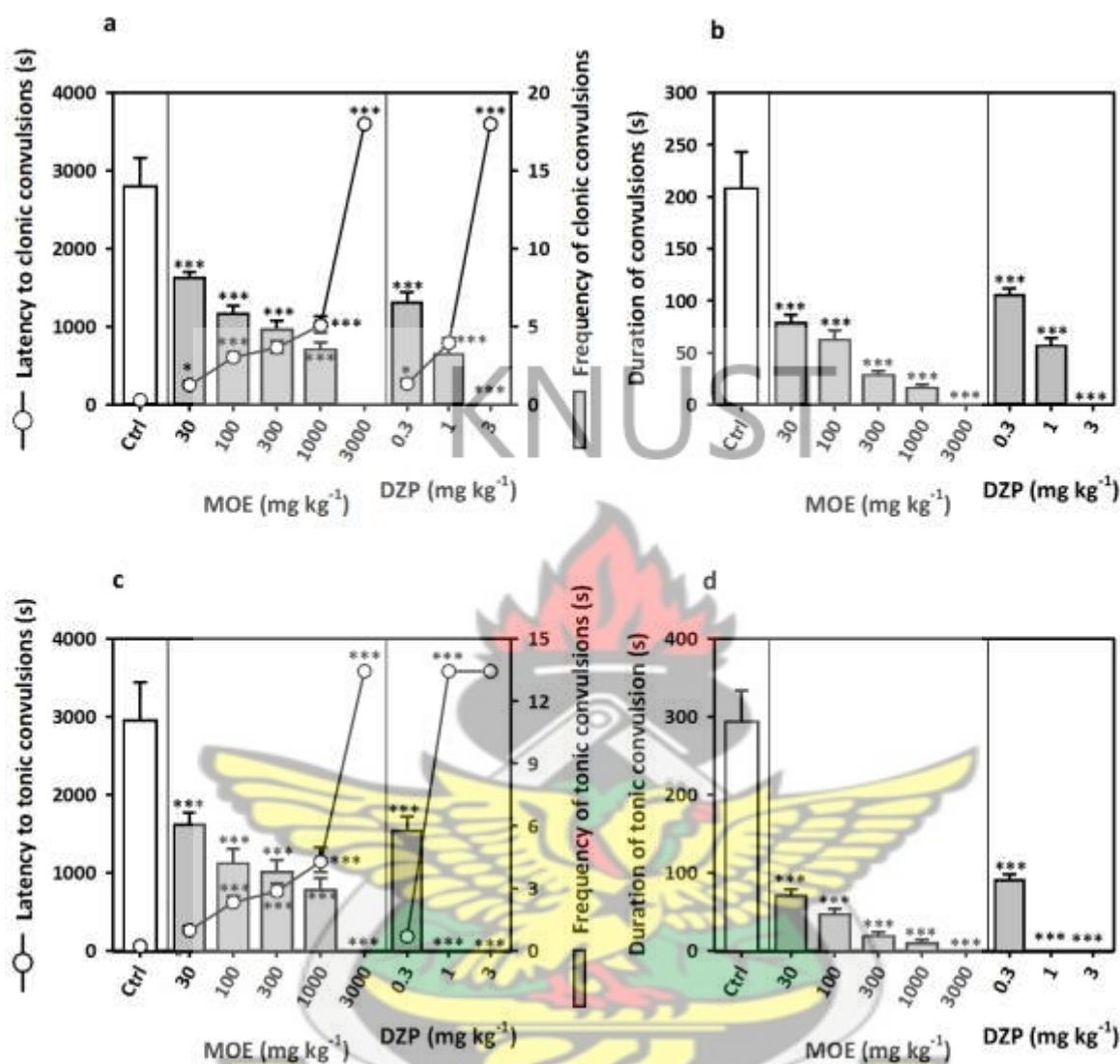


Figure 3.3 Effect of MOE (30 - 3000 mg kg<sup>-1</sup>, *p.o.*) and diazepam (0.3 - 3 mg kg<sup>-1</sup>, *i.p.*) on: (a) the latency to clonic and (c) tonic convulsions; (a and c) frequency of clonic and tonic convulsions, and (b and d) duration of clonic and tonic convulsions in the pentylenetetrazole-induced seizure test in mice. Data was presented as mean  $\pm$  S.E.M. (n=7); \*\*\*P<0.001; \*\*P<0.01; compared to vehicle-treated group (One-way ANOVA followed by Newman Keuls' test).

### 3.3.5 Pentobarbital interaction test

In the barbiturate-induced sleeping time test, the extract demonstrated a significant CNS depressant effect by causing a dose dependent increase in the duration of sleep ( $F_{7,48}=22.02$ ,  $P<0.0001$ ) (Figure 3.4a) at all doses tested except at 30 mg kg<sup>-1</sup>. It however did not affect the onset of sleep compared to the control group (Figure 3.4a). Diazepam (8 mg kg<sup>-1</sup>, *p.o.*), a reference CNS depressant caused a similar effect as the extract while caffeine (16 mg kg<sup>-1</sup>,



*p.o.*), the CNS stimulant, delayed onset of sleep and significantly reduced the sleeping time (Figure 3.4). MOE and diazepam had no effect on latency to sleep in phenobarbitone pretreated mice (Figure 3.5a). On the contrary caffeine reduced the latency to sleep in phenobarbitone pretreated mice (Figure 3.5a). MOE ( $F_{7,48}=22.02$ ,  $P<0.0001$ ) and diazepam significantly reduced the duration of sleep induced in mice pretreated with phenobarbitone for 2 days (Figure 3.5b). Decrease in sleep latency was reversed by MOE at doses of 1000 and 3000 mg  $\text{kg}^{-1}$  when mice were pretreated with various doses of extract for 2 days (Figure 3.6a). This treatment reversed the increase in sleep duration in untreated mice ( $F_{7,48}=22.02$ ,  $P<0.0001$ ) (Figure 3.6b).

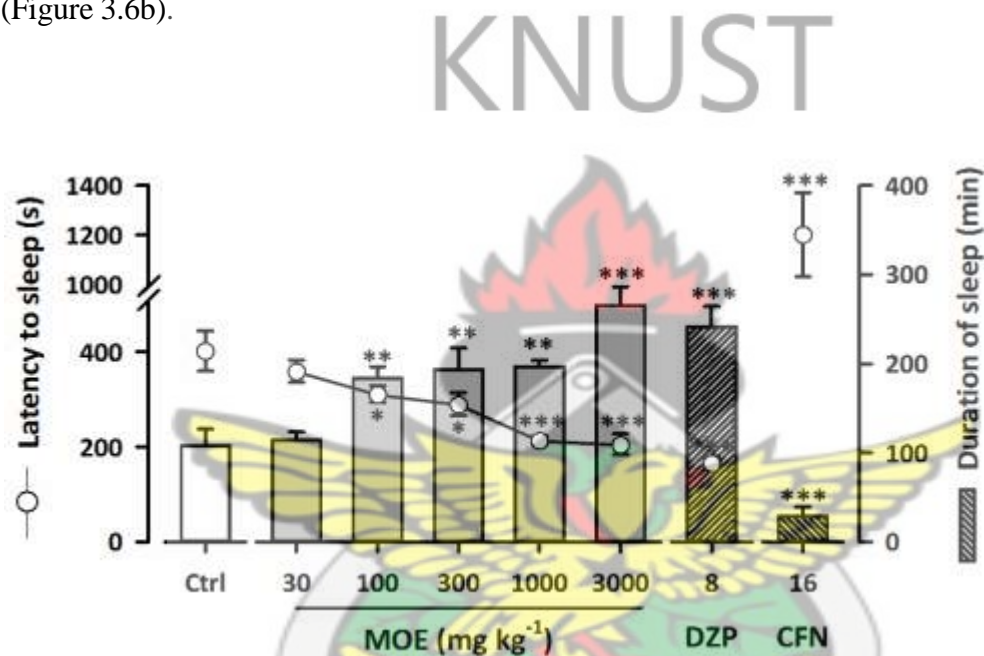


Figure 3.4 Effect of MOE (30 - 100 mg  $\text{kg}^{-1}$ , *p.o.*) and diazepam (8 mg  $\text{kg}^{-1}$ , *p.o.*) and caffeine (16 mg  $\text{kg}^{-1}$ , *p.o.*) on the latency to sleep and duration of sleep, in the pentobarbitone-induced sleeping test. Data was presented as mean  $\pm$  S.E.M. ( $n=7$ ); \*\*\* $P<0.001$ ; \*\* $P<0.01$ ; compared to vehicle-treated group (One-way ANOVA followed by Newman Keuls' test).

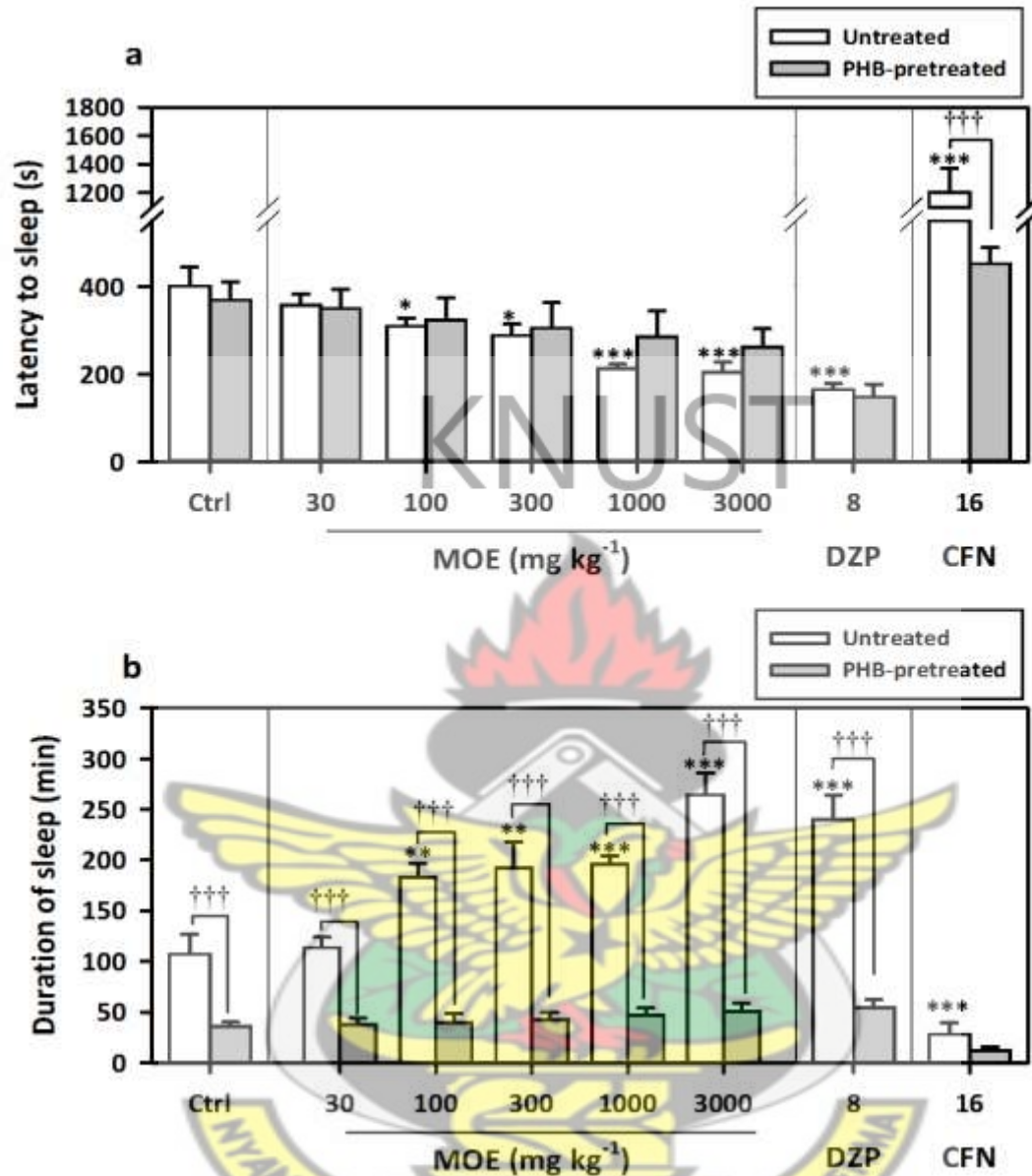


Figure 3.5 Effect of MOE (30 - 100 mg kg<sup>-1</sup>, *p.o.*), diazepam (8 mg kg<sup>-1</sup>, *p.o.*) and caffeine (16 mg kg<sup>-1</sup>, *p.o.*) on (a) the latency to sleep in the pentobarbitone induced sleeping test. Data was presented as mean ± S.E.M. (n=7); \*\*\**P*<0.001; \*\**P*<0.01; compared to vehicle-treated group (One-way ANOVA followed by Newman Keuls' test). (b) duration of sleep, after pre-treating with phenobarbitone in the pentobarbitone-induced sleeping test. Data was presented as mean ± S.E.M. (n=7); †††*P*<0.001; comparison between dose and treatment (Two-way ANOVA followed by Bonferroni's test).

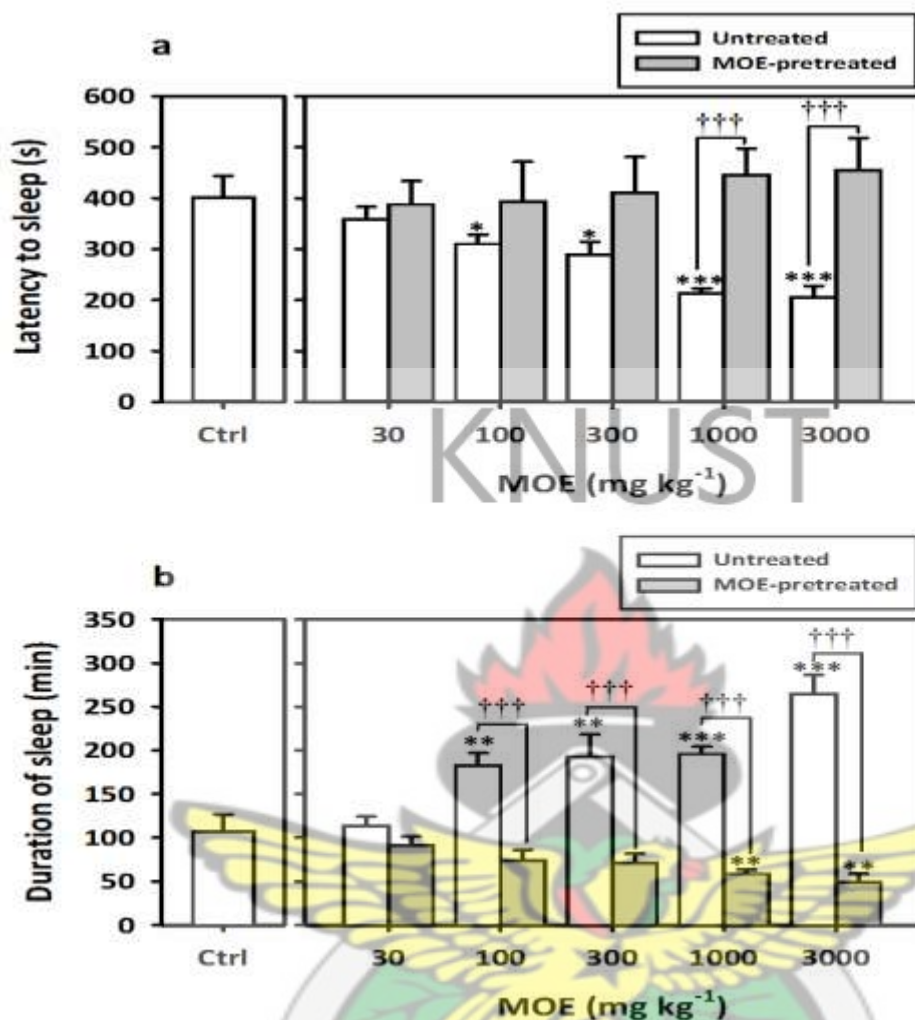


Figure 3.6 Effect of phenobarbitone on (a) the latency to sleep. Data was presented as mean  $\pm$  S.E.M. (n=7); \*\*\* $P$ <0.001; \*\* $P$ <0.01; compared to vehicle-treated group (One-way ANOVA followed by Newman Keuls' test). (b) duration of sleep, after pre-treatment with extract in the pentobarbitone-induced sleeping test. Data was presented as mean  $\pm$  S.E.M. (n=7); ††† $P$ <0.001; comparison between dose and treatment (Two-way ANOVA followed by Bonferroni's test).

### 3.3.6 Tail immersion test

MOE exhibited significant analgesic activity by increasing the % maximal possible effect (MPE) in the tail immersion test. From the time course curve, the analgesic activity peaked at 1 h after drug treatment and reduced by the second hour although analgesic activity was still maintained. One-way ANOVA followed by Newman Keuls' test of the AUC indicated that MOE exhibited analgesic effect ( $F_{6,42}=9.069$ ,  $P$ <0.0001) (Figure 3.7b). Similar result was obtained for morphine (10 mg kg<sup>-1</sup>), the reference analgesic drug used.

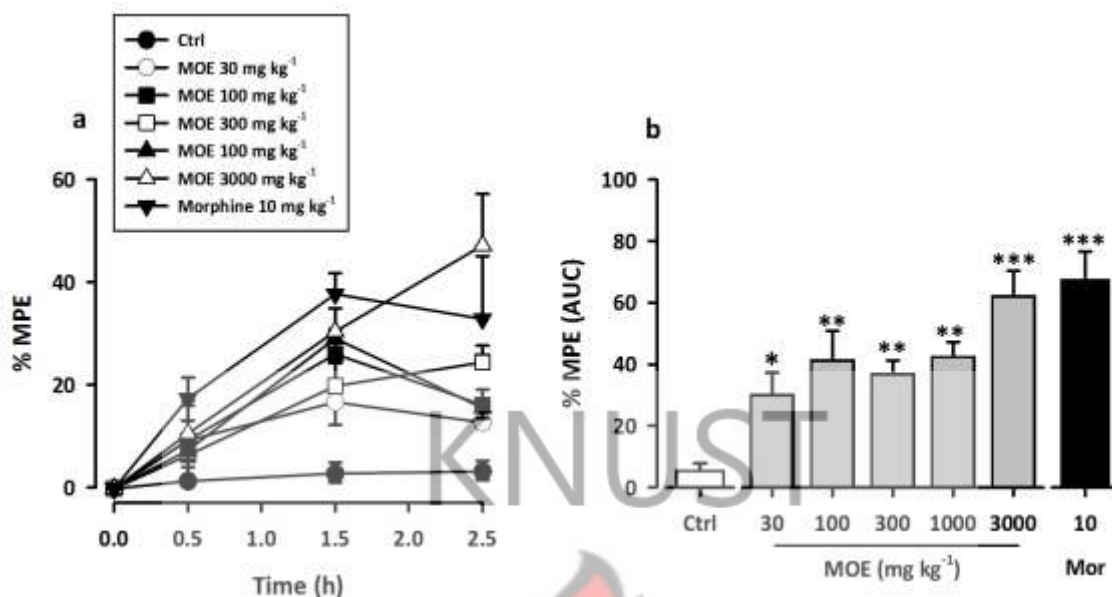


Figure 3.7 Effect of MOE (30 - 100 mg kg<sup>-1</sup>, *p.o.*) and morphine (10 mg kg<sup>-1</sup>, *i.p.*) on (a) the time course curve of the tail immersion test and (b) the area under the curve (AUC) in mice. Data are presented as mean  $\pm$  S.E.M. (n=7). \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls' test).

### 3.4 DISCUSSION

Central effects of the hydroalcoholic extract of the leaves of *Mallotus oppositifolius* was investigated in the core CNS battery test. The extract caused sedation and reduced spontaneous locomotor activity. It showed analgesic, anticonvulsant properties and induces hepatic microsomal enzymes. Furthermore, hepatic microsomal enzymes metabolized the extract.

In the Irwin test, MOE exhibited sedation and motor impairment at higher doses without impairing respiration. This is indicative of possible CNS depressant activity. The Irwin test, one of the core battery tests, is used to estimate the minimum lethal dose of a test substance, the dose range for CNS responses, and the primary effects on behavior and physiological functions (Irwin, 1968; Porsolt *et al.*, 2002; Roux *et al.*, 2005; Lynch *et al.*, 2011). The results of this test are used to predict potential therapeutic activity and to select doses for subsequent tests of efficacy. Data from the Irwin test are also used to assess the risks associated with the use of this agent (Irwin, 1968; Iezhitsa *et al.*, 2002). It is possible that MOE may have potential use in CNS conditions where there is CNS excitation such as in convulsions, epilepsy or anxiety. In fact the plant is used traditionally for managing these conditions. To corroborate this, the extract showed anticonvulsant activity in the PTZ convulsive threshold test. At all doses, the extract delayed the onset of clonic and tonic convulsions; and also reduced the frequency and duration of the clonic and tonic convulsions. Any substance that delays the onset of and the



duration of clonic or tonic clonic convulsion induced by pentylenetetrazole is described as an anticonvulsant (Vellucci and Webster, 1984; De Sarro *et al.*, 1999; Sayyah *et al.*, 2004). Caution should however be exercised since at the highest dose of 6000 mg kg<sup>-1</sup> of body weight, there were visible signs of convulsion and death in the Irwin test. Out of seven mice used, three died after 24 hours at the highest dose, suggesting that the lethal dose-50 (LD<sub>50</sub>) is approximately 6000 mg kg<sup>-1</sup>.

Drugs that act on the CNS may enhance, inhibit or not affect locomotor activity in mice (Kimmel *et al.*, 2000; Wiley and Martin, 2003; El-Mas and Abdel-Rahman, 2004; Kawaura *et al.*, 2010). MOE reduced spontaneous activity in mice in the activity meter test. Locomotion can be decreased as a result of sedation, drug-induced motor impairment by test substance (Brooks *et al.*, 1999; Betarbet *et al.*, 2002; Henry *et al.*, 2002; Porsolt *et al.*, 2002). In order to ascertain whether reduction in spontaneous activity was by drug-induced motor impairment or sedation, the rotarod test was performed. From the results, it was clear that there was significant motor impairment at the dose that reduced spontaneous activity in the activity meter test. Since sedation was also observed at the dose of 3000 mg kg<sup>-1</sup> in the Irwin test, it is possible that sedation may also contribute to the reduction in activity. Diazepam, a CNS depressant, reduced spontaneous activity and impaired motor coordination at the dose used while caffeine, a CNS stimulant increased the locomotor activity (El Yacoubi *et al.*, 2000; Solinas *et al.*, 2002; Wiley *et al.*, 2002; Dunne *et al.*, 2007). Many CNS depressant compounds can cause reduction in spontaneous locomotor activity in laboratory animals. Nearly all the neuroleptic agents used in psychiatry diminish spontaneous locomotor activity in all species including man (Baldessarini *et al.*, 1990; Simon *et al.*, 2000; Kinkead and Nemeroff, 2002).

Sleep-enhancing effect of substances can readily be detected in the barbiturate-induced sleeping time test by substances, which do not induce sleep even at high doses when administered alone (Montalto de Mecca *et al.*, 2000; Porsolt *et al.*, 2002; Nayak *et al.*, 2004). There is also a high correlation between the effects observed in this procedure and those observed in more complex tests and in man (Andre *et al.*, 1984; Renton, 1985; Zhao *et al.*, 2006). In the present study though MOE enhanced the duration of sleep in mice profoundly, this was not apparent in the Irwin test further supporting the fact that sleep enhancing effect of substances can be readily detected in the pentobarbitone sleep test. The prolongation of pentobarbitone induced sleep further supports the central depressant activity of the extract.

Pretreatment of mice with phenobarbitone for two days reduced the increase in sleep duration induced by the extract or diazepam. This suggests that the extract is metabolized by the hepatic enzymes (Conney, 1967; Kushikata *et al.*, 2003). Some drugs which are metabolized by the

liver can either induce or inhibit hepatic enzyme metabolism (Conney, 1967; Brockmoller and Roots, 1994; Faught, 2001; Back *et al.*, 2003; Park *et al.*, 2005). Hence, the possible effect of the extract on hepatic enzymes was assessed by pre-treating mice with the extract for two days before administering pentobarbitone. It is evident that the extract was a hepatic enzyme inducer since it reduced the sleep duration when compared to the group that received only pentobarbitone or extract and pentobarbitone. Drugs that are either metabolized by liver enzymes or induce hepatic enzymes are potential targets for drug-drug interactions. This pharmacokinetic effect of substances may result in toxicity or reduced therapeutic effect as a result of decrease in the effective concentration of drug in the plasma. On the other hand, beneficial synergistic effects can be observed through these interactions (George *et al.*, 1995; Park *et al.*, 2005).

Analgesic activity was observed from 100 - 6000 mg kg<sup>-1</sup> in the Irwin test. This observation in the Irwin test was further confirmed in the tail immersion test, an acute thermal pain model (Le Bars *et al.*, 2001; Simmons *et al.*, 2002; Jones *et al.*, 2005). This supports the traditional use of the plant in managing headache and other forms of pain. Morphine, the reference analgesic, also gave similar results. Thermal nociception models such as hot plate and the tail immersion tests are used to evaluate central analgesic activity. The tail immersion model is sensitive to drugs that act spinally or supraspinally (Ramabadran *et al.*, 1989; Pal *et al.*, 1999; Muhammad *et al.*, 2012). It is possible that the analgesic effect of MOE may be due to spinal or supraspinal pathways. That MOE has sedative properties may confound the test because most pain models are based on behavioural measures which involve motor coordination (Jarvis *et al.*, 2002; Kehl *et al.*, 2003; Nassar *et al.*, 2005; McGowan *et al.*, 2009; Zhao *et al.*, 2011). However in this study, doses required for analgesia were much lower than those that caused motor impairment.

### 3.5 CONCLUSION

From the core CNS battery tests, MOE exhibited significant CNS depressant, analgesic and anticonvulsant effect. The LD<sub>50</sub> is estimated to be approximately 6000 mg kg<sup>-1</sup>. The extract reduced spontaneous activity and impaired motor coordination at high doses. MOE is a hepatic enzyme inducer and metabolized by the hepatic enzymes.

## *Chapter 4*

# **ANTICONVULSANT EFFECT AND ISOBOLOGRAPHIC ANALYSIS OF DRUG INTERACTION**

### **4.1 INTRODUCTION**

Epilepsy is a heterogeneous neurological disorder that is characterized by sudden, transitory and unprovoked convulsive and non-convulsive seizures with or without loss of consciousness (McNamara and Puranam, 1996; Fisher *et al.*, 2005). Worldwide, there are over 50 million people afflicted by this disorder, approximately 50% of which can be effectively treated with the currently available anti-epileptic drugs. For another 20%, their seizures are inadequately controlled or controlled at the expense of substantial adverse effects (White, 2003; Jacoby *et al.*, 2005; Reid *et al.*, 2012). Over the last decade at least ten new antiepileptic drugs have been introduced into the market for the treatment of epilepsy with improved standard of care for a large number of patients in the form of reduced adverse events, a lower propensity for drug–drug interactions, and improved efficacy (Hachad *et al.*, 2002; Perucca, 2002; Schmidt, 2002; Bialer, 2006). Unfortunately, there still remain approximately 20–30% of the patients with refractory epilepsy (Brodie, 2001; Sander, 2003). The quest to satisfy the needs of this category of patients underscores the need to search for newer anti-epileptics.

Since prehistoric times, humans have attempted to alleviate ailments or injuries with the aid of plant parts or herbal preparations. Ancient civilizations have recorded various prescriptions of this kind. In the herbal formularies of medieval times numerous plants were promoted as remedies (Cragg *et al.*, 1997; Goldman, 2001). It is estimated that about 80% of the population in developing states use herbal remedies for primary health care. In these nations conditions like epilepsy, pain, etc. are usually treated using herbs (Mahady, 2001; Spinella, 2001; Kamatenesi-Mugisha and Oryem-Origa, 2005). One of such plants that have been used in treating epilepsy is *Mallotus oppositifolius* (Burkill, 1985).

Traditionally the leaves are used for treating convulsion and epilepsy (Burkill, 1985). Preliminary screening revealed anticonvulsant effect of the extract against PTZ-induced seizures (refer to section 3.3.4). Hence the present study was conducted to further explore the anticonvulsant potential of the hydroalcoholic extract of the leaves of *M. oppositifolius* in acute chemoconvulsant (pentylenetetrazole, picrotoxin, and strychnine) and electroshock (maximal electroshock seizure test) models. The pilocarpine model of *status epilepticus*, a human temporal lobe epilepsy model representing 70% of refractory partial seizures was also used in characterizing the anticonvulsant effect of the extract. The possible role of the benzodiazepine/GABA<sub>A</sub> receptor complex and the potassium channels in the mechanism of action of the extract was investigated.



It is common for patients in developing countries to combine herbal drugs with orthodox drugs (Zhou *et al.*, 2007). With regards to therapeutic outcomes this may be beneficial or deleterious (Camerino *et al.*, 2007; Zhou *et al.*, 2007). Isobolographic analysis is a tool used in investigating whether drug combinations produces synergistic effect, additive or antagonistic effect (2006). Therefore an isobolographic analysis of the drug interaction between the extract and diazepam against PTZ-induced seizures was performed and the possible mechanism of action investigated.

## 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

Diazepam (DZP), pentylenetetrazole (PTZ), picrotoxin (PTX), strychnine (STR), 4-aminopyridine and pilocarpine were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA. Flumazenil (FLZ) from Roche (Brazil), carbamazepine (Tegretol<sup>®</sup>, Novartis, Basel, Switzerland).

### 4.2.3 Pentylenetetrazole-induced seizure test

The method used was adapted from that described by (Swinyard and Kupferberg, 1985). Male ICR mice were divided into seven groups (n=5). The extract (10, 30 and 100 mg kg<sup>-1</sup>, *p.o.*) was administered to three groups while diazepam (0.1, 0.3 and 1.0 mg kg<sup>-1</sup>, *i.p.*) was given to three other groups and the last group received 10 ml kg<sup>-1</sup>, *p.o.* of the vehicle (distilled water) to serve as control. All other procedures were similar to earlier procedure described in 3.2.8.

### 4.2.4 Picrotoxin-induced seizure test

The procedure used was the same as in the case of pentylenetetrazole-induced seizure test except that mice were administered picrotoxin, 3.2 mg kg<sup>-1</sup> intraperitoneally (Swinyard, 1969; Avallone *et al.*, 2000; Mackenzie *et al.*, 2002; Ngo Bum *et al.*, 2004) 30 minutes and 1 hour after treatment with diazepam and MOE, respectively. The latency to myoclonic jerks, latency to clonic convulsions and the frequency and duration of clonic convulsions were recorded from the videos for each mouse as in pentylenetetrazole-induced convulsions.

### 4.2.5 Maximal electroshock seizure test

The method used has been previously described by (Toman *et al.*, 1946) and modified by (Swinyard and Kupferberg, 1985). Male ICR mice were grouped into seven groups (n=5). Three groups were treated with the extract (10, 30 and 100 mg kg<sup>-1</sup>, *p.o.*), three other groups treated with carbamazepine (10, 30 and 100 mg kg<sup>-1</sup>, *p.o.*) and the last grouped administered distilled water (10



ml kg<sup>-1</sup>, *p.o.*), to serve as control. After 1 hour of oral drug treatments, tonic convulsions of the hind limb extremities of mice were induced by passing alternating electrical current (50 Hz, 60 mA and 0.2 s) through the ear electrodes. This was the maximal current (60 mA) that induced tonic hind limb extension in all the trial mice and it was determined previously before commencement of the experiment. The duration of tonic hind limb extension seizures was determined in each dose group.

#### 4.2.6 Strychnine-induced seizure test

This method has been described previously (Lehmann *et al.*, 1988). In brief, strychnine (STR) seizures were induced in male mice by the i.p. injection of 0.5 mg kg<sup>-1</sup> of the STR nitrate after one hour of extract (10 - 100 mg kg<sup>-1</sup>) or 30 minutes of diazepam (0.1 - 1.0 mg kg<sup>-1</sup>) administration. The latency to myoclonic jerks, the frequency and duration of convulsions were recorded for extract treated groups and the diazepam group compared with the control group (vehicle).

#### 4.2.7 Pilocarpine-induced *status epilepticus*

In this experiment, seizures were induced by injection of pilocarpine (300 mg kg<sup>-1</sup>, i.p.) to male ICR mice, 10 in each group. MOE (10 - 100 mg kg<sup>-1</sup>) or diazepam (1 - 10 mg kg<sup>-1</sup>) was administered one hour or thirty minutes respectively before pilocarpine injection. To reduce peripheral autonomic effects produced by pilocarpine, the animals were pretreated with n-butylbromide hyoscine (1 mg kg<sup>-1</sup>, 30 min before pilocarpine administration). After the injection of the pilocarpine, the animals were placed separately into the transparent plexiglass testing chamber and video recordings made as described in the pentylenetetrazole-induced seizure experiments. The latency to and duration of clonic-tonic seizures were tracked using The JWatcher software version 1.0. The ED<sub>50</sub> (a measure of anticonvulsant potency) and E<sub>max</sub> (a measure of efficacy) were calculated from the dose response curves plotted.

#### 4.2.8 Possible Mechanisms

##### 4.2.8.1 Involvement of GABAergic mechanisms

To investigate the probable involvement of GABA<sub>A</sub> receptors in the anticonvulsant mechanism of the extract, the effects of a selective benzodiazepine receptor antagonist, flumazenil on the anticonvulsant activity of MOE was studied. Six groups of eight mice each were selected. The first four groups received MOE (100 mg kg<sup>-1</sup>, *p.o.*), diazepam (0.3 mg kg<sup>-1</sup>, i.p.), flumazenil (2 mg kg<sup>-1</sup>) and normal saline 30 min before the administration of PTZ (85 mg kg<sup>-1</sup>, i.p.). The last two groups were given flumazenil (2 mg kg<sup>-1</sup>, i.p.) 5 min before the administration of MOE (100 mg kg<sup>-1</sup>, *p.o.*) or diazepam (0.3 mg kg<sup>-1</sup>, i.p.) and 65 min or 35 min before the injection of PTZ (85 mg kg<sup>-1</sup>, i.p.)

respectively. The dose of flumazenil was selected based on the work of Rolland *et al.*, (2001). The latency to, the frequency and duration of clonic convulsions were tracked for each mouse.

#### 4.2.8.2 Involvement of potassium channels

The 4-aminopyridine induced seizure test described by Yamaguchi and Rogawski, (1992) was used. Eighty mice were randomly divided into groups of 10 mice each. The first group received normal saline (10 mg kg<sup>-1</sup> body weight, i.p.); the second, third and fourth groups were given 10, 30 and 100 mg kg<sup>-1</sup> body weight *p.o.* of the extract, respectively, and the last three groups received 100, 300 and 600 mg kg<sup>-1</sup> body weight of sodium valproate *p.o.* respectively. One hour later, mice in all the groups received 10 mg kg<sup>-1</sup> body weight s.c. of 4-aminopyridine (4-AP). Ability of the extract/drug to protect the mice from lethality within a one hour observation period and the latencies to clonic as well as tonic convulsions were considered as indices of anticonvulsant activity.

#### 4.2.9 Isobolographic analysis of drug interaction between extract and diazepam

Drugs given in combination may produce effects that are greater than or less than the effect predicted from their individual potencies. Isobolographic analysis is a tool consisting of a graph (isobologram), constructed on a coordinate system which is composed of the individual drug doses, a straight 'line of additivity' that is employed to distinguish additive from synergistic and antagonistic interactions.

ED<sub>50</sub>s of diazepam and the extract were obtained from the pentylenetetrazole-induced seizure test for isobolographic analysis. Mice were grouped into eight (n=7). Group I mice were treated with 0.22 mg kg<sup>-1</sup> diazepam i.p. (ED<sub>50</sub> of diazepam) and then received 85 mg kg<sup>-1</sup> of pentylenetetrazole s.c 30 minutes afterwards. Group II mice were treated with 15.37 mg kg<sup>-1</sup> extract *p.o.* (ED<sub>50</sub> of MOE) and then received 85 mg kg<sup>-1</sup> of pentylenetetrazole s.c 1 h afterwards. Group III-VI mice were treated with a combination of diazepam and extract respectively as follows; (0.22 + 15.37) mg kg<sup>-1</sup>,  $\frac{0.22 + 15.37}{2}$  mg kg<sup>-1</sup>,  $\frac{0.22 + 15.37}{4}$  mg kg<sup>-1</sup>,  $\frac{0.22 + 15.37}{8}$  mg kg<sup>-1</sup> followed by administration of 85 mg kg<sup>-1</sup> of pentylenetetrazole. Group VII-IX mice were treated 1 mg kg<sup>-1</sup> flumazenil and after 15 min a combination of diazepam and extract (0.22 + 15.37) mg kg<sup>-1</sup>,  $\frac{0.22 + 15.37}{2}$  mg kg<sup>-1</sup>,  $\frac{0.22 + 15.37}{4}$  mg kg<sup>-1</sup>, followed by administration 85 mg kg<sup>-1</sup> of pentylenetetrazole. Isobologram (a Cartesian plot of pairs of doses that, in combination, yield a specified level of effect) was then built by connecting the theoretical ED<sub>50</sub> of diazepam plotted on the abscissa with that of extract plotted on the ordinate to obtain the additivity line. For each drug mixture, the ED<sub>50</sub> (experimental) and its associated 95% confidence intervals were determined by linear regression analysis of the log

dose–response curve (and compared by a ‘t’-test to a theoretical additive ED<sub>50</sub>) obtained from the calculation;

$$Z_{add} = f (ED_{50}) \text{ of diazepam} + (1-f) ED_{50} \text{ of extract}$$

Where f is the fraction of the each component in the mixture and the variance (Var) of Z<sub>add</sub> was calculated as:

$$\text{Var } Z_{add} = f^2 (\text{Var } ED_{50} \text{ of diazepam}) + (1-f)^2 \text{Var } ED_{50} \text{ of extract.}$$

From these variances S.E.M’s are calculated and resolved according to the ratio of the individual drugs in the combination. A supra-additive or synergistic effect is defined as the effect of a drug combination that is higher and statistically different (ED<sub>50</sub> significantly lower) than the theoretically calculated equieffect of a drug combination in the same proportion. If the ED<sub>50</sub>s are not statistically different, the effect of the combination is additive and additivity means that each constituent contributes with its own potency to the total effect. The degree of interaction was calculated using fractional analysis by dividing the experimental ED<sub>50</sub> (Z<sub>mix</sub>) by the theoretical ED<sub>50</sub> (Z<sub>add</sub>). A value close to 1 is considered as additive interaction. Values lower than 1 are an indication of the magnitude of supra-additive or synergistic interactions (Z<sub>mix</sub>/Z<sub>add</sub><1), and values higher than 1 correspond to sub-additive or antagonistic interactions (Miranda *et al.*, 2007).

#### 4.2.10 Rotarod test

The effect of the extract on motor coordination was determined using the Ugo Basile Rotarod bar (model 7600, Comerio, Varese, Italy) ICR mice were divided into 7 groups of 5 animals each after an initial screening to obtain a baseline. This screening involved placing each mouse on the Rotarod prior to treatment, any mouse that fell off before the cut off time of 2 min was excluded from the experiment. The extract (10, 30 and 100 mg kg<sup>-1</sup>, *p.o.*) were administered to groups A, B, C whilst the reference diazepam (0.1, 0.3, 1 mg kg<sup>-1</sup>, *i.p.*) were administered to groups D, E and F respectively. Group G received distilled water (10 ml kg<sup>-1</sup>, *i.p.*) and served as the control group. The animals were placed on the Rota rod bar prior to treatment and at 1, 2, 3 and 4 h after treatment. The time in seconds for the mouse to fall off within the cut off time of 120 s was noted.

#### 4.2.11 Statistical analysis

All data are presented as mean ± SEM. Data were analyzed using one-way analysis of variance (ANOVA) with drug treatment as a between-subjects factor. Whenever ANOVA was significant, further comparisons between vehicle- and drug-treated groups were performed using the Newman Keuls’ test. In analyzing the possible role of GABAergic mechanisms in the anticonvulsant effect of the extract, Two-way ANOVA with the Bonferroni’s *post hoc* test (treatment × dose) was performed. In all the tests GraphPad Prism for Windows Version 5 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. P < 0.05 was considered significant. Doses



for 50% of the maximal effect (ED<sub>50</sub>) and E<sub>max</sub> for each drug were determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation:

$$Y = \frac{a + (b - a)}{1 + 10^{(\text{LogED}_{50} - X)}}$$

Where, X is the logarithm of dose and Y is the response. Y starts at a (the bottom) and goes to b (the top) with a sigmoid shape. The fitted midpoints (ED<sub>50</sub>s) of the curves were compared statistically using F test (Miller, 2003; Motulsky and Christopoulos, 2003).

Survival curves were plotted for the 4-aminopyridine test by plotting percentage survival against time. Analysis of survival curves was done with the log-rank test.

Isobolographic calculations were performed with the program Pharm Tools Pro (version 1.27, the McCary Group Inc.). Results are presented as mean ± S.E.M. or as ED<sub>50</sub> values with 95% confidence limits (95% CL). The statistical analysis of the isobolograms was performed according to Tallarida (2006) and the statistical difference between experimental and theoretical values was assessed by the Student's t test for independent means, and the *P* values < 0.05 were considered significant.

## 4.3 RESULTS

### 4.3.1 PTZ-induced seizures

The extract (10 - 100 mg kg<sup>-1</sup>) exhibited a significant anticonvulsant effect in this model. MOE caused a dose dependent delay in the onset of myoclonic jerks ( $F_{3,16}=22.63$ ,  $P<0.0001$ ) (Figure 4.1a) and clonic convulsions in mice ( $F_{3,16}=31.78$ ,  $P<0.0001$ ) (Figure 4.1a). It also significantly decreased the frequency ( $F_{3,16}=10.89$ ,  $P<0.0001$ ) (Figure 4.1b) and duration of clonic convulsions ( $F_{3,16}=16.54$ ,  $P<0.0001$ ) (Figure 4.1b), in the same animal model. Diazepam (0.1 - 1.0 mg kg<sup>-1</sup>), used as the reference anticonvulsant, showed similar results as the extract (Figure 4.1c, d). Comparing the ED<sub>50</sub> and E<sub>max</sub> values, diazepam was more potent and more efficacious than the extract in reducing the duration of seizures (Table 4.3). MOE was more efficacious than diazepam though less potent in reducing the frequency of seizures (Table 4.2) and delaying onset of convulsions (Table 4.1).



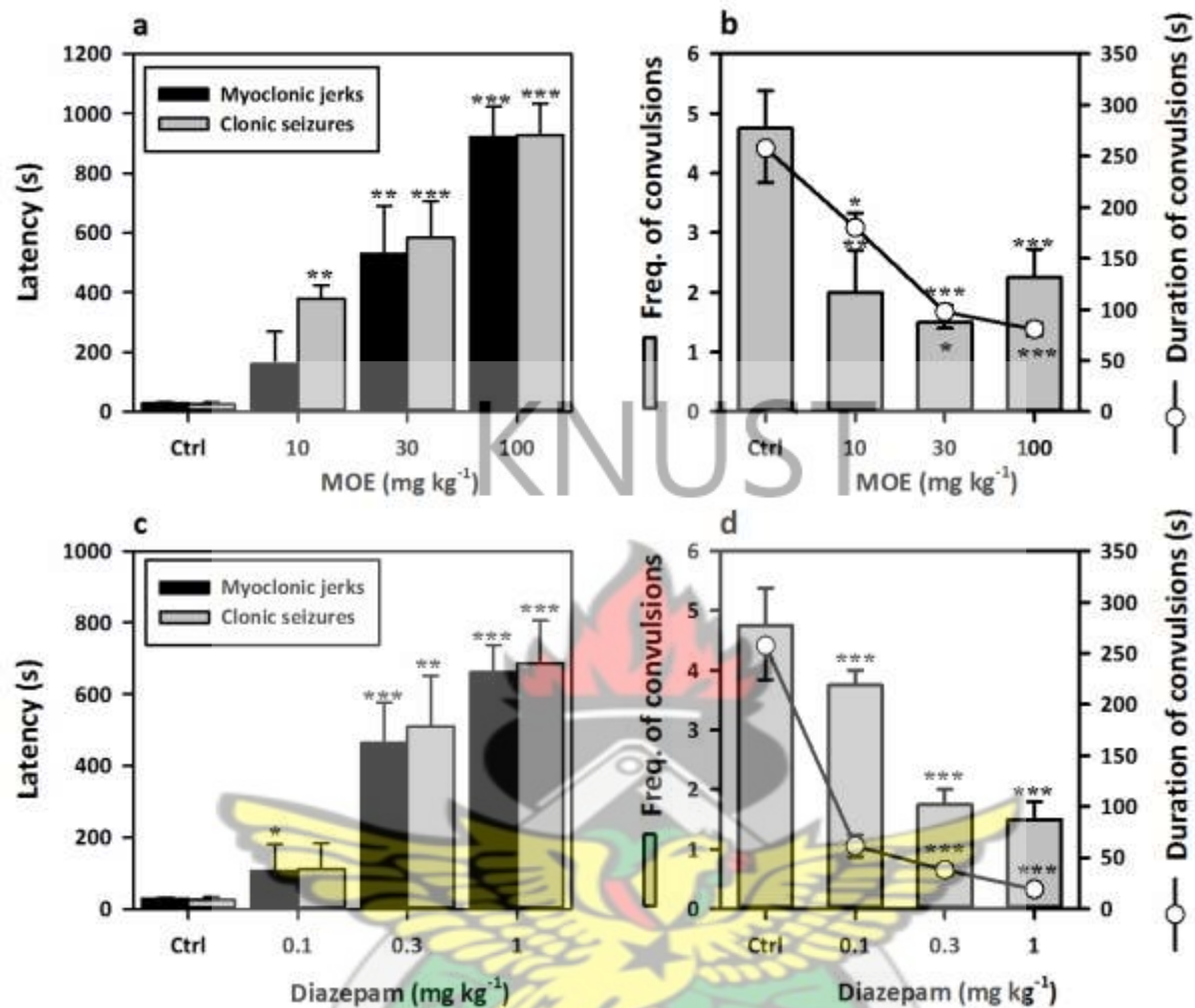


Figure 4.1 Effect of MOE (10 - 100 mg kg<sup>-1</sup>, *p.o.*) and diazepam (0.1 - 1.0 mg kg<sup>-1</sup>, *i.p.*) on (a, c) the latencies to myoclonic jerks and clonic convulsions respectively; (b and d) frequency and duration of clonic convulsions respectively, in the pentylenetetrazole-induced seizure test in mice. Data was presented as mean  $\pm$  S.E.M. (n=5); \*\*\**P*<0.001; \*\**P*<0.01; \**P*<0.05; compared to control group (One-way ANOVA followed by Newman Keuls' test).

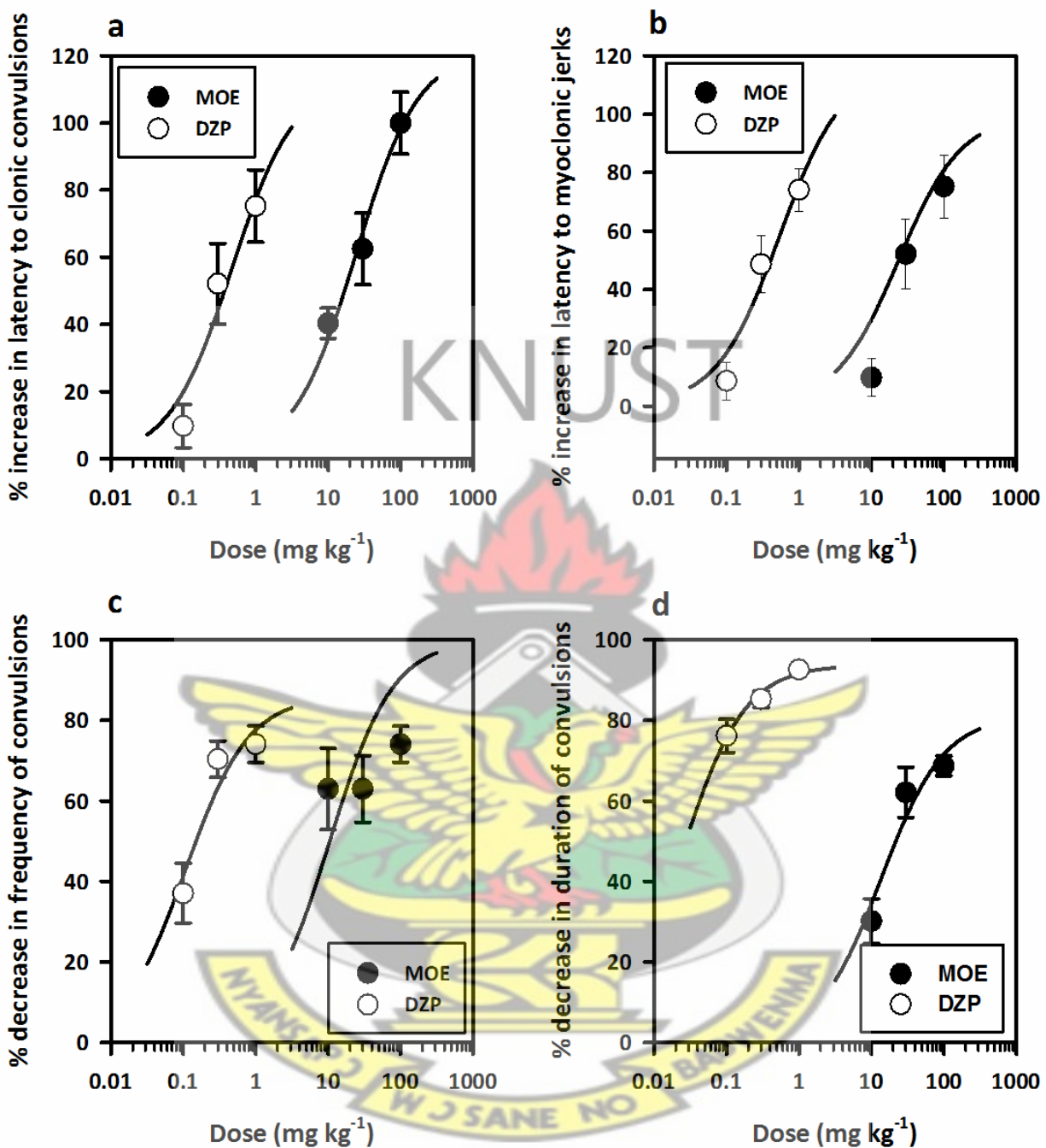


Figure 4.2 Dose–response curves for the anticonvulsant activity induced by the administration of MOE (10 - 100 mg kg<sup>-1</sup>) and diazepam (0.1 - 1 mg kg<sup>-1</sup>) in the pentylenetetrazol- induced seizure test in mice. (a) % increase in latency to clonic convulsions; (b) % increase in latency to myoclonic convulsions; (c) % decrease in frequency convulsions; (d) % decrease in duration of convulsions. Each point is the mean  $\pm$  S.E.M. of 5 animals.

### 4.3.2 Picrotoxin-induced seizures

Extract treated groups exhibited a significant anticonvulsant effect in this model. MOE (10 - 100 mg kg<sup>-1</sup>) caused a profound dose dependent delay in the onset of myoclonic jerks ( $F_{3,16}=157.0$ ,  $P<0.0001$ ) (Figure 4.3a) and clonic convulsions in mice ( $F_{3,16}=97.82$ ,  $P<0.0001$ ) (Figure 4.3a). The extract also decreased the frequency ( $F_{3,16}=8.667$ ,  $P=0.0012$ ) (Figure 4.3b) and duration of convulsions ( $F_{3,16}=22.63$ ,  $P<0.0001$ ) (Figure 4.3b) significantly. Diazepam (0.1 - 1.0 mg kg<sup>-1</sup>), the reference anticonvulsant showed similar results as the extract. Diazepam was more efficacious and potent than MOE (Figure 4.4) in reducing the frequency and duration of clonic convulsions but less efficacious in delaying the onset of seizures (Table 4.2, Table 4.3 and Table 4.4).

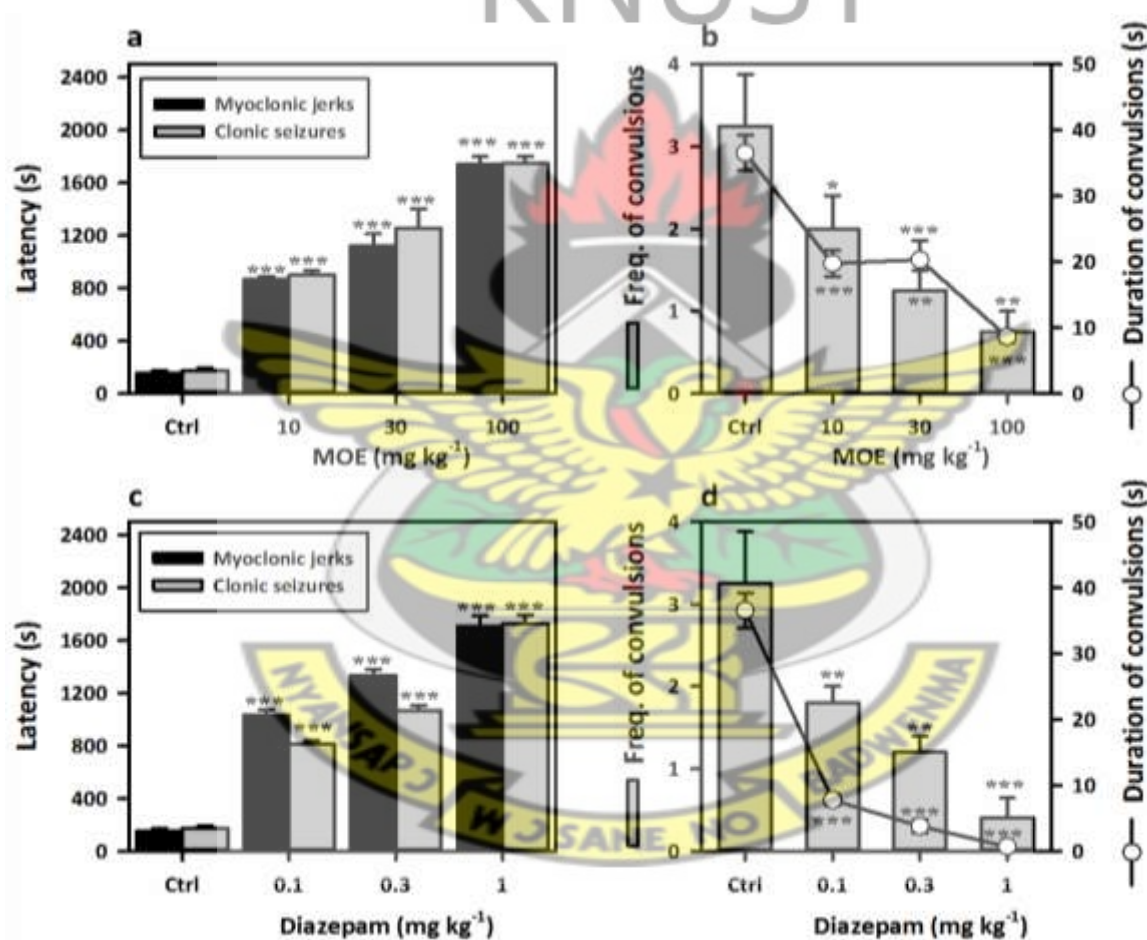


Figure 4.3 Effect of MOE (10 - 100 mg kg<sup>-1</sup>, *p.o.*) and diazepam (0.1 - 1 mg kg<sup>-1</sup>, *i.p.*) on (a, c) the latencies to myoclonic jerks and clonic convulsions; (b and d) frequency and duration of convulsions, in the picrotoxin-induced seizure test in mice. Data was presented as mean  $\pm$  S.E.M. (n = 5); \*\*\* $P<0.001$ ; \*\*  $P<0.01$ ; compared to control group (One-way ANOVA followed by Newman Keuls' test).

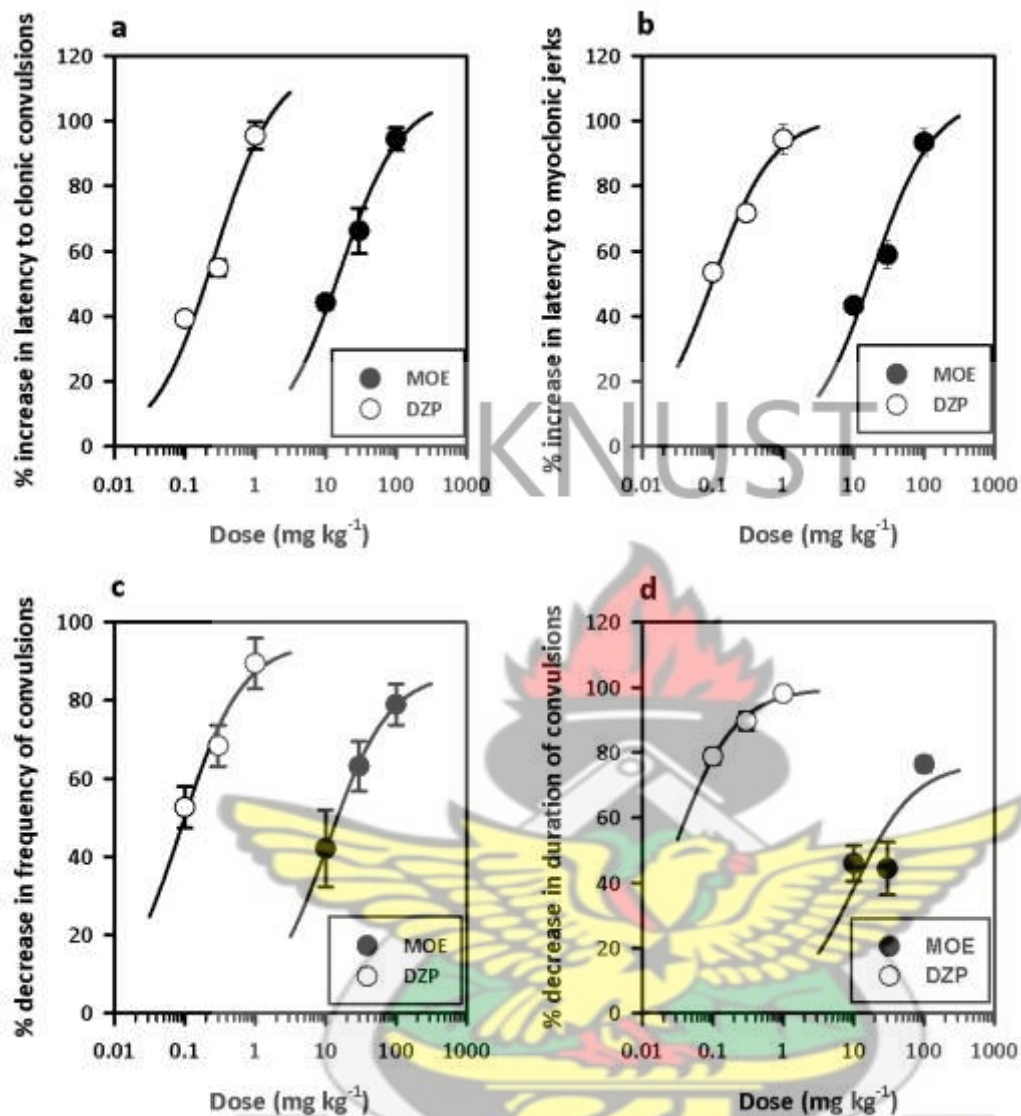


Figure 4.4 Dose–response curves for the anticonvulsant activity induced by the administration of MOE (10 - 100 mg kg<sup>-1</sup>) and diazepam (0.1 - 1 mg kg<sup>-1</sup>) in the picrotoxin-induced seizure test in mice. Each point is the mean  $\pm$  S.E.M. of 5 animals.

#### 4.3.3 Maximal electroshock seizure (MES) test

Though MOE (10 - 100 mg kg<sup>-1</sup>, *p.o.*) could not prevent tonic limb extensions (TLEs) it caused a significant reduction in the duration of tonic limb extensions at all dose levels ( $F_{3,19}=132.9$ ,  $P<0.0001$ ) (Figure 4.5). Carbamazepine [CBZ], (10 - 100 mg kg<sup>-1</sup>), the reference anticonvulsant significantly reduced the duration of TLEs. At 100 mg kg<sup>-1</sup>, CBZ totally abolished the TLEs in mice. From the table of value of ED<sub>50</sub>s and E<sub>max</sub>, oral administration of carbamazepine was more efficacious and potent than the extract in the reducing the duration of convulsions (Table 4.3, Figure 4.6).



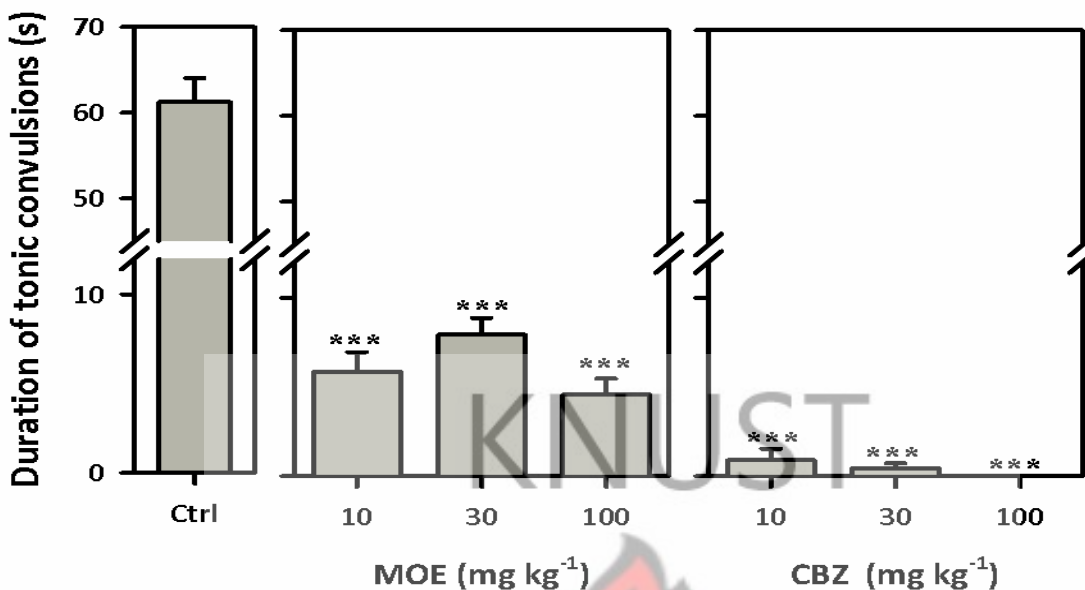


Figure 4.5 Effect of MOE (10 - 100 mg kg<sup>-1</sup>, *p.o.*) and carbamazepine (10 - 100 mg kg<sup>-1</sup>, *p.o.*) on the duration of tonic limb extensions maximal electroshock seizure test in mice. Data was presented as mean  $\pm$  S.E.M. (n = 5); \*\*\**P* < 0.001; compared to control group (One-way ANOVA followed by Newman Keuls' test).

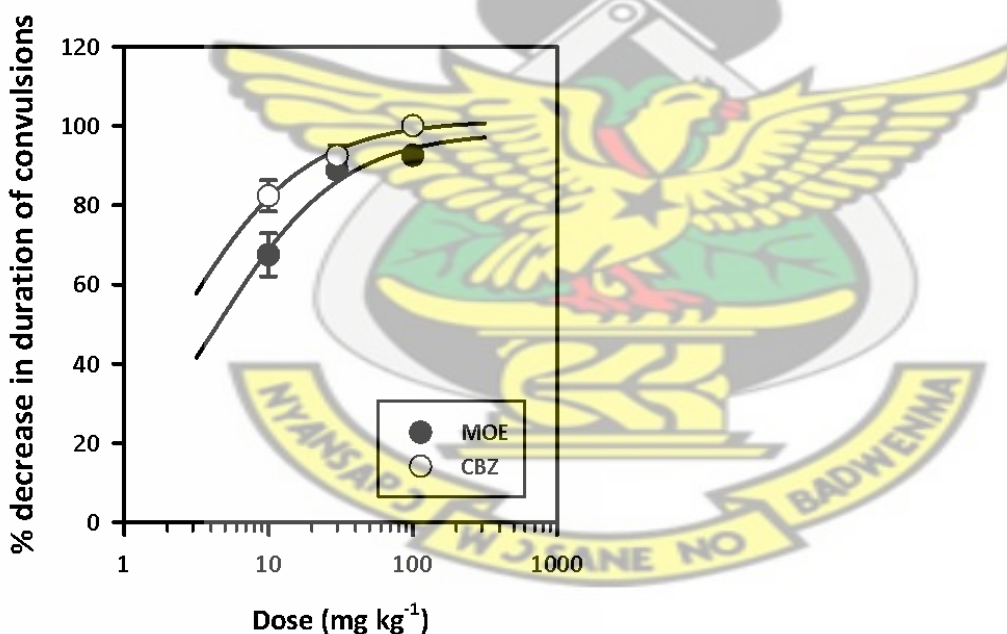


Figure 4.6 Dose–response curves for the anticonvulsant activity induced by the administration of MOE (10 - 100 mg kg<sup>-1</sup>) and carbamazepine (10 - 100 mg kg<sup>-1</sup>) in the maximal electroshock seizure test in mice. Each point is the mean  $\pm$  S.E.M. of 5 animals.

#### 4.3.4 Strychnine-induced convulsions

Extract (10 - 100 mg kg<sup>-1</sup>) showed significant anticonvulsant property. It dose dependently decreased the frequency ( $F_{3,25}=11.91$ ,  $P<0.0001$ ) (Figure 4.7a) and duration of convulsions

( $F_{3,36}=12.07$ ,  $P<0.0001$ ) (Figure 4.7c). MOE also significantly delayed the onset of myoclonic jerks ( $F_{3,25}=11.85$ ,  $P<0.0001$ ) (Figure 4.7a). Diazepam (0.1 - 1 mg kg<sup>-1</sup>) showed similar results as the extract but was more potent and efficacious than the extract in reducing the duration of convulsions (Table 4.3, Figure 4.8c).

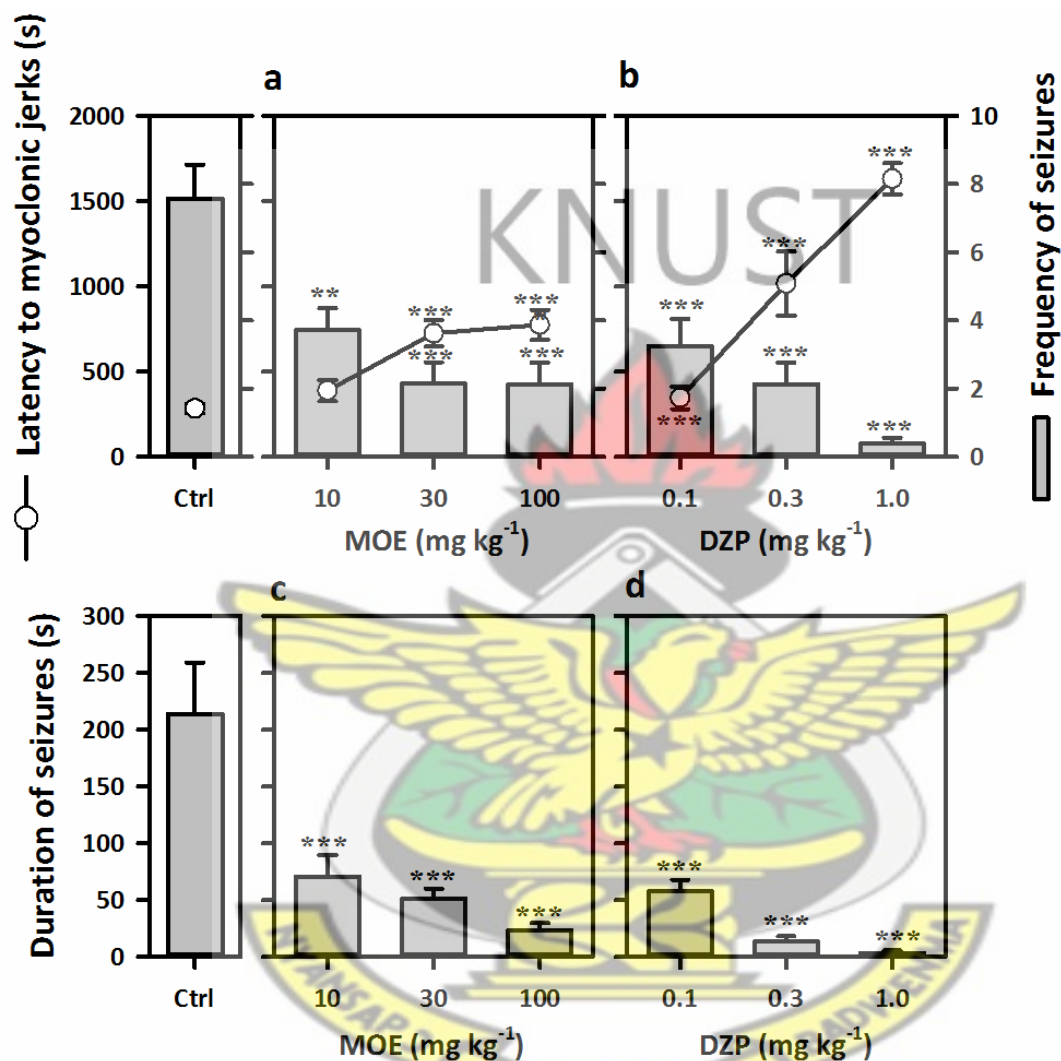


Figure 4.7 Effect of MOE (10 - 100 mg kg<sup>-1</sup>, *p.o.*) and diazepam (0.1 - 1 mg kg<sup>-1</sup>, *i.p.*) on (a, b) the latency to clonic convulsions and frequency of convulsions; (c, d) duration of convulsions, in strychnine-induced seizure test in mice. Data was presented as mean  $\pm$  S.E.M. (n = 8); \*\*\* $P<0.001$ ; \*\* $P<0.01$ ; compared to control group (One-way ANOVA followed by Newman Keuls' test).

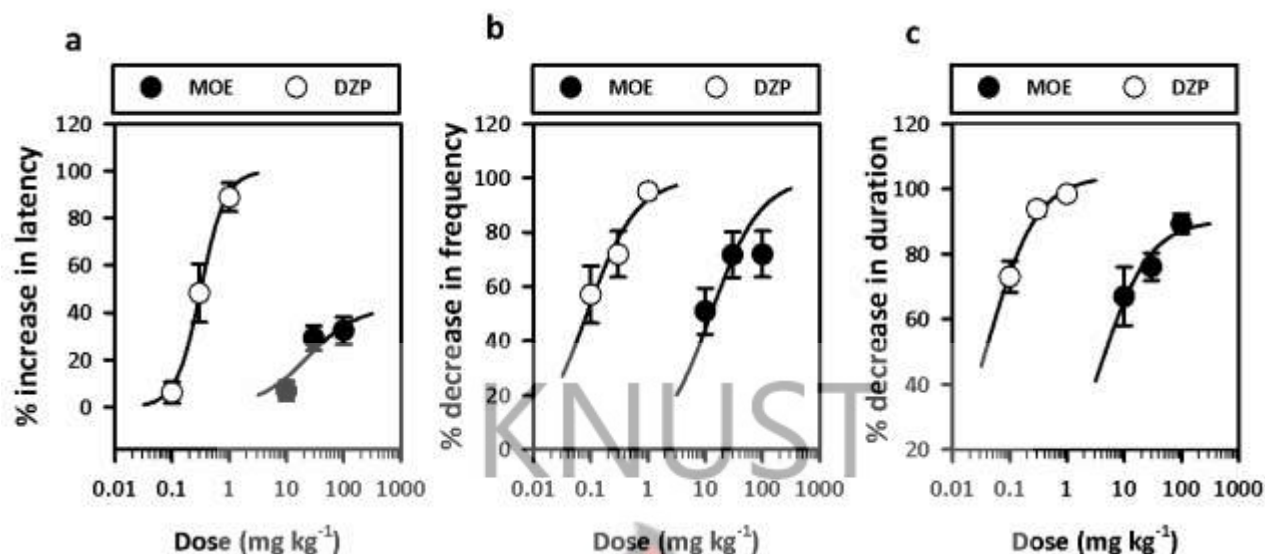


Figure 4.8 Dose–response curves for the anticonvulsant activity induced by the administration of MOE (10 - 100 mg kg<sup>-1</sup>) and diazepam (0.1 - 1.0 mg kg<sup>-1</sup>) in the strychnine-induced seizure test in mice. (a) % increase in latency to convulsions; (b) % decrease in frequency of convulsions; (c) % decrease in duration of convulsions. Each point is the mean  $\pm$  S.E.M. of 8 animals.

#### 4.3.5 Pilocarpine-induced *status epilepticus*

Oral administration of MOE was effective against pilocarpine induced *status epilepticus*. One-way ANOVA followed by Newman Keuls' test revealed that the extract significantly delayed the onset of clonic convulsions ( $F_{3,36}=25.72$ ,  $P<0.0001$ ) (Figure 4.9) and the duration of clonic convulsions ( $F_{3,36}=250.5$ ,  $P<0.0001$ ) (Figure 4.9).  $ED_{50}$  and  $E_{max}$  values calculated from the dose response curves (Figure 4.10) demonstrated that MOE was less potent than diazepam in reducing the duration of convulsions and delaying the onset of convulsions but their efficacies were comparable (Table 4.3; Table 4.4 and Figure 4.10).

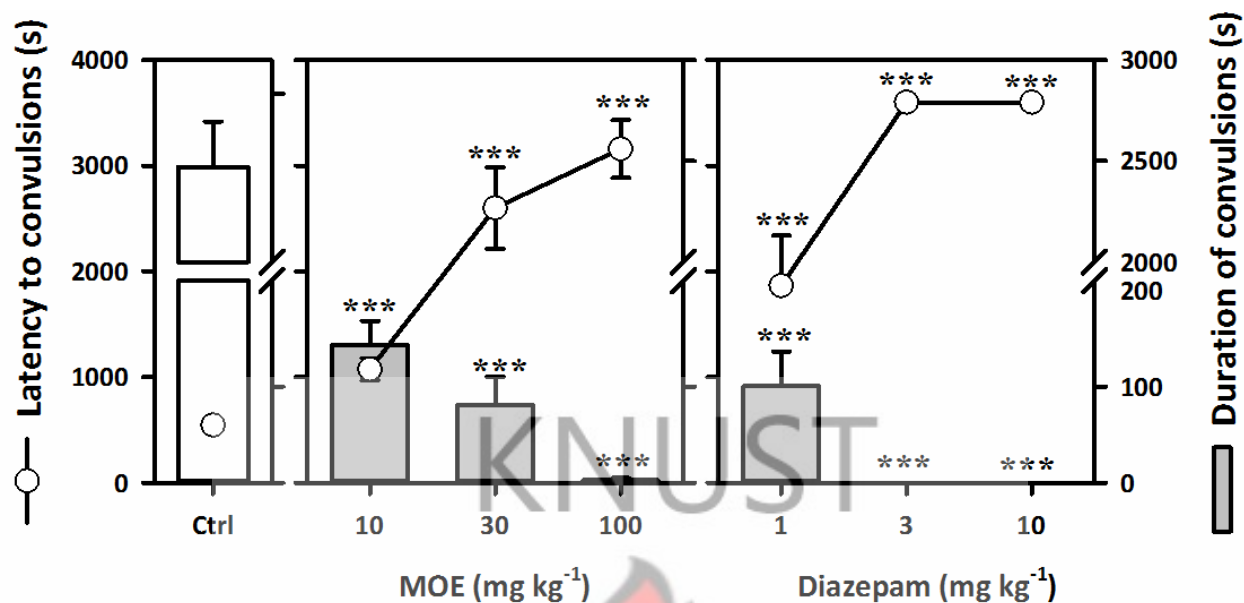


Figure 4.9 Effect of MOE (10 - 100 mg kg<sup>-1</sup>, *p.o.*) and diazepam (1 - 10 mg kg<sup>-1</sup>, *i.p.*) on the latency to and duration of clonic convulsions in the pilocarpine-induced *status epilepticus* in mice. Data was presented as mean  $\pm$  S.E.M. (n = 10); \*\*\**P* < 0.001; \*\* *P* < 0.01; compared to control group (One-way ANOVA followed by Newman Keuls' test).

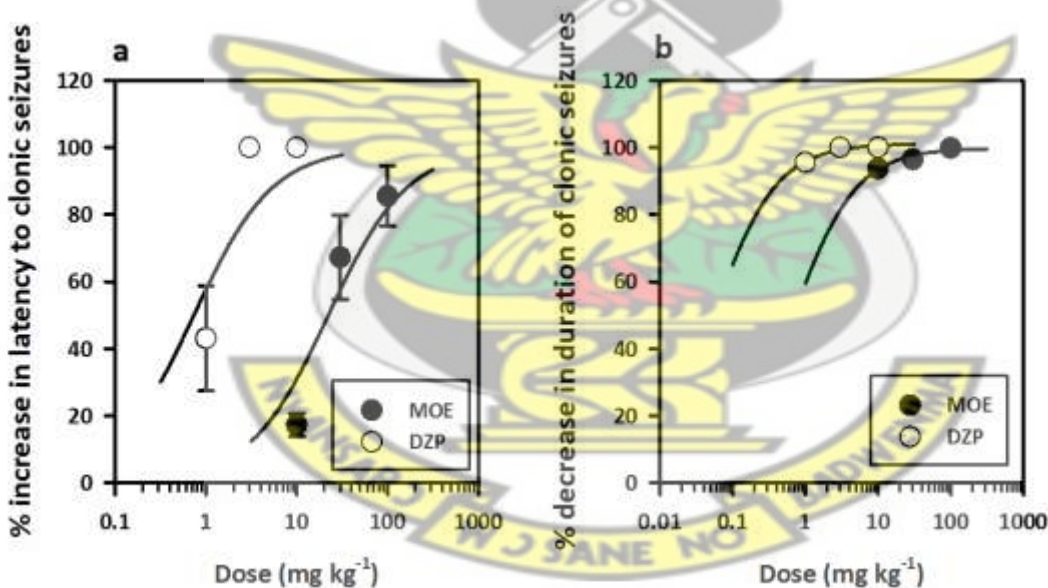


Figure 4.10 Dose-response curves for the anticonvulsant activity induced by the administration of MOE (10 - 100 mg kg<sup>-1</sup>) and diazepam, (1 - 10 mg kg<sup>-1</sup>) in the pilocarpine-induced *status epilepticus* in mice. (a) % Increase in latency to clonic convulsions; (b) % Decrease in duration of clonic convulsions. Each point is the mean  $\pm$  S.E.M. of 10 animals.

#### 4.3.6 Involvement of GABAergic mechanisms

MOE alone delayed onset of convulsions and decreased frequency and duration of convulsions similar to diazepam alone (Figure 4.11a, b and c). Flumazenil alone (2 mg kg<sup>-1</sup>) did not alter the onset, frequency or duration of convulsions. Pretreatment with flumazenil, inhibited the



anticonvulsant effect of extract and diazepam. It reversed the delay in convulsion onset by 90.99%, the decrease in frequency by 78.41% and the decrease in duration of convulsions by 87.66% (Figure 4.11a, b and c).

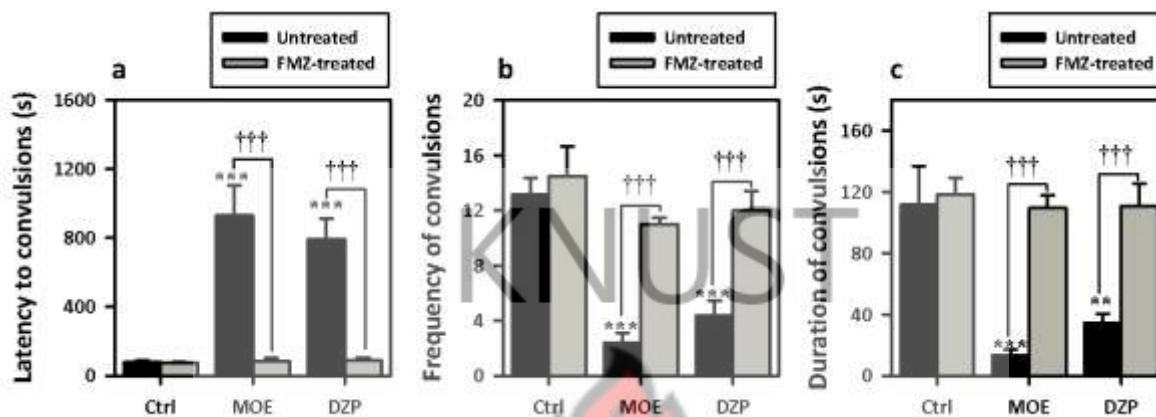


Figure 4.11 Effect of MOE (10 - 100 mg kg<sup>-1</sup>, *p.o.*) and diazepam (0.1 - 1.0 mg kg<sup>-1</sup>, *i.p.*) on the effect of flumazenil pretreatment on the (a) latency to (b) frequency and (c) duration of clonic convulsions in the pentylenetetrazole induced seizure test in mice. Data was presented as mean  $\pm$  S.E.M. (n = 8); \*\*\* $P$ <0.001; \*\* $P$ <0.01; compared to control group (One-way ANOVA followed by Newman Keuls' test). ††† $P$ <0.001, comparison between treated and untreated group (Two-way ANOVA followed by Bonferroni's test).

#### 4.3.7 Involvement of potassium channels: The 4-aminopyridine induced seizure test

Oral dose of MOE (10-100 mg kg<sup>-1</sup>), showed profound anticonvulsant effect. One-way ANOVA showed that MOE dose dependently delayed the onset of clonic ( $F_{3,36}=3.764$ ,  $P=0.0190$ ) (Figure 4.12) and tonic convulsions ( $F_{3,36}=3.857$ ,  $P=0.0172$ ) (Figure 4.12). Valproate, the reference anticonvulsant (100, 300, 600 mg kg<sup>-1</sup>) showed similar effects as the extract. Valproate was more potent than the extract in delaying the onset of convulsions (Table 4.2, Figure 4.14). From the survival curves, percentage survival decreased with time in the extract-treated group. The percentage survival however increased dose dependently (Figure 4.13).

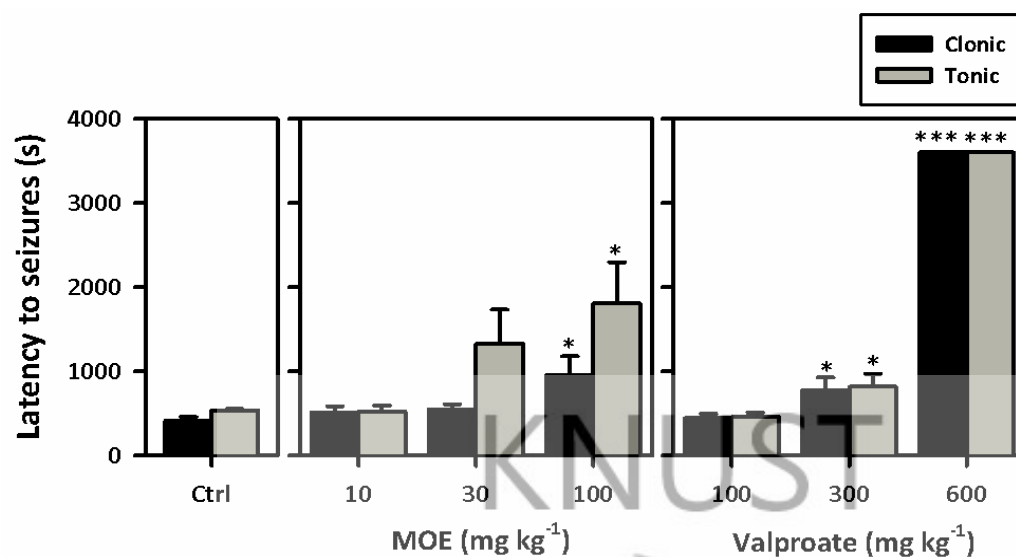


Figure 4.12 Effect of MOE (10 - 100 mg kg<sup>-1</sup>, *p.o.*) and sodium valproate (100 - 600 mg kg<sup>-1</sup>, *p.o.*) on the latency to clonic and tonic convulsions in the 4-aminopyridine induced seizure test in mice. Data was presented as mean  $\pm$  S.E.M. (n = 10); \*\*\**P*<0.001; \**P*<0.05; compared to vehicle-treated group (One-way ANOVA followed by Newman Keuls' test).

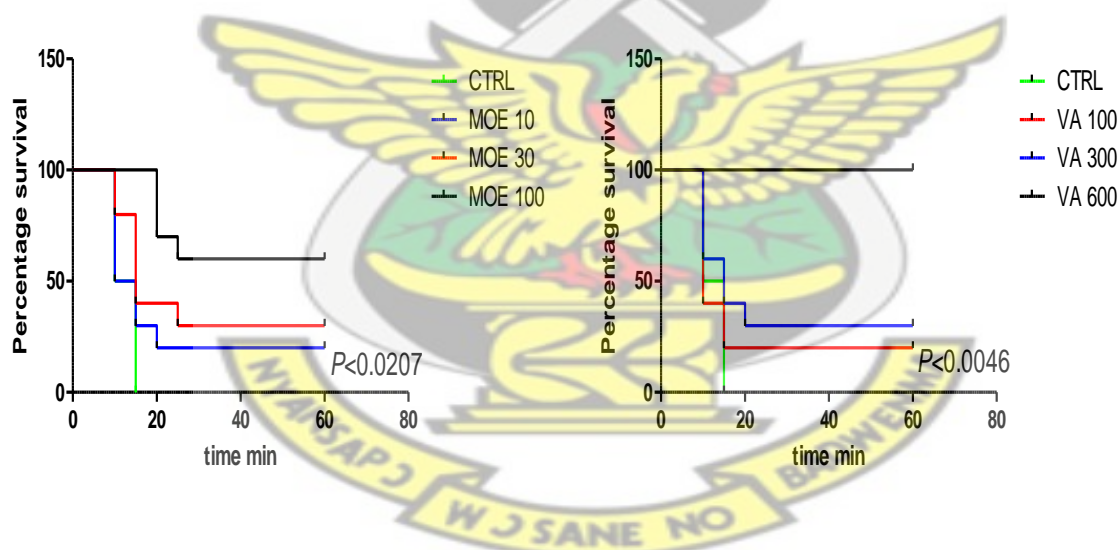


Figure 4.13 A Kaplan-Meier estimates of the percentage survival of mice for extract (MOE 10 – 100 mg kg<sup>-1</sup>) and sodium valproate (VA 100 – 600 mg kg<sup>-1</sup>). Each point is the mean  $\pm$  S.E.M. of 10 animals.

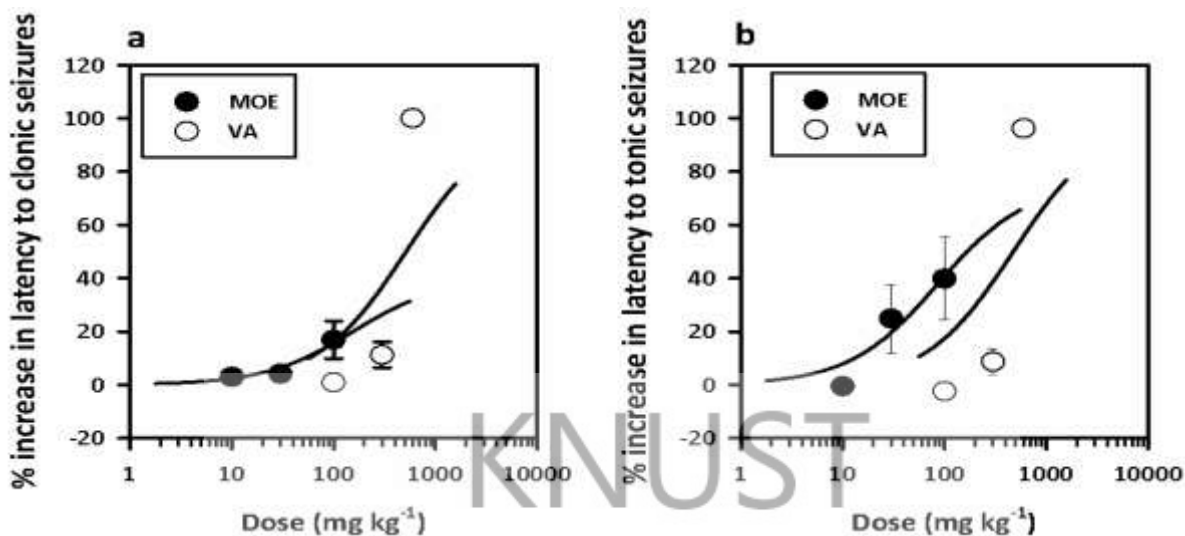


Figure 4.14 Dose–response curves for the anticonvulsant activity induced by the administration of MOE (10 - 100 mg kg<sup>-1</sup>) and sodium valproate (100 - 600 mg kg<sup>-1</sup>) in the 4-aminopyridine induced seizure test in mice. (a) % increase in latency to clonic convulsions; (b) % increase in latency to tonic convulsions. Each point is the mean  $\pm$  S.E.M. of 10 animals.

#### 4.3.8 Isobolographic analysis of extract and diazepam

Fractions of the extract and diazepam combination dose dependently decreased the frequency and duration of convulsions in the PTZ induced seizure test (Figure 4.15). Flumazenil failed to reverse the decline in the frequency and duration of convulsions by the fraction of extract and diazepam combination (Figure 4.15). The experimental ED<sub>50</sub> ( $Z_{mix}$ ) obtained by non-linear regression analysis was  $5.49 \pm 2.92$  indicating potentiation of anticonvulsant effect (Figure 4.16a). The interaction index (a measure of the degree of potentiation) displayed as the  $Z_{mix}$  lying below the line of additivity of the isobologram was 0.70 indicating synergism (Figure 4.16b).

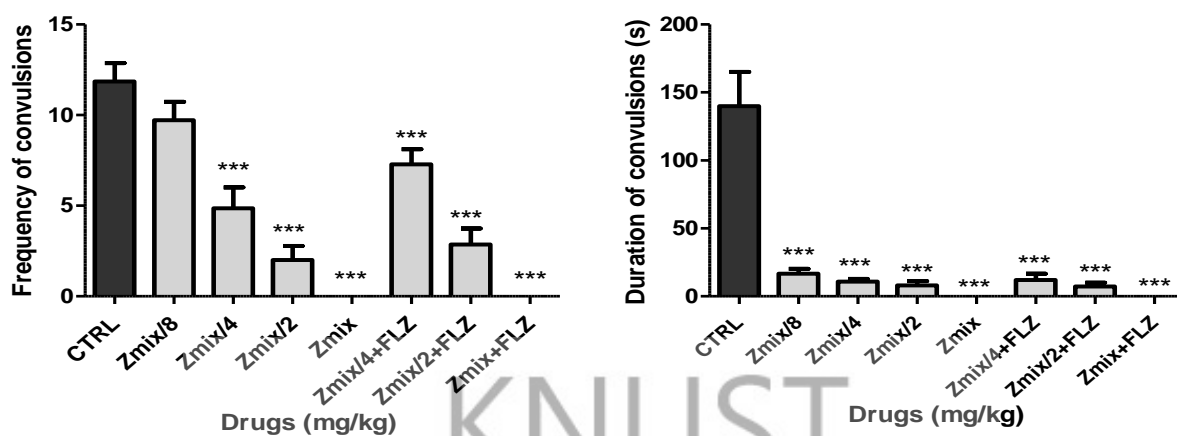


Figure 4.15 Effect of fractions of extract and diazepam combinations and their antagonism with flumazenil on frequency and duration of convulsions in the PTZ test. Each point is the mean  $\pm$  S.E.M. of 7 animals. \*\*\* $P < 0.001$ ; (One-way ANOVA followed by Newman Keuls' test).

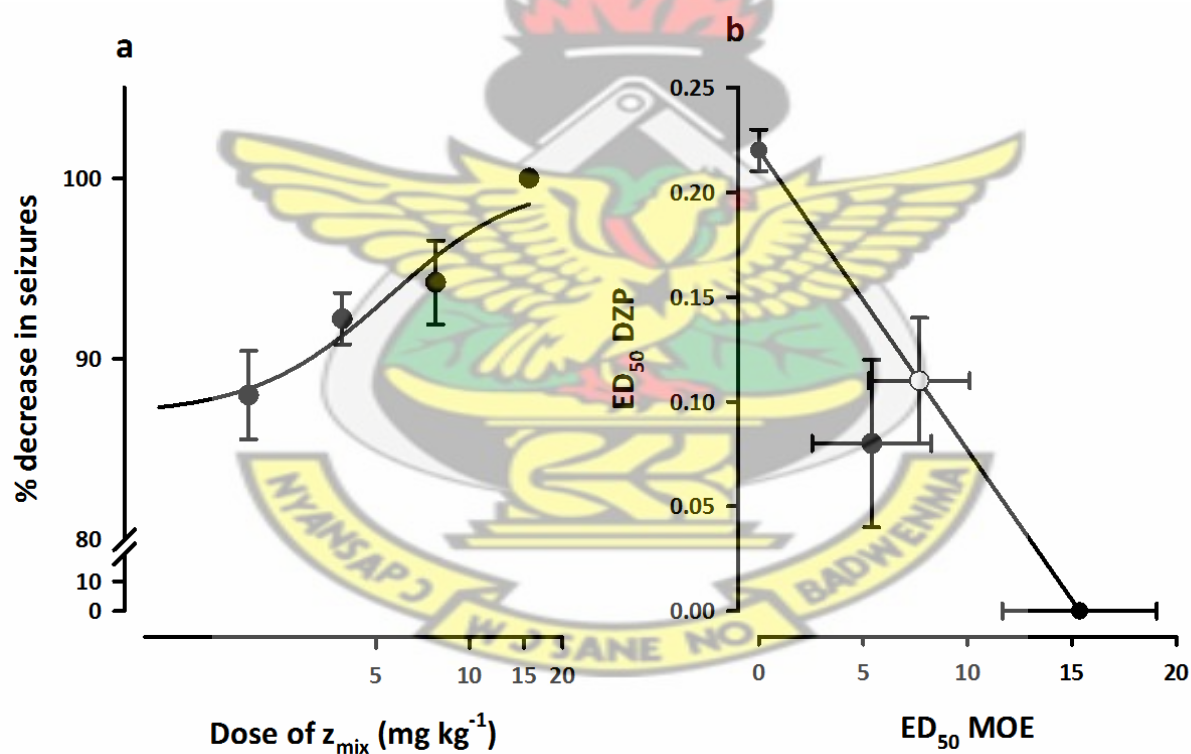


Figure 4.16 (a) Dose response curve for fractions of extract and diazepam combinations in the PTZ seizure test. (b) Isobologram for the combination of extract and diazepam in the PTZ seizure test. Filled circle represent the theoretical  $ED_{50} \pm S.E.M$  and open circle for the experimental  $ED_{50} \pm S.E.M$ .



Table 4.1 ED<sub>50</sub> and E<sub>max</sub> Values of the Latency to Convulsions

Drug (mg kg <sup>-1</sup> )	STR		PTZ		PTX		4-AP	
	ED <sub>50</sub>	E <sub>max</sub>	ED <sub>50</sub>	E <sub>max</sub>	ED <sub>50</sub>	E <sub>max</sub>	ED <sub>50</sub>	E <sub>max</sub>
MOE	23.7±26.8	42.4	24.2±12.5	122	18.7±4.8	107.5	86.1±59.6	75.7
DZP	0.3±0.1	100	0.5±0.1	113.6	0.1±0.0	101.2		
VA	-	-	-	-	-	-	47.51±154.6	100

Values are expressed as mean ± S.E.M. (n=5). The values were obtained from experiments described earlier. ED<sub>50</sub> ± S.E.M. were obtained by least-square nonlinear regression as described in material and methods.

Table 4.2 ED<sub>50</sub> and E<sub>max</sub> Values of the frequency of Convulsions

Drug (mg kg <sup>-1</sup> )	STR		PTZ		PTX	
	ED <sub>50</sub>	E <sub>max</sub>	ED <sub>50</sub>	E <sub>max</sub>	ED <sub>50</sub>	E <sub>max</sub>
MOE	12.6±3.9	100	15.4±3.7	100	10.9±6.1	87.2
DZP	0.1±0.1	99.8	0.2±0.0	85.9	0.1±0.0	94.8

Values are expressed as mean ± S.E.M. (n=5). The values were obtained from experiments described earlier. ED<sub>50</sub> ± S.E.M. were obtained by least-square nonlinear regression as described in material and methods.

Table 4.3 ED<sub>50</sub> and E<sub>max</sub> Values of the Duration of Convulsions.

Drug (mg kg <sup>-1</sup> )	MES		STR		PTZ		PTX	
	ED <sub>50</sub>	E <sub>max</sub>	ED <sub>50</sub>	E <sub>max</sub>	ED <sub>50</sub>	E <sub>max</sub>	ED <sub>50</sub>	E <sub>max</sub>
MOE	4.3±1.2	98.4	3.8±2.2	90.3	13.6±5.8	81.3	10.1±6.7	76.9
DZP	-	-	2.4±0.7	103.9	0.3±0.1	93.8	0.3±0.1	99.7
CBZ	2.4±0.7	101.4	-	-	-	-	-	-

Values are expressed as mean ± S.E.M. (n=5). The values were obtained from experiments described earlier. ED<sub>50</sub> ± S.E.M. were obtained by least-square nonlinear regression as described in material and methods.

#### 4.3.9 Rotarod

MOE did not impair motor coordination at all dose levels. Diazepam, however, significantly impaired motor coordination at 1 mg kg<sup>-1</sup> (Table 4.4).

Table 4.4 showing the latency for mice to fall off the rotarod

Drug (mg kg <sup>-1</sup> )	Latency to fall (s)				
	0 h	1 h	2 h	3 h	4 h
Ctrl	108.6±11.4	120.0±0.0	98.9±14.1	120.0±0.0	109.8±10.2
MOE					
10	101.6±9.9	76.1±13.6	67.5±15.5	71.0±21.9	102.2±11.4
30	119.6±33.6	111.6±8.4	97.0±15.0	97.0±17.4	50.0±11.7*
100	109.1±9.8	87.2±21.5	105.1±14.9	76.6±18.5	85.9±14.0
DZP					
0.1	112.0±5.8	96.1±10.4	82.6±4.9	97.0±9.4	100.6±11.8
0.3	110.5±5.8	81.5±10.2	75.6±6.1	60.3±12.3***	82.5±17.5
1.0	109.0±8.7	65.7±12.3**	50.1±11.6**	35.1±13.5***	65.8±12.7*

Values are expressed as mean ± S.E.M. (n=5). \*\*\* $P < 0.001$ ; \*\*  $P < 0.01$

#### 4.4 DISCUSSION

From the studies carried out, it is evident that oral administration of *Mallotus oppositifolius* leaf extract (MOE) has anticonvulsant effect in both acute generalized seizure models—pentylenetetrazole-, picrotoxin- and maximal electroshock-, strychnine-, 4-aminopyridine -induced seizure tests and partial seizure model—the pilocarpine-induced *status epilepticus*.

The maximal electroshock test is the most widely used animal model in antiepileptic drug discovery because seizure induction is simple and the predictive value for detecting clinically effective antiepileptic is high (White, 1997; Holmes, 2007; Castel-Branco *et al.*, 2009). Though the extract did not totally abolish tonic hind limb extension at all the doses used in the maximal electroshock seizure test it significantly reduced the duration of the tonic hind limb extension. Tonic hind limb extension is the universal feature of maximal electroshock in mice, rats, rabbits, cats, monkeys and humans (Raza *et al.*, 2001; Giardina *et al.*, 2005). Abolishing tonic hind limb extension in electroshock test predicts the ability of testing material to prevent the spread of seizure discharge from the epileptic focus and its effectiveness in this model correlates well with suppressing generalized tonic-clonic seizures and indicates the ability of the testing material to inhibit or prevent seizure discharge within brainstem substrate (Krall *et al.*, 1978; Porter *et al.*, 1984; Raza *et al.*, 2001). All the currently available drugs that are clinically effective in generalized tonic-clonic seizures (phenytoin, carbamazepine, phenobarbitone, valproate, lamotrigine, oxycarbamazepine, etc.) are effective in this model (Macdonald and Kelly, 1995; Castel-Branco *et al.*, 2009). Thus the extract may not be effective in completely attenuating generalised tonic-clonic seizures but may reduce the duration of these seizures.

An agent that prevents or delays the onset of clonic and tonic-clonic convulsion induced by PTZ, a GABA receptor antagonist, in animals is an anticonvulsant (Vellucci and Webster, 1984;

Amabeoku and Chikuni, 1993; Sayyah *et al.*, 2004). In the present study, the extract showed anticonvulsant effect against PTZ-induced seizures by delaying the onset of myoclonic jerks and clonic convulsions in mice. It also caused profound decrease in the frequency and duration of the clonic convulsions. This result confirmed an earlier report of the effectiveness of MOE against PTZ seizures in the preliminary CNS screening tests (Kukuia *et al.*, 2012). PTZ-induced seizure model identifies compounds that can raise seizure threshold in the brain (Raza *et al.*, 2001; White, 2003; Mandhane *et al.*, 2007). Antiepileptics effective in the therapy of generalized seizures of *petit mal* type (absence or myoclonic) i.e. phenobarbitone, valproate, ethosuximide and benzodiazepines suppress PTZ-induced seizures (Loscher *et al.*, 1991; Kaminski *et al.*, 2001; White, 2003; Akula *et al.*, 2009). Thus the extract may be effective against generalized absence/myoclonic seizures.

MOE also showed anticonvulsant activity against seizures induced by picrotoxin, a GABA<sub>A</sub> receptor antagonist. PTZ and picrotoxin induce convulsions in rodents by blocking the chloride-ion channels linked to GABA<sub>A</sub> receptors, thus preventing the entry of chloride ions into the brain and consequently inhibitory transmission in the brain (Loscher and Schmidt, 1988; Mehta and Ticku, 2001). GABAergic neurotransmission plays an important role in stress, anxiety (Zwanzger and Rupprecht, 2005), pain (Rode *et al.*, 2005) and epilepsy (Perucca, 2005). Benzodiazepines and many barbiturates potentiate the inhibitory action of GABA<sub>A</sub> receptors, reducing neuronal excitability and increasing the threshold for convulsions (Loscher and Schmidt, 2006). Since MOE was effective against both pentylenetetrazole and picrotoxin-induced seizures, a possible interaction with GABAergic mechanisms was investigated by pretreating mice with flumazenil, a benzodiazepine receptor antagonist (File and Pellow, 1986; Przegalinski *et al.*, 2000), in the PTZ-induced seizure test. The reversal of the anticonvulsant effect of MOE by flumazenil in this test, confirms possible interaction with the GABA/benzodiazepine receptor complex or pathway.

Together with the GABA<sub>A</sub> receptors, the glycine receptor is responsible for mediating fast inhibitory neurotransmission in the mature central nervous system making it a potential target for antiepileptic drugs (Lopez-Corcuera *et al.*, 2001; Bowery and Smart, 2006; Webb and Lynch, 2007). Strychnine causes convulsions by antagonizing the activity of strychnine sensitive glycine receptors and increasing postsynaptic excitability and ongoing activity in the brainstem and spinal cord (Curtis *et al.*, 1967; Werman *et al.*, 1967; Wang *et al.*, 2001; Wood *et al.*, 2002). Since MOE delayed the onset of convulsions, reduced the frequency and duration of convulsions induced by strychnine, an interaction of the extract with glycine receptors/pathways is plausible. It is possible the extract may contain compounds that activate glycinergic inhibitory neurotransmission.

The importance of potassium ion channels as an inhibitory mechanism in epilepsy is well-known (Lerche *et al.*, 2001; George, 2004; Parthasarathi *et al.*, 2006). Moreover, it has been established



that blocking these potassium channels and preventing them from opening in response to membrane depolarization or deleting these channels will lower seizure threshold and can produce seizures (Zhang *et al.*, 2010; N'Gouemo, 2011). 4-aminopyridine (4-AP) is a known potassium channel blocker that produces seizures by increasing the release of glutamate and calcium while preventing GABAergic neurotransmission (Yamaguchi and Rogawski, 1992; Lohle *et al.*, 2008; Leung *et al.*, 2011). The effectiveness of the extract against 4-AP induced seizures is an indication that the anticonvulsant activity of the extract may depend on activation of potassium channels or conductance. It is also possible that the extract may be indirectly enhancing potassium conductance through its activation of GABAergic neurotransmission since activation of GABA neurotransmission can result in enhancement of potassium conductance. Retigabine, a newly approved drug for the treatment of epilepsy with broad-spectrum anticonvulsant profile in animal seizure and epilepsy models, functions through its ability to activate potassium currents (Rostock *et al.*, 1996; Rundfeldt, 1997). So far it is the only potassium channel opener that has been approved as an anticonvulsant. That MOE possibly interacts with potassium channels is an important finding since MOE may be another source of anticonvulsant compounds that will enhance potassium conductance. Rottlerin (mallotoxin), which has been found in the leaves of *M. oppositifolius* (Oliver, 1960; Adekunle and Ikumapayi, 2006), is a potent activator of the large conductance voltage and  $\text{Ca}^{2+}$  activated  $\text{K}^{+}$  channels which are implicated in epilepsy (Zakharov *et al.*, 2005; Soltoff, 2007; Wu *et al.*, 2007). It is possible that the anticonvulsant effect observed in this model may be due to the presence of rottlerin in the leaves.

Systemic administration of pilocarpine, a nonselective muscarinic agonist, is an animal model of intractable epilepsy (Turski *et al.*, 1989; Matsui *et al.*, 2000; Eglen *et al.*, 2001; Mirza *et al.*, 2003; Wirtshafter, 2006). Histological studies have shown important similarities between this model and temporal lobe epilepsy in humans; thus drugs effective in this model are potential candidates for managing temporal lobe epilepsy (Isokawa and Mello, 1991; Liu *et al.*, 1994; Wall *et al.*, 2000; Szyndler *et al.*, 2005; Perez-Mendes *et al.*, 2011). MOE exhibited potent anticonvulsant effect against *status epilepticus* induced by pilocarpine. It is therefore possible that the extract may have potential value in the management of temporal lobe epilepsy and/or other partial seizures. Muscarinic receptor stimulation is presumed to be responsible for the onset of pilocarpine-induced seizures, whereas the effect of glutamate on NMDA receptors sustains seizure activity and causes neuronal damage (Jope *et al.*, 1986; Clifford *et al.*, 1987; Turski *et al.*, 1989; Smolders *et al.*, 1997). By delaying the onset of clonic seizures in this model, it is possible that the extract may have some antimuscarinic properties and by reducing the duration of seizures, it is likely it has antagonistic effect on glutamatergic neurotransmission. It is possible that activation of potassium conductance (which can lead to inhibition in the release of glutamate) by the extract may support the antiglutamatergic theory proposed for the extract.

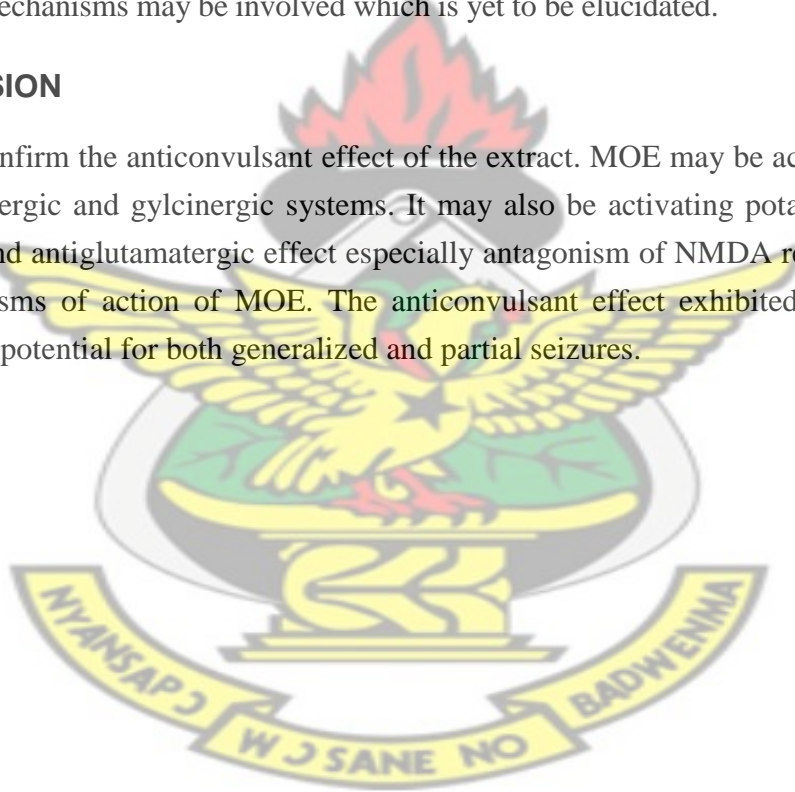


Triterpenic steroids, saponins and alkaloids have been reported to possess anticonvulsant activity in some experimental seizure models such as PTZ (Chauhan *et al.*, 1988; Kasture *et al.*, 2002). It is possible that one or more of the secondary metabolites in the plant—alkaloids, saponins and sterols may be responsible for the observed anticonvulsant effect of the extract (Farombi *et al.*, 2001; Adekunle and Ikumapayi, 2006, Kukuia *et. al.*, 2012).

Isobolographic analysis of the drug interaction between diazepam and MOE in the PTZ induced seizures showed a significant potentiating effect suggesting that a combination of the two drugs have beneficial anticonvulsant effect. Though flumazenil pretreatment inhibited the anticonvulsant effect of the extract and diazepam individually, interestingly it failed to reverse their anticonvulsant effect when administered together. This suggests that the mechanism of action of the combination of extract and diazepam may not be due to activation of the benzodiazepine/GABA receptor complex. Other mechanisms may be involved which is yet to be elucidated.

#### 4.5 CONCLUSION

These findings confirm the anticonvulsant effect of the extract. MOE may be acting possibly by activating GABAergic and glycinergic systems. It may also be activating potassium channels. Antimuscarinic and antiglutamatergic effect especially antagonism of NMDA receptors are also possible mechanisms of action of MOE. The anticonvulsant effect exhibited makes MOE a likely therapeutic potential for both generalized and partial seizures.



## *Chapter 5* **ANTIDEPRESSANT EFFECT**

### **5.1 INTRODUCTION**

Depression is an extremely common pathological complex with psychological, neuroendocrine and pathological symptoms (Newcomer *et al.*, 1990; Holmes, 2003). It is a leading cause of disability worldwide and has a very significant impact on morbidity, mortality and health care cost (Alonso *et al.*, 2004; Ustun *et al.*, 2004; Gilmour and Patten, 2007).

Perturbations in monoaminergic neurotransmission especially serotonin and noradrenaline neurotransmission are considered major culprits for the observed symptoms of depression, explaining why currently used antidepressants more or less selectively interact with the monoaminergic systems (Pacher *et al.*, 2001; Slattery *et al.*, 2004; Berton and Nestler, 2006; Yacoubi *et al.*, 2011). Unfortunately the efficacies of these medications are unsatisfactory and multiple side effects are common (Gumnick and Nemeroff, 2000; Nomura, 2003; Poleszak *et al.*, 2011). Furthermore, these drugs have slow onset of action, often requiring at least 2-4 weeks of administration before producing clinical improvement in the symptoms (Tran *et al.*, 2003; Adell *et al.*, 2005; Gourion, 2008; Machado-Vieira *et al.*, 2009). Additionally, about 30- 40% of patients have conditions refractory to current medications (Fava and Kendler, 2000; Belmaker and Agam, 2008). These reasons accentuate the need for novel therapeutic agents with less side effects and faster onset of action and wider patient spectrum (Cryan *et al.*, 2002; Nestler *et al.*, 2002). Also both preclinical and clinical studies support the role of NMDA receptor antagonists as possible therapeutic agents for depression (Trullas and Skolnick, 1990; Poleszak *et al.*, 2004; Siwek *et al.*, 2009). For instance ketamine and memantine have demonstrated rapid and profound antidepressant effects clinically (Berman *et al.*, 2000; Zarate *et al.*, 2012). Numerous behavioural studies have further demonstrated that antagonists and partial agonists at the glycine co-agonist site of the NMDA receptor have antidepressant potentials with less severe side effects (Trullas and Skolnick, 1990; Vamvakides, 1998). While the search of newer antidepressants continue, renewed interest in medicinal plants e.g. *Mallotus oppositifolius* for the treatment of many CNS disorders has been on the ascendancy (Briskin, 2000; Akhondzadeh and Abbasi, 2006; Darwish and Aburjai, 2010). Despite the use of *Mallotus oppositifolius* in treating psychiatric disorders, there is no scientific data on its antidepressant effect. Thus the present study investigated the effect of the hydroalcoholic leaf extract of the plant in acute antidepressant models- the forced swim (FST) and tail suspension tests (TST).

Investigations were carried out to ascertain whether MOE would exhibit antidepressant effect with chronic administration in the open space swim test. Though predictive of antidepressant activity, the FST and TST are unlike human depression (Cryan *et al.*, 2005; Stone *et al.*, 2008). The open

space swim test, a chronic model was chosen because it has closer resemblance to human depression while maintaining its predictive and construct validity (Sun and Alkon, 2003; Stone and Lin, 2011). Major depressive disorder (depression) itself and some antidepressants induce significant weight changes (loss or gain) and alter cognitive function among others (Fava, 2000; Dixon *et al.*, 2003; Nebes *et al.*, 2003; Anderson *et al.*, 2006; Biringer *et al.*, 2007). Hence the effect of the extract on cognitive function using the Morris water maze, and weight changes in the depressed rodents was examined.

The effects of the extract on the monoaminergic system (serotonin and noradrenaline) as well as the glycine/NMDA receptor complex were investigated in order to elucidate the possible mechanism(s) of action of the extract.

## **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 Chemicals**

Fluoxetine hydrochloride (Prozac®), was purchased from Eli Lilly and Co., Basingstoke, England. Imipramine hydrochloride (Tofranil®) and desipramine hydrochloride, D-serine, D-cycloserine  $\alpha$ -methyldopa, reserpine, para-chlorophenylalanine were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA.

### **5.2.3 Acute antidepressant model**

#### **5.2.3.1 Forced Swimming test**

The FST was based on that described by Porsolt *et al.*, (1977). Mice were divided into ten groups of five animals each and received the vehicle (water), extract (10, 30 or 100 mg kg<sup>-1</sup>, *p.o.*), or the reference drugs fluoxetine (3, 10 or 30 mg kg<sup>-1</sup>, *p.o.*) and imipramine (3, 10 or 30 mg kg<sup>-1</sup>, *p.o.*). Thirty minutes after i.p. and 1 h after oral administration of the test compounds, mice were gently dropped individually into transparent cylindrical polyethylene tanks (25 cm high, 10 cm internal diameter) containing water (25 to 28°C) up to a level of 20 cm and allowed to swim for 5 min. Mice placed in the tank engage in escape directed activities until they assume an immobile state which represents a state of helplessness (depression). Each session was recorded by a video camera suspended approximately 100 cm above the cylinders. An observer scored the duration of immobility (when it floated upright in the water and made only small movements to keep its head above water), during the 5 min test, from the videotapes with the aid of the public domain software

JWatcher Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia. Available at <http://www.jwatcher.ucla.edu/>).

#### **5.2.3.2 Tail suspension test**

The TST was carried out as previously described Steru *et al.*, (1985). Mice were allowed to acclimatize to the room for 3.5-4 h before the test. Groups of ten mice were treated with MOE (10, 30 or 100 mg kg<sup>-1</sup>, *p.o.*), fluoxetine (3, 10 or 30 mg kg<sup>-1</sup>, *p.o.*) and imipramine (3, 10 or 30 mg kg<sup>-1</sup>, *p.o.*) or vehicle. One hour after oral administration of the test compounds, mice were individually suspended by the tail from a horizontal bar (distance from floor is 30 cm) using adhesive tape (distance from tip of tail is 1 cm). Mice hanged by the tail engage in escape directed activities until they become immobile which represent a state of helplessness (depression). Duration of immobility, defined as the absence of all movement except for those required for respiration, was recorded by an observer for 5 min from video recordings of the test as described above for forced swimming test. Mice that climbed up on their tails during the test session were gently pulled down and testing continued. Mice that continued to climb their tails were excluded from the study.

#### **5.2.4 Chronic depression**

##### **5.2.4.1 Open space swim test**

The method described by Stone and Lin (2011) with some slight modifications was used. Swimming was carried out in rat tub cages (28×26×41 cm, w×h×l) filled with 13 cm high tepid tap water (32–34 °C). Mice were swum individually for 15 min/day on 4 consecutive days in order to induce a depressive state characterized by the decrease in mobility and distance travelled by the mice. Drug treatment started from day 5, through days 7, 10, 14, 18. All swim sessions were videotaped from above. No special procedures were used to dry or warm the animals as they rapidly dried themselves with no observable episodes of shivering. The distance swum was rated as the number of quadrants of the tub entered and duration of immobility from the total time the animal was observed to float, which is defined as drifting with the tail fully extended and no motion observed in the tail or limbs.

##### **5.2.4.2 Tail suspension test**

The tail suspension test was performed 24 h after the last treatment in the open space swim test to ascertain if the behavioural effect will be sustained. The procedure used was similar to that described in section 5.2.3.2.



#### **5.2.4.3 Spatial learning and memory task- Morris water maze**

Effects of the induced depressive behavior on spatial learning and memory were evaluated with the Morris water maze task (Sun and Alkon, 2004), in an 85 cm-diameter pool ( $24 \pm 1$  °C). The maze was divided into four quadrants. Naïve control mice were allowed to swim for 1 min in the pool without a hidden platform, the same day when the other groups were on their third day of depressive behavior induction. Twenty-four hours later, all mice were swum in two sessions a day for 4 days to find a hidden platform (5 cm diameter). The platform was centred in one of the quadrants and submerged about 2 cm below the water surface. At the start of all trials, mice were placed in the water facing the maze wall, using different starting positions, and allowed to swim until they found the platform where they remained for 20 s. A mouse that failed to find the platform within 1 min was guided there, with the maximum latency of 60 s scored. The swimming activity was observed for latency to find the platform.

#### **5.2.4.4 Weight variation**

Weights of mice were taken with a sensitive electronic balance when chronic depression was being induced and every 3 days during the treatment period. The change in weight was calculated to ascertain drug-induced changes in the weight of the mice.

#### **5.2.5 Possible Mechanisms**

Based on current knowledge of the mode of action of antidepressants and the pathophysiology of depression, the contribution of the adrenergic, serotonergic and glutamatergic systems to the antidepressant effect of the extract were investigated.

##### **5.2.5.1 Involvement of noradrenergic systems**

Mice were pretreated with reserpine and/or  $\alpha$ -methyl dopa ( $\alpha$ -MD) in order to investigate the possible role of noradrenergic system in the actions of MOE. The doses of  $\alpha$ -MD and reserpine were chosen based on work done by others (van Giersbergen *et al.*, 1990; O'Leary *et al.*, 2007, Woode *et al.*, 2010). To deplete newly synthesized pools of noradrenaline (NA) and dopamine (DA), mice were treated with a single dose of  $\alpha$ -MD ( $400 \text{ mg kg}^{-1}$ , i.p.) 3.5 hours before behavioural testing. To deplete vesicular pools of NA and DA, mice were treated with a single dose of reserpine ( $1 \text{ mg kg}^{-1}$ , s.c.) 24 h before behavioral testing. In an effort to deplete both the vesicular and cytoplasmic pools of NA and DA, mice were pretreated with a combination of reserpine ( $1 \text{ mg kg}^{-1}$ , s.c., 24 h before behavioral testing) and  $\alpha$ -MD ( $200 \text{ mg kg}^{-1}$ , i.p., 3.5 h before behavioural testing), respectively.

### 5.2.5.2 Involvement of serotonineric systems

Mice were grouped into twenty (20) groups,  $n=5$ . *p*CPA (200 mg  $\text{kg}^{-1}$ , i.p.) was administered once daily for 3 consecutive days to 10 groups of animals. On the fourth day, group 1 received saline, groups 2- 4 received MOE (10, 30 and 100 mg  $\text{kg}^{-1}$ ); groups 5- 7 received fluoxetine (3, 10 and 30 mg  $\text{kg}^{-1}$ , *p.o.*), groups 8- 10 received imipramine (3, 10 and 30 mg  $\text{kg}^{-1}$ , *p.o.*). The remaining 10 groups received which did not undergo pretreatment, received extract (10, 30 and 100 mg  $\text{kg}^{-1}$ , *p.o.*), fluoxetine (3, 10 and 30 mg  $\text{kg}^{-1}$ , *p.o.*), imipramine (3, 10 and 30 mg  $\text{kg}^{-1}$ , *p.o.*) or saline on the day of experiment. The tail suspension and modified forced swim tests were used. After the tail suspension sessions, mice were taken through the modified forced swimming test. The modified forced swimming test followed the same procedure described above except that the depth of water was changed to 20 cm. For tail suspension, immobility period was scored whilst for the modified swimming test mean immobility counts, mean swimming and mean climbing counts were scored. Swimming is defined as the active horizontal movement of the mice in the swim tank and this behaviour is sensitive to serotonin-based drugs; climbing is the vertical movement of the paw of the mice along the walls of the tank and it is sensitive to adrenergic drugs.

In a separate experiment, the effects of the extract (10 - 100 mg  $\text{kg}^{-1}$ , *p.o.*) and fluoxetine (3 -30 mg  $\text{kg}^{-1}$ , *p.o.*) on 5-hydroxytryptamine (5-HTP) (200 mg  $\text{kg}^{-1}$ ) induced head twitch response were studied. Seven groups of mice ( $n= 6$ ) were used. The first 3 groups were given the extract, the next three fluoxetine and the last group received vehicle and after 1 h or 30 min for the drug treated groups or vehicle treated respectively, 5-HTP was administered and the number of head twitches every 5 min were counted for 30 min and also the total number of head twitches for the 30 min period.

### 5.2.5.3 Involvement of glycine/NMDA neurotransmission

Mice were divided into 2 groups, A and B. Groups A and B were further subdivided into 6 groups each ( $n=8$ ). Shortly five groups of mice from group A received D-cycloserine (2.5 mg  $\text{kg}^{-1}$ , i.p.) and 30 min after the first three groups received an oral dose of the extract (10- 100 mg  $\text{kg}^{-1}$ ) with the last two groups receiving either fluoxetine (10 mg  $\text{kg}^{-1}$ ) or desipramine (10 mg  $\text{kg}^{-1}$ , i.p.). The sixth group received only D-cycloserine. Five groups of mice from group B received D-serine (600 mg  $\text{kg}^{-1}$ ) and 30 min after the first three groups received an oral dose of the extract (10 - 100 mg  $\text{kg}^{-1}$ ) with the last two groups receiving either fluoxetine (10 mg  $\text{kg}^{-1}$ , *p.o.*) or desipramine (10 mg  $\text{kg}^{-1}$ , i.p.). The sixth group from group B received only D-serine. The forced swim and tail suspension tests were used as described above to investigate the antidepressant mechanism. The rotarod test was used to assess whether there was any locomotor impairment after extract, D-serine or D-cycloserine administration. Pedalling behaviour was defined as the continuous movement of

the paw of the mice without moving the body while curling was defined as the raising of the head of the mouse towards its hind paws.

### 5.2.6 Statistics

GraphPad Prism for Windows version 4.03 (GraphPad Software, San Diego, CA, USA) was used for all data and statistical analyses.  $P < 0.05$  was considered statistically significant. In all the tests, a sample size of ten animals ( $n=10$ ) were used. The time-course curves were subjected to two-way (treatment  $\times$  time) repeated measures analysis of variance (ANOVA) with Bonferroni's post hoc test. Total immobility time, distance travelled, and time taken to find the hidden platform and change in weight for each treatment was calculated in arbitrary unit as the area under the curve (AUC). Differences in AUCs were analysed by ANOVA followed by Student-Newman-Keuls' post hoc 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:

$$Y = \frac{a + (b - a)}{1 + 10^{(Log ED_{50} - X)}}$$

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 and Tail suspension tests

MOE ( $10 - 100 \text{ mg kg}^{-1}$ , *p.o.*), administered 60 min before the test period, significantly decreased the frequency of immobility ( $F_{3,19}=21.47$ ,  $P < 0.001$ ) (Figure 5.1a) and immobility periods of mice ( $F_{3,19}=143.4$ ,  $P < 0.001$ ) (Figure 5.1d) in a dose dependent manner in the FST. In the TST both frequency ( $F_{6,54}=0.486$ ,  $P=0.8159$ ) (Figure 5.3a, b, c), and duration ( $F_{6,52}=25.57$ ,  $P < 0.001$ ) (Figure 5.3d, e, f) of immobility decreased, indicating significant antidepressant activity. In both TST and FST, the order of antidepressant efficacy calculated from the dose response curves (Figure 5.2 and Figure 5.4), with regards to duration of immobility was imipramine > fluoxetine > MOE. From the  $ED_{50}$  values calculated, MOE was more potent in reducing duration of immobility than fluoxetine and imipramine in the TST but was the least potent in the FST (Table 5.1)

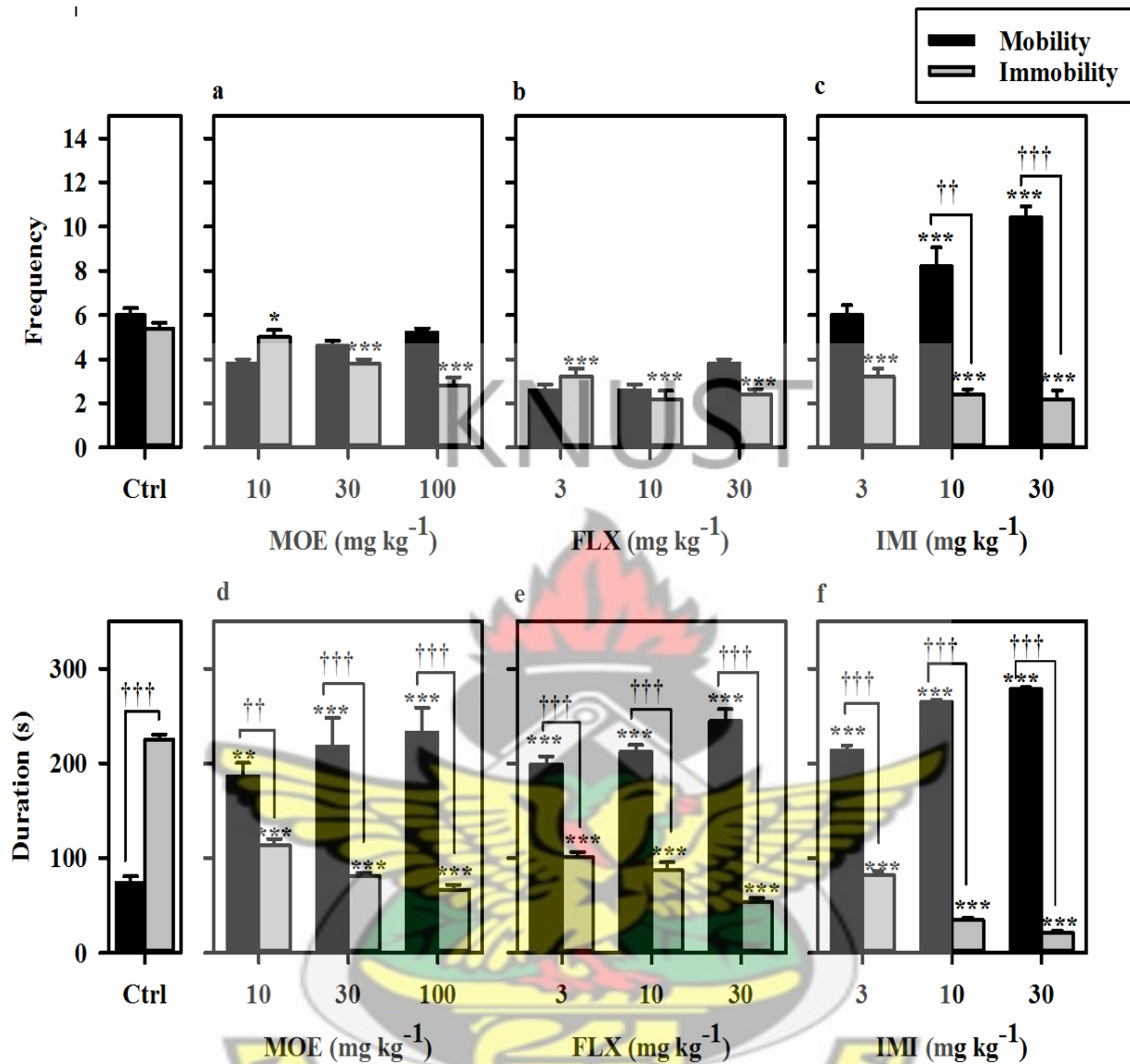


Figure 5.1 Effects of extract, MOE (10 - 100 mg kg<sup>-1</sup>), fluoxetine, FLX (3 - 30 mg kg<sup>-1</sup>) and imipramine, IMI (3 - 30 mg kg<sup>-1</sup>) treatment on (a, b, c) the frequency of mobility and immobility and (d, e, f) duration of mobility and immobility in the FST. Data are presented as group Means  $\pm$  SEM. Significantly different from control: \*\*\* $P$ <0.001; \*\* $P$ <0.01; (One-way ANOVA followed by Newman Keuls' test). ††† $P$ <0.001, comparison between effect and dose (Two-way ANOVA followed by Bonferroni's test).



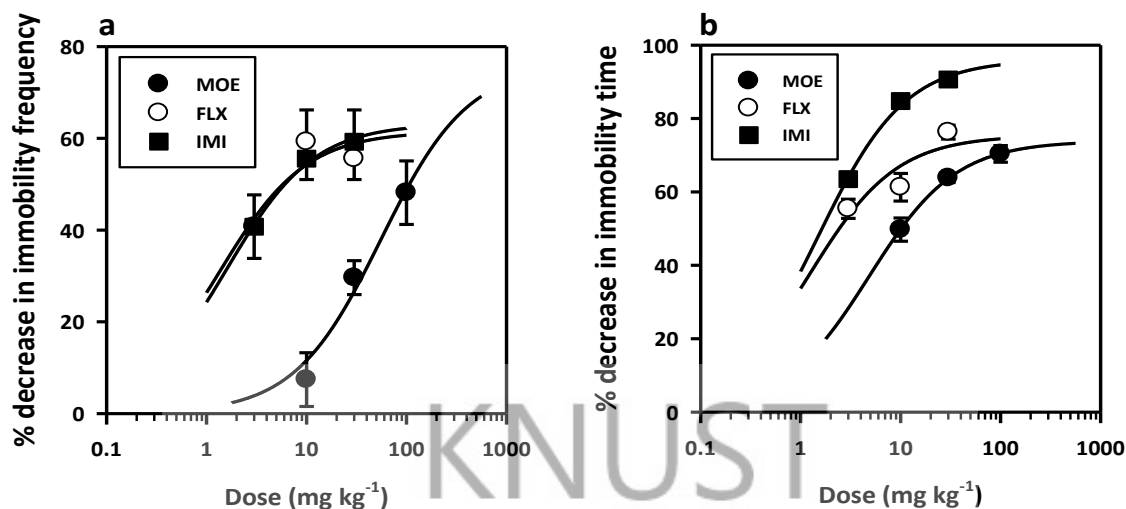


Figure 5.2 Dose–response curves of the extract, MOE (10 - 100 mg kg<sup>-1</sup>), fluoxetine, FLX (3 - 30 mg kg<sup>-1</sup>) and imipramine, IMI (3 - 30 mg kg<sup>-1</sup>) showing (a) % decrease in frequency and (b) % decrease in immobility in the forced swimming test in mice. Each point is the mean  $\pm$  S.E.M. of 5 animals.

Table 5.1 ED<sub>50</sub> and E<sub>max</sub> values of drugs used in the forced swim (FST) and tail suspension tests (TST)

Test		Frequency		Duration (s)	
		ED <sub>50</sub>	E <sub>max</sub>	ED <sub>50</sub>	E <sub>max</sub>
FST	MOE	55.7±13.6	76.0	4.8±1.1	73.9
	FLX	1.3±1.3	61.5	1.2±0.5	75.4
	IMI	1.6±1.3	63.2	1.5±0.1	96.1
TST	MOE	82.6±13.3	40.0	1.2±1.0	59.1
	FLX	7.2±6.9	39.8	3.1±1.7	82.1
	IMI	32.3±9.2	100	1.7±0.9	85.8

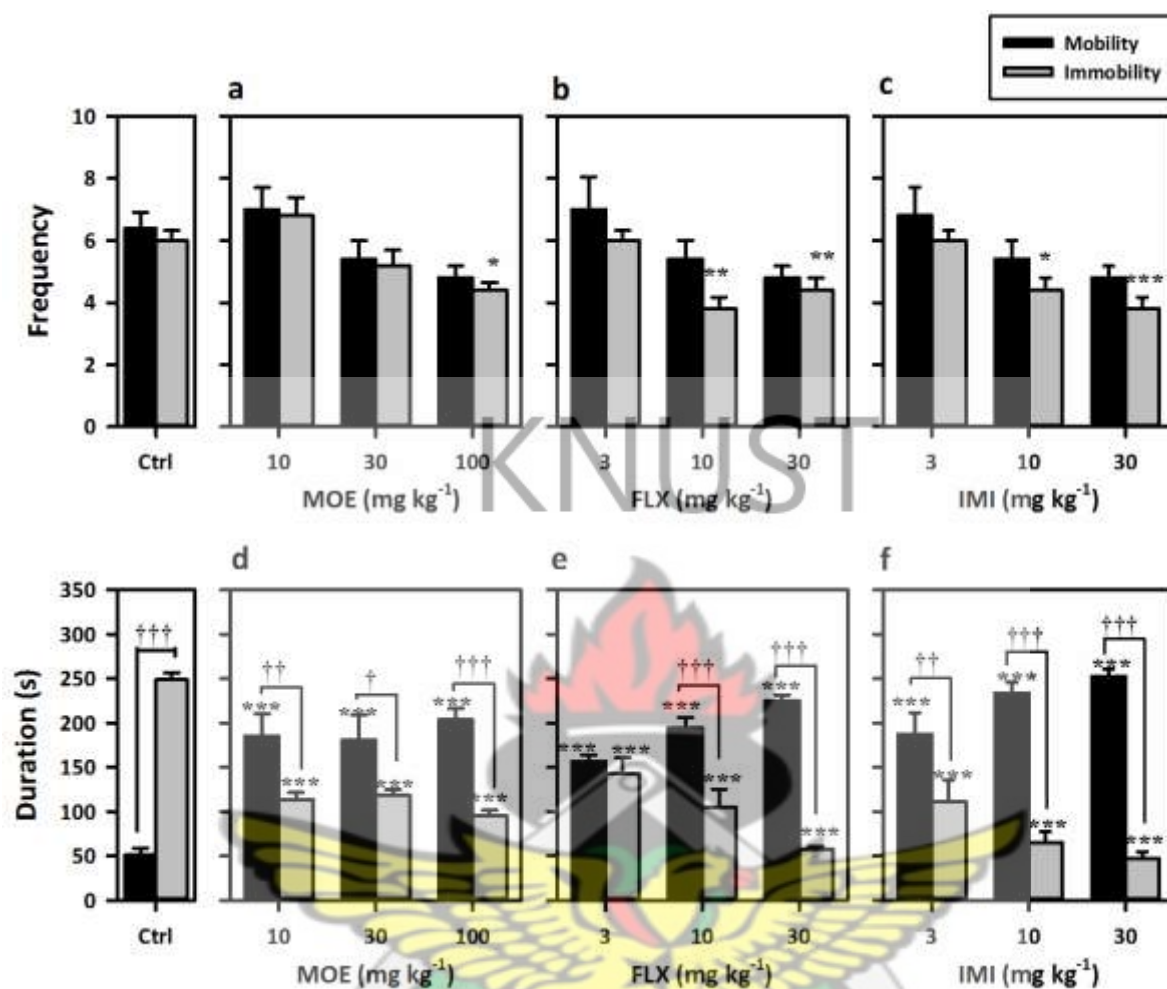


Figure 5.3 Effect of the extract, MOE (10, 30 and 100 mg kg<sup>-1</sup>), fluoxetine, FLX (3, 10 and 30 mg kg<sup>-1</sup>) and imipramine, IMI (3, 10 and 30 mg kg<sup>-1</sup>) treatment on the (a, b, c) frequency of mobility and immobility; and (d, e, f) duration of mobility and immobility in the TST. Data are presented as group Means  $\pm$  SEM. \*\*\* $P$ <0.001; \*\* $P$ <0.01; compared to vehicle-treated group (One-way ANOVA followed by Newman Keuls' test). ††† $P$ <0.001, comparison effect and dose (Two-way ANOVA followed by Bonferroni's test).

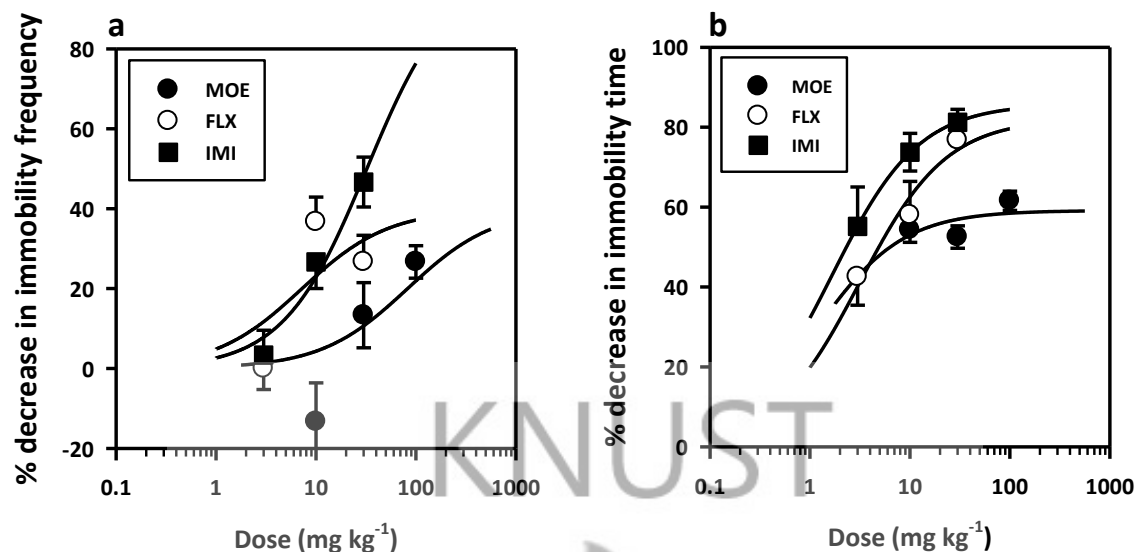


Figure 5.4 Dose–response curves of the extract, MOE (10 - 100 mg kg<sup>-1</sup>), fluoxetine, FLX (3 - 30 mg kg<sup>-1</sup>) and imipramine, IMI (3 - 30 mg kg<sup>-1</sup>) showing (a) % decrease in frequency and (b) % decrease in immobility in the tail suspension test in mice. Each point is the mean  $\pm$  S.E.M. of 5 animals.

### 5.3.2 The open space swim test

Extract showed significant antidepressant effect in mice exposed to the open space swim test. From the time course curve, decrease in immobility by extract was observed after first day of treatment and this was sustained till the fourteenth day of treatment ( $F_{3,216}=34.48$ ,  $P<0.0001$ ) (Figure 5.5a). In contrast MOE increased distance travelled by mice ( $F_{3,216}=4.05$ ,  $P<0.0001$ ) (Figure 5.6a). The graph showing the area under the curve for the extract treated group also showed a dose-dependent decrease in immobility period ( $F_{3,36}=369.8$ ,  $P<0.0001$ ) (Figure 5.5b). One way ANOVA showed that distance travelled was significantly increased in extract-treated groups at all dose levels ( $F_{3,36}=28.42$ ,  $P<0.0001$ ) (Figure 5.6b). Both imipramine (Figure 5.5c, d) and fluoxetine (Figure 5.5e, f), the standard antidepressant used, showed a decrease in immobility time after day 7 of treatment but similar effect as extract with regards to distance travelled (Figure 5.6c - f). Though fluoxetine and imipramine were more potent than the extract, the extract was more efficacious in reducing immobility time which is the more reliable index for mice. With regards to distance travelled MOE was less efficacious and potent than fluoxetine and imipramine. See (Figure 5.7 and Table 5.2).

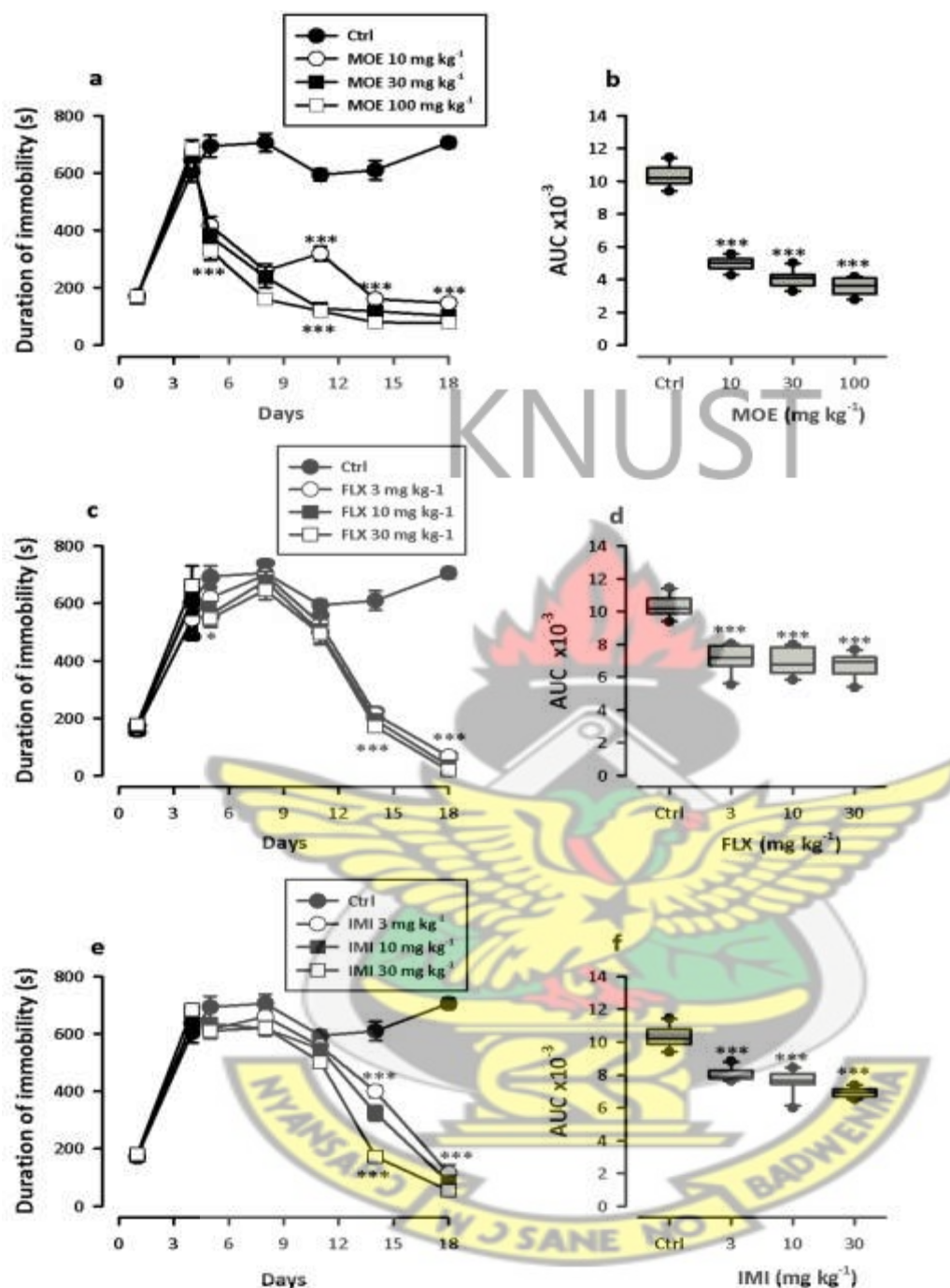


Figure 5.5 Effects of extract, MOE (10 - 100 mg kg<sup>-1</sup>), fluoxetine, FLX (3 - 30 mg kg<sup>-1</sup>) and imipramine, IMI (3 - 30 mg kg<sup>-1</sup>) treatment on the duration of immobility in the open space swim test. Data are presented as both (a, c, e) a time course curve and the (b, d, f) Mean  $\pm$  SEM of their areas under the curves (AUCs). Significantly different from control: \*P<0.05, \*\*\*P<0.001 by Newman Keuls' test.



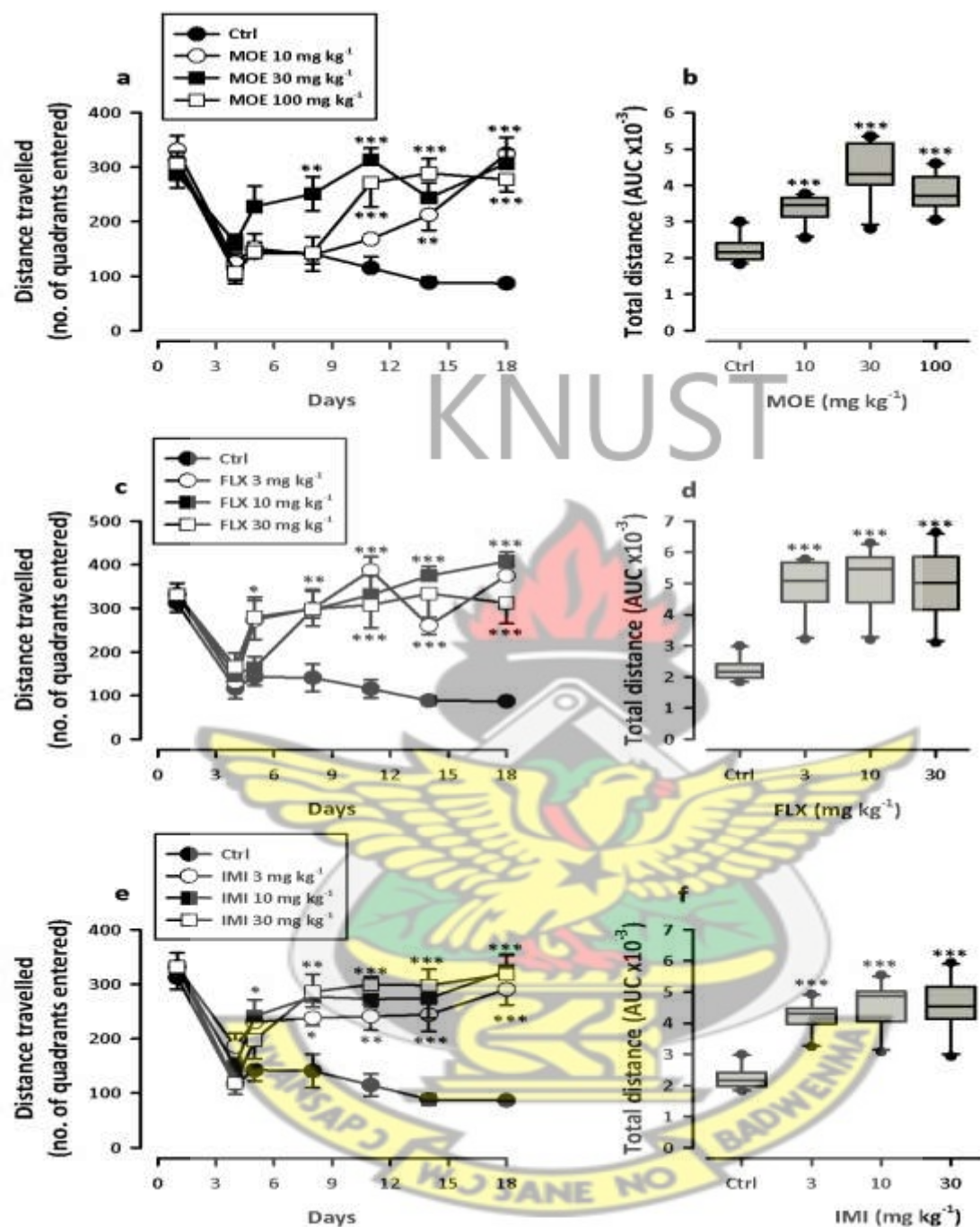


Figure 5.6 Effects of extract, MOE (10 - 100 mg kg<sup>-1</sup>), fluoxetine, FLX (3 - 30 mg kg<sup>-1</sup>) and imipramine, IMI (3 - 30 mg kg<sup>-1</sup>) treatment on the distance travelled in the open space swim test. Data are presented as both (a, c, e) a time course curve and the (b, d, f) Mean  $\pm$  SEM of their areas under the curves (AUCs). 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. The horizontal line within the box is the median. Significantly different from control: \* $P$ <0.05, \*\* $P$ <0.001, \*\*\* $P$ <0.001 by Newman Keuls' test.

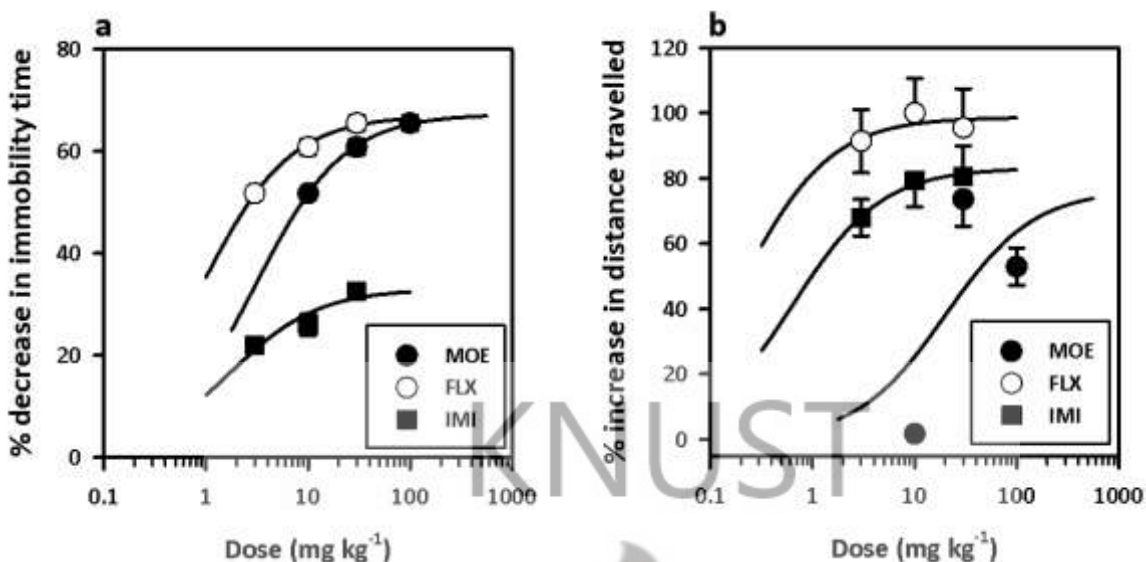


Figure 5.7 Dose–response curves showing the effect of extract, MOE (10 - 100 mg kg<sup>-1</sup>), fluoxetine, FLX (3 - 30 mg kg<sup>-1</sup>) and imipramine, IMI (3 - 30 mg kg<sup>-1</sup>) on (a) % decrease in immobility time and (b) % increase in distance travelled in the open space swim test in mice. Each point is the mean  $\pm$  S.E.M. of 10 animals.

Table 5.2 ED<sub>50</sub> and E<sub>max</sub> values of drugs used in the open space swim test.

Parameters	MOE		FLX		IMI	
	ED <sub>50</sub>	E <sub>max</sub>	ED <sub>50</sub>	E <sub>max</sub>	ED <sub>50</sub>	E <sub>max</sub>
Immobility time	3.0 $\pm$ 0.6	67.3	0.9 $\pm$ 0.2	67.0	1.7 $\pm$ 0.6	32.9
Distance travelled	20.2 $\pm$ 6.0	76.5	0.2 $\pm$ 2.7	98.7	0.7 $\pm$ 0.2	83.1

### 5.3.3 Spatial memory and learning in the Morris water maze task.

Extract improved spatial memory and learning task in mice exposed to open space swim test. From the time course curve, the extract significantly reduced latency for the mice to find the hidden platform from the third day ( $F_{4,180}=23.41$ ,  $P<0.0001$ ) (Figure 5.8a). Two Way ANOVA revealed that treatment had effect on the behavioural response ( $F_{4,45}=3.37$ ,  $P<0.0169$ ). The graph showing the area under the curve (AUC) revealed that the extract dose dependently reduced latency to find hidden platform ( $F_{4,45}=3.139$ ,  $P<0.0233$ ) (Figure 5.8b). Spatial memory and learning was improved in naïve mice that were not exposed to the open space swim test. Similar results as the extract were obtained for fluoxetine. In contrast imipramine did not show any memory

improvement. See (Figure 5.8c - f). Fluoxetine was the most efficacious in improving memory, followed by the extract then imipramine (Figure 5.9).

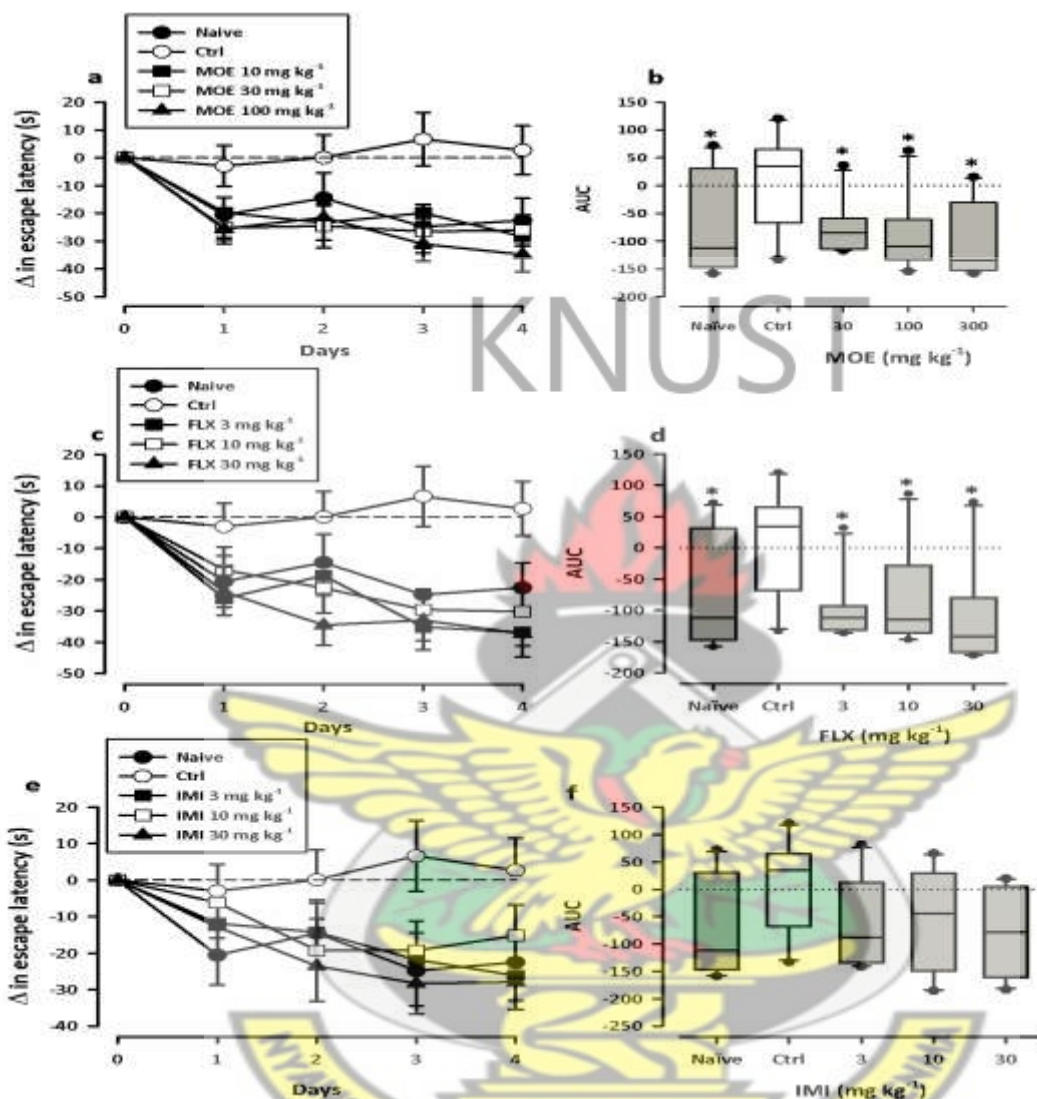


Figure 5.8 Effects of MOE (10 - 100 mg kg<sup>-1</sup>), fluoxetine, FLX (3 - 30 mg kg<sup>-1</sup>) and imipramine, IMI (3 - 30 mg kg<sup>-1</sup>) treatment on the spatial learning and memory in the Morris water maze test. Data are presented as both (a, c, e) a time course curve and (b, d, f) the Mean  $\pm$  SEM of their areas under the curve (AUCs). 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. The horizontal line within the box is the median. Significantly different from control: \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001 by Newman Keuls' test.

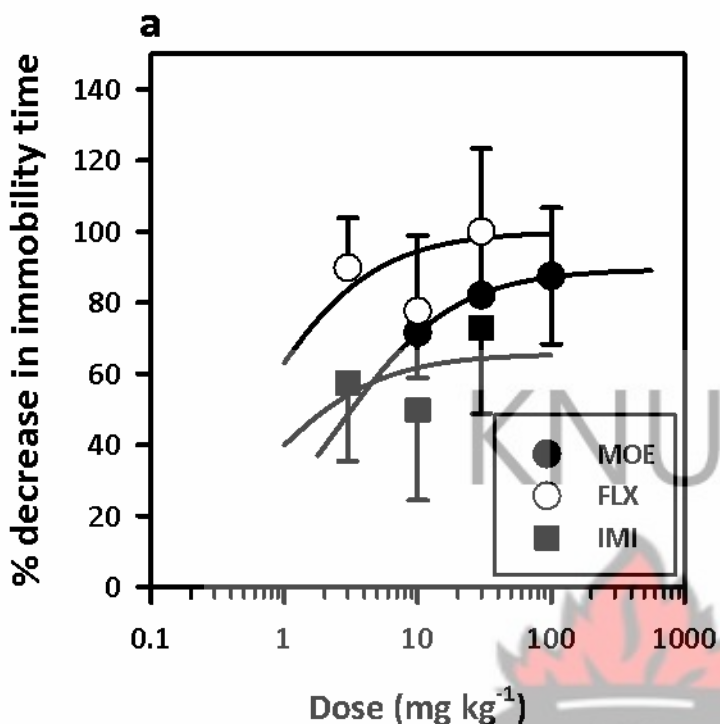


Figure 5.9 Dose–response curves showing the effect of MOE (10 - 100 mg kg<sup>-1</sup>), fluoxetine (3 - 30 mg kg<sup>-1</sup>) and imipramine (3 - 30 mg kg<sup>-1</sup>) on (a) % decrease in the escape latency in the Morris water maze test in mice. Each point is the mean  $\pm$  S.E.M. of 10 animals.

Table 5.3 ED<sub>50</sub> and E<sub>max</sub> values of drugs used in the Morris water maze test.

Drug	Escape Latency	
	ED <sub>50</sub>	E <sub>max</sub>
MOE	2.5 $\pm$ 1.1	89.4
FLX	0.6 $\pm$ 0.3	100
IMI	0.7 $\pm$ 0.4	65.7

#### 5.3.4 Weight variation

Extract did not have any significant effect on the weight of mice during induction of chronic depression and after the 2 weeks period of treatment (Figure 5.10a, b). Imipramine significantly reduced the weight of the mice at all dose levels whilst the lowest dose of fluoxetine significantly reduced the weight of the mice after the 2 week period (Figure 5.10c - f).



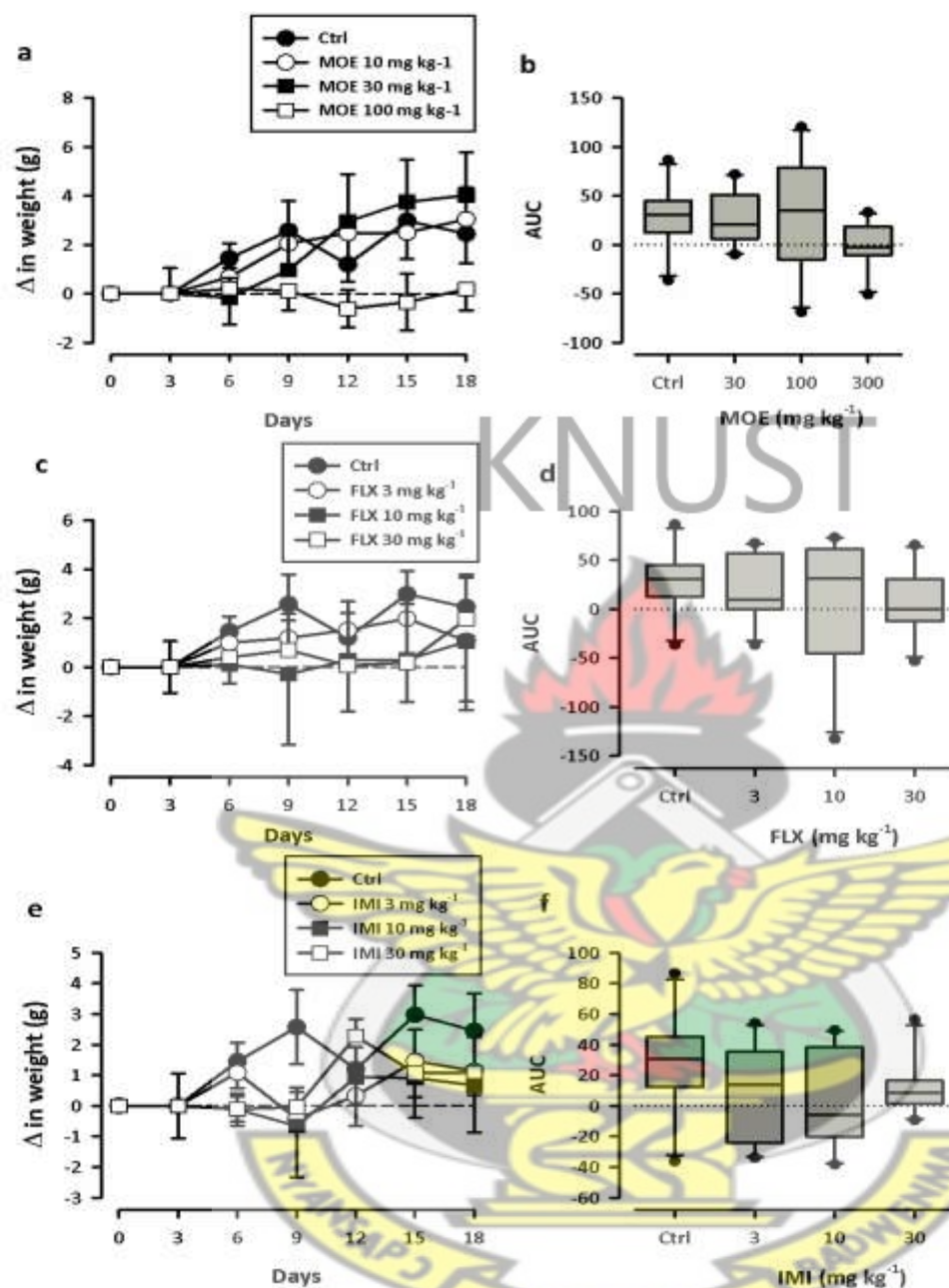


Figure 5.10 Effect of extract, MOE (10 - 100 mg kg<sup>-1</sup>), fluoxetine, FLX (3 - 30 mg kg<sup>-1</sup>) and imipramine, IMI (3 - 30 mg kg<sup>-1</sup>) treatment on weight changes. Data are presented as both (a, c, e) a time course curve and (b, d, f) the Mean  $\pm$  SEM of their AUCs (n= 10). 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. The horizontal line within the box is the median.

### 5.3.5 Tail suspension test (TST).

The extract maintained a significant antidepressant effect 24 hours after treatment day fourteen in the TST. It demonstrated a dose dependent decrease in both the absolute immobility period and %

immobility period at all dose levels (Figure 5.11a, d). Similar results were obtained for fluoxetine and imipramine (Figure 5.11b, c, e, and f).

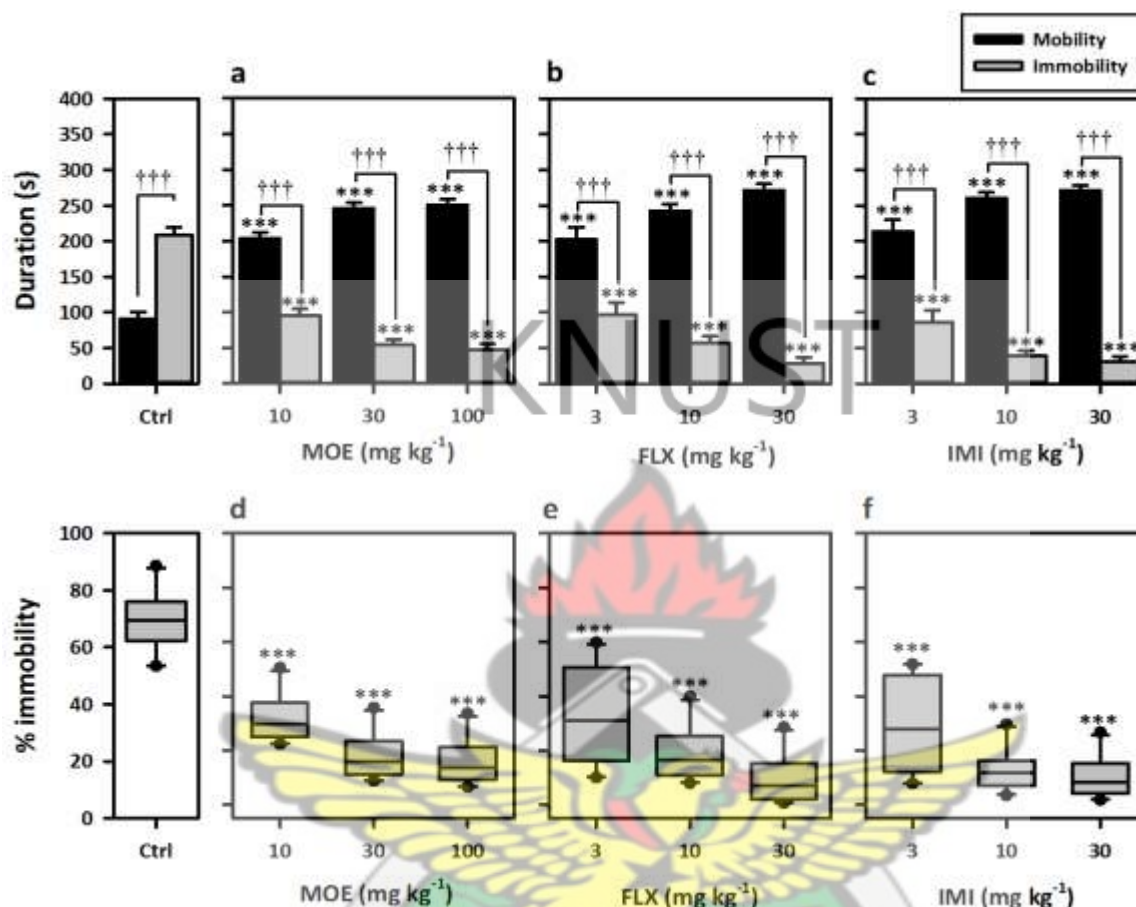


Figure 5.11 Effect of (a) the extract, MOE (10 - 100 mg kg<sup>-1</sup>), (b) fluoxetine, FLX (3 - 30 mg kg<sup>-1</sup>) and (c) imipramine, IMI (3 - 30 mg kg<sup>-1</sup>) treatment on duration of mobility and immobility. (d - f) % Immobility of the extract, MOE, fluoxetine, FLX and imipramine, IMI treatment respectively. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; compared to vehicle-treated group; (One-way ANOVA followed by Newman Keuls' test). ††† $P < 0.001$  comparison between immobility and mobility (Two-way ANOVA followed by Bonferroni's test). Data are presented as group Means  $\pm$  SEM of 10 animals.

### 5.3.6 Involvement of noradrenergic mechanisms

Pretreatment with either reserpine (1 mg kg<sup>-1</sup>, s.c.) alone,  $\alpha$ -methyldopa (400 mg kg<sup>-1</sup>, *p.o.*) alone or a concomitant administration of reserpine (1 mg kg<sup>-1</sup>, s.c.) and  $\alpha$ -methyldopa (200 mg kg<sup>-1</sup>, *p.o.*) did not reverse the decrease in immobility caused by the extract, MOE (10 -100 mg kg<sup>-1</sup>, *p.o.*) in the forced swim test (FST) (Figure 5.12a, d, g). Results obtained for fluoxetine (FLX) treated groups (3 - 30 mg kg<sup>-1</sup>, *p.o.*) were similar to that of the extract treated groups (Figure 5.12b, e, h). In contrast, the antidepressant effect of imipramine (IMI) was reversed by either reserpine alone,  $\alpha$ -methyldopa alone or a concomitant administration of reserpine and  $\alpha$ -methyldopa (Figure 5.12c, f, i).

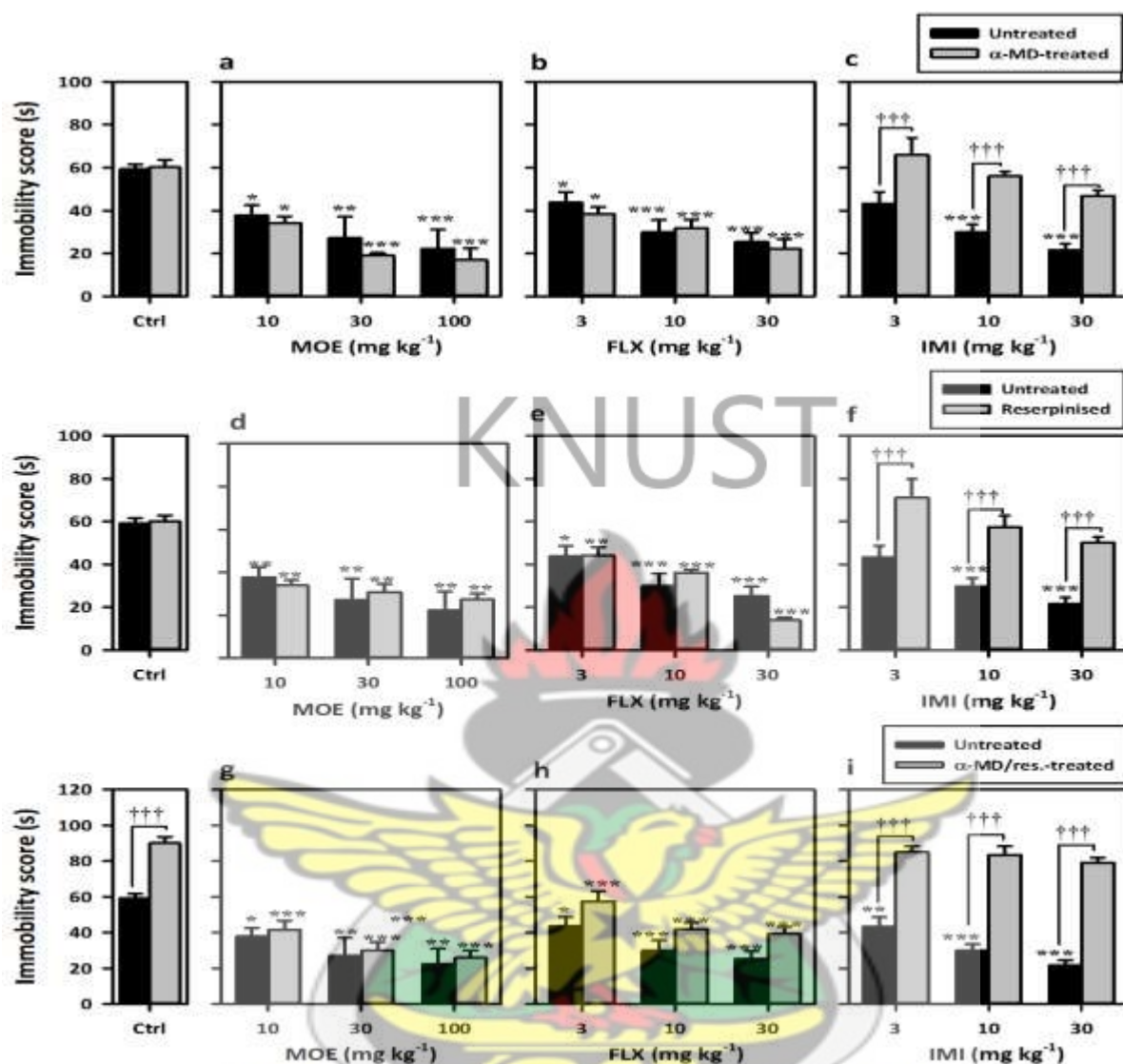


Figure 5.12 Effects of (a - c) reserpine alone; (d - f)  $\alpha$ -methyl dopa,  $\alpha$ MD alone or (g - i) both reserpine and  $\alpha$ -methyl dopa, on duration of immobility of MOE (10 - 100 mg kg<sup>-1</sup>, *p.o.*), fluoxetine (3 - 30 mg kg<sup>-1</sup>, *p.o.*) and imipramine (3 - 30 mg kg<sup>-1</sup>, *p.o.*), treatment in the FST. Data are presented as Mean  $\pm$  SEM of 5 animals. Significantly different from control: \*\**P*<0.01, \*\*\**P*<0.001 by One Way ANOVA followed by Newman Keuls' test. †*P*<0.05, ††*P*<0.01, †††*P*<0.001; Significant difference between treatment and dose (Two Way ANOVA with Bonferonni *post hoc* test).

### 5.3.7 Involvement of serotonergic mechanism

Pretreatment of mice with *p*CPA (200 mg kg<sup>-1</sup>) abolished the antidepressant effect of MOE (10 - 100 mg kg<sup>-1</sup>, *p.o.*) and fluoxetine, FLX (3 - 30 mg kg<sup>-1</sup>, *p.o.*) but not imipramine, IMI (3-30 mg kg<sup>-1</sup>, *p.o.*) in the FST. The mean counts for immobility ( $F_{7,32}=14.63$ ;  $P<0.0001$ ) (Figure 5.13a), swimming ( $F_{7,32}=44.74$ ;  $P<0.0001$ ) (Figure 5.13d) and climbing ( $F_{7,32}=1.121$ ;  $P=0.3742$ ) (Figure 5.13g) in the extract treated group after *p*CPA treatment did not show any difference when

compared with the control. Similar results as above were observed for FLX-treated groups but not imipramine (Figure 5.13b - c, e - f, h - i). In an attempt to investigate the possible involvement of 5-HT<sub>2A</sub> receptor activation in the antidepressant action of the extract, mice were given 5-hydroxytryptophan after extract pretreatment to induced head twitch responses. It was observed from the time course curve that the extract as well as fluoxetine increased the head twitch responses significantly for the period of 30 minutes (Figure 5.14a, c). Response peaked after 15 minutes. One-way ANOVA followed by Newman Keuls' test of the areas under the curve (AUCs) showed a dose dependent increase in the head twitch response for both extract and fluoxetine (Figure 5.14b, d). From the dose response curve, fluoxetine ( $ED_{50}=6.472\pm0.27$ ) was more potent in activating 5-HT<sub>2A</sub> receptors than MOE ( $ED_{50}=41.30\pm4.51$ ) though their efficacies were comparable (Figure 5.15).





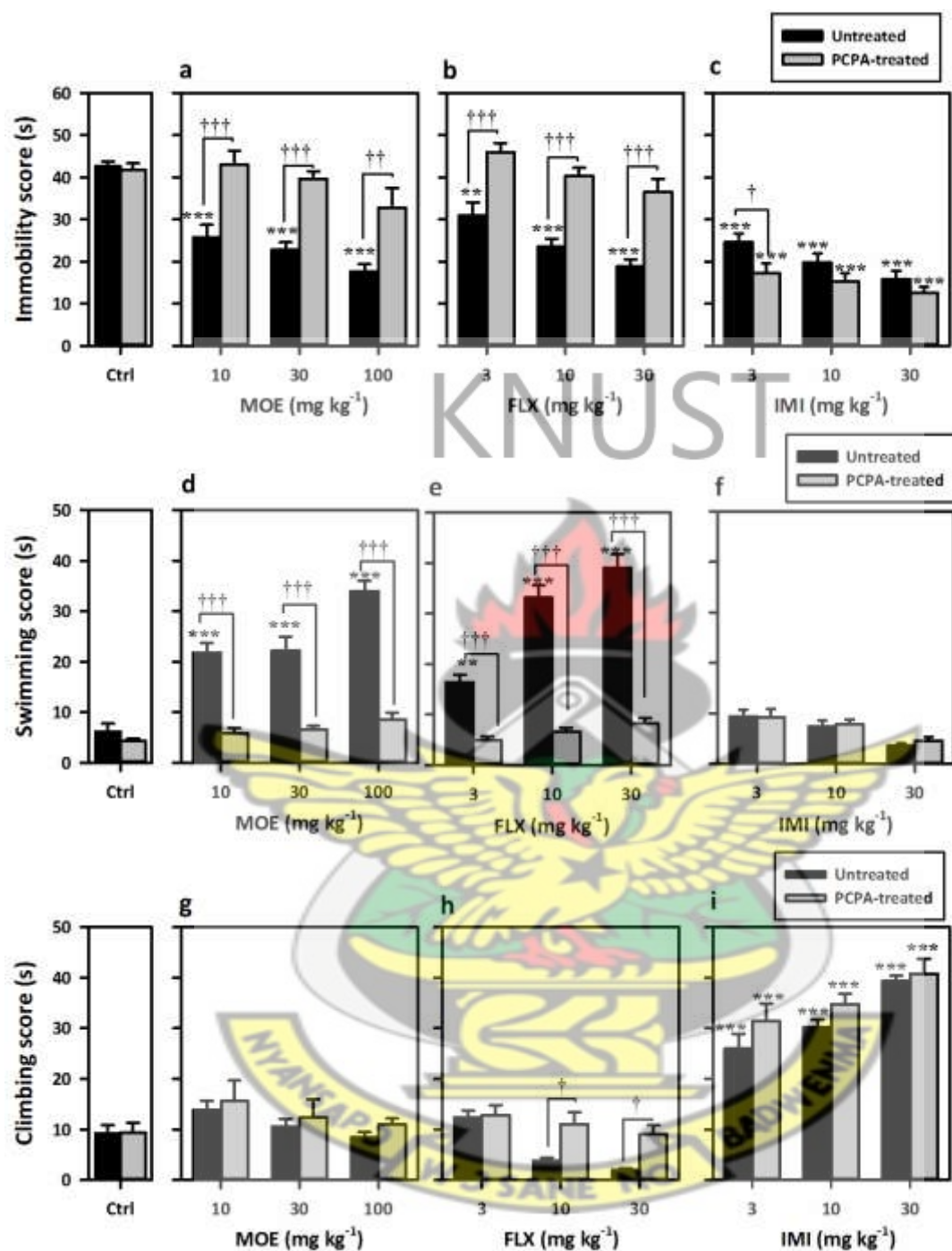


Figure 5.13 Effects of *p*CPA (200 mg kg<sup>-1</sup>) pretreatment on the (a - c) mean immobility counts; (d - f) swimming counts and (g - i) climbing counts of oral doses of extract, MOE (10 - 100 mg kg<sup>-1</sup>), fluoxetine, FLX (3 - 30 mg kg<sup>-1</sup>) and IMI (3 - 30 mg kg<sup>-1</sup>) treated groups in the FST. Data are presented as mean  $\pm$  SEM. Significantly different from control: \*\* $P$ <0.01, \*\*\* $P$ <0.001 by Newman Keuls' test. ††† $P$ <0.001: (Two-way ANOVA followed by Bonferroni's post; comparison between drug treatment and dos

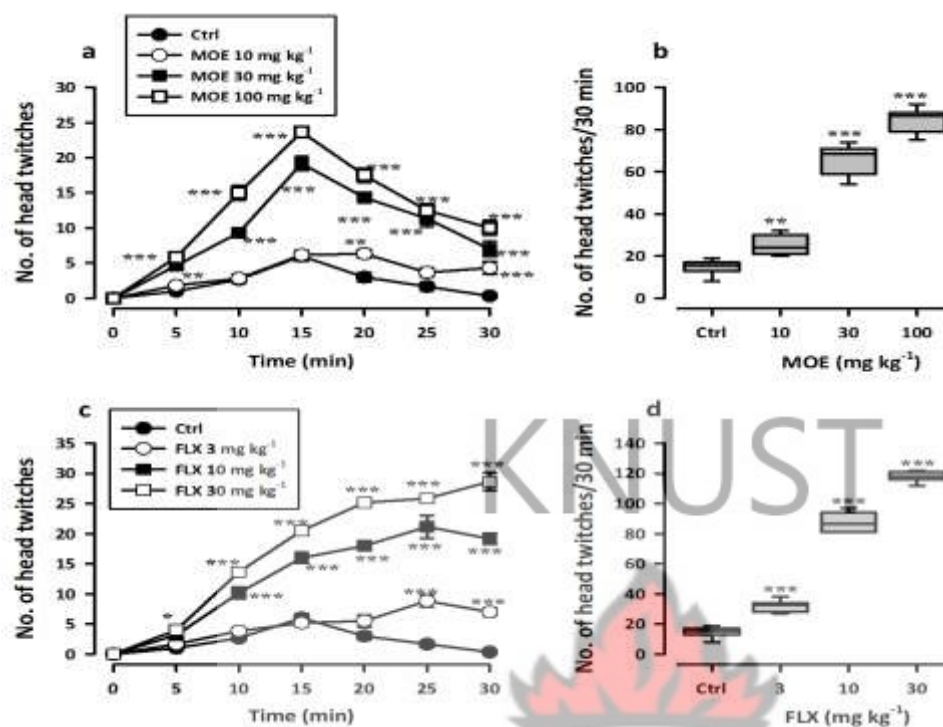


Figure 5.14 Effect of MOE (10 - 100 mg kg<sup>-1</sup>, *p.o.*) and fluoxetine (3 - 30 mg kg<sup>-1</sup>, *p.o.*) on the time course curve of head twitch response test (a and c) and their corresponding AUCs (b and d) in the same test represented as box plots. Data was presented as mean  $\pm$  S.E.M. (n=6); \*\*\**P*<0.001; \*\**P*<0.01; compared to vehicle-treated group (One-way ANOVA followed by Newman Keuls' test). †††*P*<0.001: (Two-way ANOVA followed by Bonferroni's test; comparison between drug treatment and dose). 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.

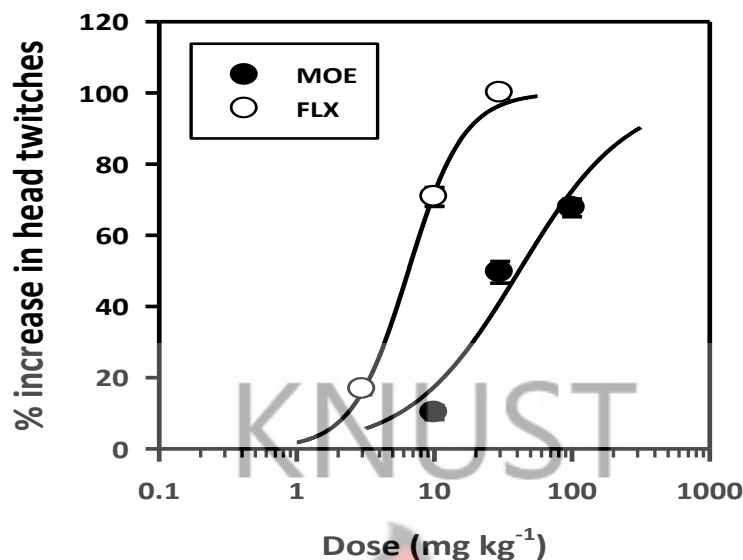


Figure 5.15 Dose–response curves of % increase in head twitches by oral dose of the extract, MOE (10 - 100 mg kg<sup>-1</sup>) and fluoxetine, FLX (3 - 30 mg kg<sup>-1</sup>) in response to 5-hydroxytryptophan (5-HTP) administration in mice. Each point is the mean  $\pm$  S.E.M. of 6 animals.

### 5.3.8 Involvement of glycine/NMDA receptor complex

In the tail suspension test, MOE (100 mg kg<sup>-1</sup>, p.o.), fluoxetine, FLX (10 mg kg<sup>-1</sup>, p.o.) and desipramine, DSP (10 mg kg<sup>-1</sup>, i.p.), exhibited significant antidepressant effect by decreasing mean immobility score which was reversed by D-serine, DS, (600 mg kg<sup>-1</sup>, i.p.) pretreatment (Figure 5.16a). Pretreatment with D-cycloserine, DCS, (2.5 mg kg<sup>-1</sup>, i.p.) potentiated the effect of MOE and FLX (but not DSP) by further decreasing mean immobility score (Figure 5.16b). MOE alone did not affect curling score and this was not changed by DS pretreatment (Figure 5.16e). FLX and DSP alone, caused slight increase in the curling score which was reversed by DS. Pretreatment with DCS significantly increased curling score of MOE but caused only a modest increase in both FLX and DSP treated groups (Figure 5.16f). MOE, DS, DCS, FLX and DSP alone increased swinging score. DS pretreatment partially inhibited swinging behaviour by MOE but totally in FLX and DSP treated groups (Figure 5.16c). DCS pretreatment also inhibited swinging behaviour by MOE and DSP but not FLX (Figure 5.16d). In the forced swim test, MOE, FLX and DSP, decreased immobility score and this was reversed by DS pretreatment (Figure 5.17a). Pretreatment with DCS potentiated the effect of MOE and FLX (but not DSP) by further decreasing immobility score (Figure 5.17b). MOE and FLX unlike DSP, increased swimming behaviour which was inhibited by DS but increased by DCS pretreatment (Figure 5.17c and d). Climbing scores were decreased by both MOE and FLX and this was not affected by both DS and DCS pretreatment (Figure 5.17e and f). DSP on the contrary, increased climbing score which was unaffected by DCS pretreatment but decreased by DS.

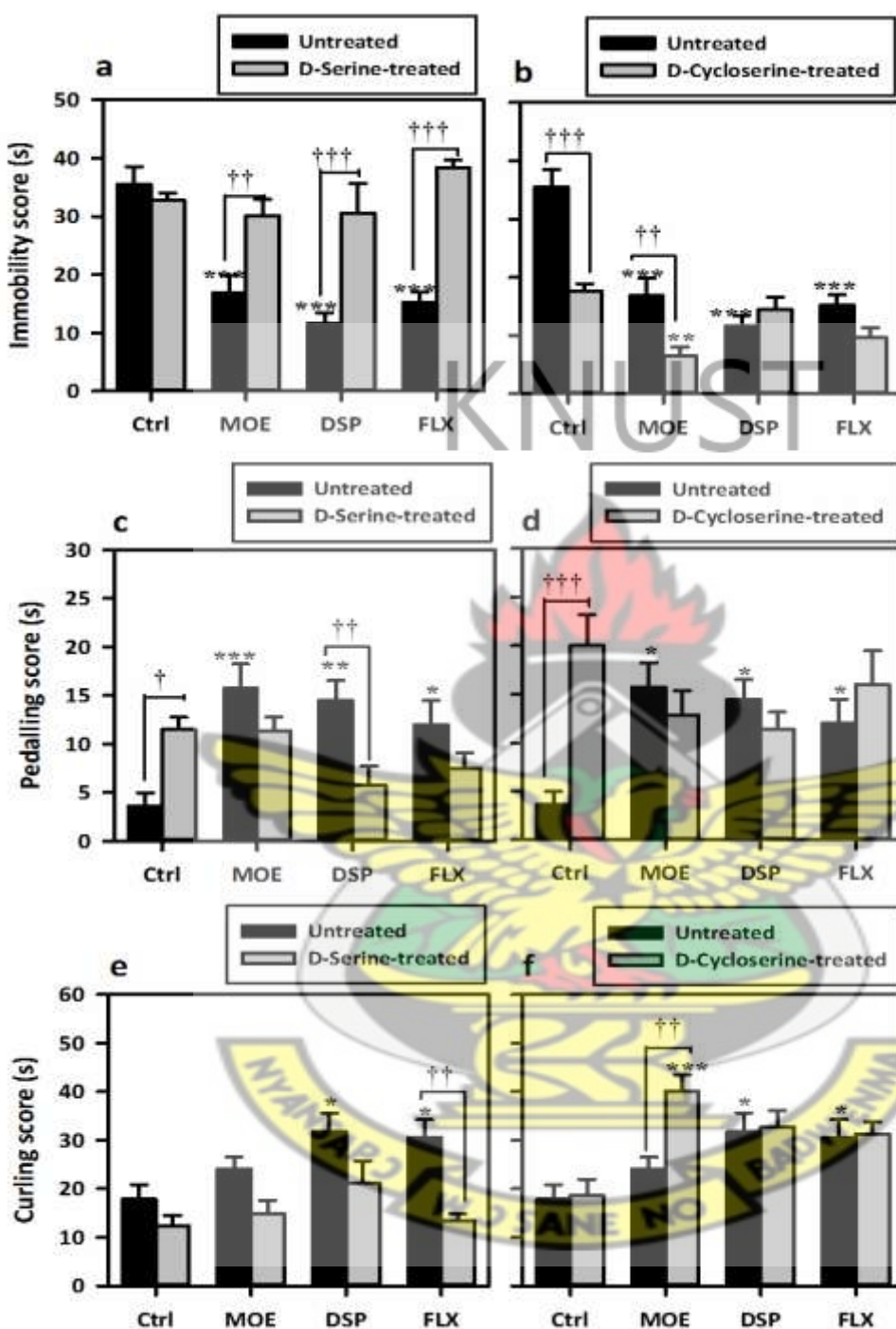


Figure 5.16 Effects of D-serine (DS) or D-cycloserine (DCS) pretreatment on (a, b) mean immobility count, (c, d) pedalling count and (e, f) curling count of extract, MOE ( $100 \text{ mg kg}^{-1}$ ), fluoxetine, FLX ( $10 \text{ mg kg}^{-1}$ ) and desipramine, DSP ( $10 \text{ mg kg}^{-1}$ ) treatment in the tail suspension test (TST). Data are presented as Means  $\pm$  SEM. Significantly different from control: \*\* $P < 0.01$ , \*\*\* $P < 0.001$  by Newman Keul's test. Significant difference between treatments: † $P < 0.05$ , †† $P < 0.01$ , ††† $P < 0.001$ .



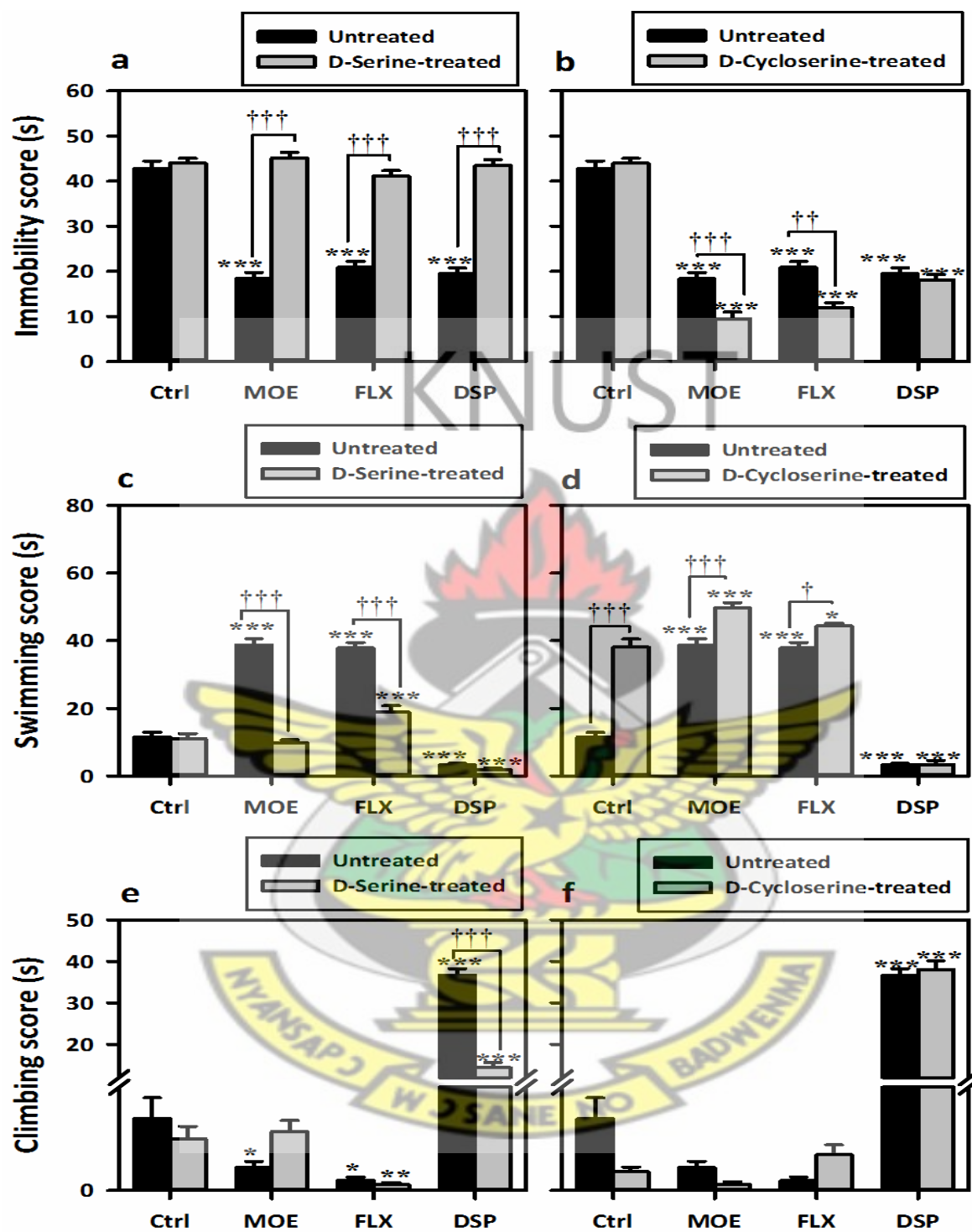


Figure 5.17 Effects of D-serine (DS) or D-cycloserine (DCS) pretreatment on (a, b) mean immobility count, (c, d) swimming count and (e, f) climbing count of extract, MOE (100 mg kg<sup>-1</sup>), fluoxetine, FLX (10 mg kg<sup>-1</sup>) and desipramine, DSP (10 mg kg<sup>-1</sup>) treatment in the forced swimming test (FST). Data are presented as Means  $\pm$  SEM. Significantly different from control: \*\* $P$ <0.01, \*\*\* $P$ <0.001 by Newman Keul's test. Significant difference between treatments: † $P$ <0.05, †† $P$ <0.01, ††† $P$ <0.001.

## 5.4 DISCUSSION

Depressed patients often have suicidal tendencies and the delay in improvement of symptomatology as observed with most antidepressants may increase mortality if medications with faster onset and sustained effect are not sought (Lucas, 2008). The goal of the present study was to ascertain first of all whether the hydroalcoholic leaf extract of *Mallotus oppositifolius* has antidepressant action and to further assess the contributions of monoamine neurotransmitters, to the behavioural effects of MOE. The possible interaction of MOE with the glycine coagonist site of NMDA receptors as a means of investigating possible effect on glutamatergic neurotransmission was also evaluated.

The results of the study demonstrated that MOE has significant antidepressant effect in both the forced swimming and the tail suspension tests. In both animal models, percentage immobility and frequency of immobility were decreased by extract treatment. Reduction in immobility has been used as the primary index for antidepressant effect of test substances in these models—almost all antidepressant in clinical use induce a decrease in immobility in rodents whilst other drugs fail to give the same response (Cryan *et al.*, 2005; Petit-Demouliere *et al.*, 2005). MOE exhibited a rapid and sustained antidepressant effect in the chronically depressed mice. The extract reduced immobility significantly and increased distanced travelled, the primary indices of antidepressant effect, in the open space swim test (Sun and Alkon, 2003; Stone and Lin, 2011).

Preclinical and clinical studies suggest that depletion of a monoamine implicated in depression pathophysiology may abolish the antidepressant effect of a substance if the substance depends on that particular monoamine for its antidepressant effect (Delgado *et al.*, 1991; 1999; O'Leary *et al.*, 2007). Hence when 5-HT was depleted by pretreating mice for 3 days with the tryptophan hydroxylase inhibitor para-chlorophenylalanine (*pCPA*), the effects of drugs that act by enhancing 5-HT neurotransmission were abolished (Eckeli *et al.*, 2000; Gavioli *et al.*, 2004) while those that act on noradrenergic pathways were not affected (Lucki *et al.*, 1994; Page *et al.*, 1999; Page and Lucki, 2002; Gavioli *et al.*, 2004). The inhibition of the antidepressant effect of MOE by *pCPA* in both TST and FST suggests that its antidepressant effect is dependent on enhancement of serotonergic neurotransmission. The lack of antidepressant effect of fluoxetine in *pCPA* treated mice is consistent with the hypothesis that fluoxetine elicits its acute behavioural effects by increasing extracellular 5-HT after blockade of the serotonin transporter (Bymaster *et al.*, 2002). The extract and fluoxetine increased swimming score which was reversed with *pCPA* pretreatment, further supporting their action on the serotonergic system (Page *et*

*al.*, 1999; Chau *et al.*, 2011). Both MOE and fluoxetine did not affect mean climbing score, suggesting that their behavioural effect may not depend on noradrenergic pathways. Further evidence suggesting that the extract enhances 5-HT neurotransmission was derived from its ability to increase head twitch responses induced by 5-HTP. The head twitch response (HTR) in rodents induced by 5-hydroxytryptophan (5-HTP), a precursor of 5-HT (Corne *et al.*, 1963), is considered as a specific behavioural model for the activation of serotonergic neuron specifically 5-HT<sub>2A</sub> receptors (Schreiber and Pick, 1995). Thus it can be inferred that MOE may be acting via activating 5-HT<sub>2A</sub> receptors. Fluoxetine also increased the frequency of HTRs. This result is consistent with a number of studies where fluoxetine elicited similar responses (Essman *et al.*, 1994; Xu *et al.*, 2006).

The present experiments also examined the role of noradrenaline and dopamine in the acute behavioural effects of the extract in the modified FST by using drugs that interfere with their neurotransmitter synthesis or release. Depletion with  $\alpha$ -methyldopa, an L-aromatic amino acid decarboxylase inhibitor that inhibits the biosynthesis of catecholamines and 5-HT (DeMuth and Ackerman, 1983) failed to attenuate the behavioural effects of MOE and fluoxetine while that of imipramine was abolished. This suggests that MOE may not affect the biosynthesis of noradrenaline or dopamine. Moreover when vesicular pools were depleted by reserpine, the decrease in immobility elicited by MOE or fluoxetine was not affected. Here again the effect of imipramine was attenuated. Reserpine is an irreversible inhibitor of 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 (Metzger *et al.*, 2002; Ji *et al.*, 2007). Treatment with reserpine therefore leads to depletion of vesicular monoamine stores- both serotonin and noradrenaline (Fukui *et al.*, 2007) suggesting both serotonin and noradrenaline might be important in the antidepressant effects of imipramine. The inability of reserpine pretreatment to reverse the antidepressant effects of the extract and fluoxetine however, seem to suggest that reserpine does not affect vesicular storage of 5-HT to the same extent as that of noradrenaline. In fact this assertion is consistent with the results obtained by O'Leary *et al.*, (2007) and Woode *et al.*, (2010)—the former demonstrating that reserpine at the dose used produced a 93 and 95% depletion of cortical noradrenaline and dopamine content respectively, and a 78% depletion of 5-HT. To inhibit both synthesis and deplete vesicular pools of noradrenaline and dopamine, mice were pretreated with both reserpine and  $\alpha$ -methyldopa. Results were similar to effects observed when mice were treated with reserpine alone. The work published by O'Leary *et al.*, (2007) indicated that when reserpine was combined with  $\alpha$ -methyl para-tyrosine AMPT (NE and DA biosynthetic inhibitor), a depletion of cortical DA (95%), NE (97%), and 5-HT (78%) was observed. The combination had only a modest



effect on NE and DA but failed to affect 5-HT. Though  $\alpha$ -methyldopa was used instead of the AMPT used by O'Leary and colleagues, the results from the combined effect of reserpine and  $\alpha$ -methyldopa did not differ significantly from when reserpine was used alone. A more recent by Woode *et al.*, (2010) used  $\alpha$ -methyldopa and similar results was observed. These results demonstrate that the antidepressant effect of MOE may not be dependent on noradrenergic neurotransmission.

Both clinical and preclinical studies support the antidepressant activity of antagonists on functional glycine/NMDA receptor complex. These compounds are thought to have lower side effect profiles compared to the competitive and non-competitive NMDA antagonists (Poleszak *et al.*, 2011). The effect of MOE on glutamatergic neurotransmission was assessed by pretreating mice with D-serine (DS), a full agonist on glycine/NMDA receptors or D-cycloserine (DCS), a partial agonist on these receptors. In both the TST and FST, DS reversed the decline in immobility by MOE and fluoxetine but DCS pretreatment potentiated the decline, demonstrating that MOE may be acting as an antagonist on the glycine/NMDA receptor complex. In contrast, the decrease in immobility of desipramine was reversed by DS but DCS had no effect on it. This suggests that the antidepressant effect of both serotonin and noradrenaline-based compounds depend on the inhibition of the glycine/NMDA receptor complex but the enhancement of antidepressant activity depends on the serotonergic pathway and not the noradrenaline pathway (Poleszak *et al.*, 2011). This explains why the antidepressant effect of the extract, fluoxetine and desipramine was abolished by D-serine but only the effect of the extract and fluoxetine (which act via serotonergic pathway) were potentiated by D-cycloserine. Moreover MOE increased curling score in the TST slightly (suggestive of opioidergic activity) though not significant. Pretreatment with DCS significantly increased curling score of MOE but did not affect both FLX and DSP treated groups. DS pretreatment partially inhibited pedalling behaviour by MOE but totally in FLX and DSP treated groups. DCS pretreatment also inhibited pedalling behaviour by MOE and DSP but not FLX. According to Berrocoso *et al.*, (2012), opioids decrease immobility score while increasing curling behaviour in mice. This suggests that the extract on its own may have little effect on opioidergic activity but may interact with opioid receptors when combined with DCS. In the FST, MOE and FLX unlike DSP increased swimming behaviour which is sensitive to selective serotonin reuptake inhibitors (SSRIs) and 5HT agonists (Cryan and Lucki, 2000; Cryan *et al.*, 2005). This behaviour exhibited by MOE and fluoxetine treated mice was inhibited by DS but increased by DCS pretreatment further supporting the theory that antidepressant effect of serotonin based drugs depend on the inhibition of the glycine/NMDA receptor complex and the enhancement of antidepressant activity depends on the serotonergic pathway



(Poleszak *et al.*, 2011). Climbing scores were decreased by both MOE and FLX and this was not affected by both DS and DCS pretreatment. This confirms their lack of noradrenergic activity. DSP, a selective noradrenergic reuptake inhibitor, on the contrary increased climbing score which was unaffected by DCS pretreatment but decreased by DS. It is worth mentioning that the extract, fluoxetine, desipramine alone or their combination with either D-serine or D-cycloserine did not impair motor coordination. Thus the behavioural effect observed can be attributed to drug treatment alone.

The enhancement of serotonergic mechanisms and inhibition of glycine/NMDA systems observed in the acute models may be responsible for the antidepressant-like effect of MOE in the open space swim test. This assumption is consistent with findings that suggest that chronic treatment with antidepressants acting via serotonergic pathways enhance serotonergic neurotransmission (Owens, 1996; Ceglia *et al.*, 2004; Faure *et al.*, 2006). Another explanation that can be attributed to the effectiveness of MOE in the chronic model is its possible effect on neurotrophic factors. Successful antidepressant treatments following chronic administration are associated with an enhancement of hippocampal neurogenesis, increase in neurotrophin levels, and a resultant increase in hippocampal function which can be measured as neuronal response or behaviour (Duman and Monteggia, 2006; Monteggia *et al.*, 2007; Sahay and Hen, 2007). This has been substantiated by the ability of chronic SSRIs administration of fluoxetine or citalopram, along with other antidepressants and electroconvulsive shock to increase hippocampal cell proliferation and increase the expression of BDNF (Nibuya *et al.*, 1995; Malberg *et al.*, 2000; Balu and Lucki, 2009). It is possible that the sustained antidepressant effect of the extract may be partly due to effect on neurotrophic factors.

According to Stone and Lin, depressed mice in the open space swim test model respond only to chronic treatment ( $\geq 2$  weeks) and not to acute or subacute treatment (Stone and Lin, 2011). While this delay in the onset of action was observed in imipramine and fluoxetine treated mice, it was not so in the extract treated groups. Antidepressant effect in this model with regards to immobility period which is deemed the more consistent index in mice (Stone *et al.*, 2008; Stone and Lin, 2011) began on the first day of treatment. The complex array of neuropharmacological changes, such as desensitization of 5-HT autoreceptors (5HT<sub>1A</sub> and 5HT<sub>1B/D</sub>) and transporters, downregulation of neurotransmitter receptors, changes in signal transduction, mobilization of neurotrophins, and increases in hippocampal neurogenesis that emerge after chronic treatment with SSRIs may be involved in their clinical efficacy and the time taken for these processes to occur may contribute to the time lag in their clinical onset of action (Caldecott-Hazard and Schneider, 1992;

Pineyro and Blier, 1999; Frazer and Benmansour, 2002; Duman and Monteggia, 2006; Tardito *et al.*, 2006; Sahay *et al.*, 2007). For a faster onset of action drugs may act as antagonist at presynaptic 5-HT<sub>1A</sub> receptors or bypass the inertia of the autoinhibitory mechanisms of the serotonin receptors (Zanardi *et al.*, 1997; Artigas *et al.*, 2001; Adell *et al.*, 2005). This quick effect has been the basis for adding buspirone, a partial agonist at 5-HT<sub>1A</sub> receptors or pindolol, as adjuncts to SSRIs in depression (Dimitriou and Dimitriou, 1998; Keks *et al.*, 2007). The inhibitory effect of the 5-HT<sub>1A</sub> autoreceptors is one of the reasons for the delay in onset of action of antidepressants affecting 5-HT systems (Blier and de Montigny, 1994; Moret, 2005). Moreover drugs that activate the 5HT<sub>4</sub> receptors demonstrated faster onset of action in vitro and in animal models that were known to respond to chronic treatment only (Lucas *et al.*, 2007; 2010). Additionally, 1-aminocyclopropanecarboxylic acid, a partial agonist on the glycine/NMDA site produced a faster onset of action than biogenic amine-based agents in the chronic mild stress model (Cherkofsky, 1995; Papp and Moryl, 1996; Poleszak *et al.*, 2011). It is therefore not surprising that the extract, which enhances serotonergic neurotransmission and antagonize glycine/NMDA system, should demonstrate a rapid onset of behavioural effect in the open space swim test. It is interesting to note that the antidepressant effect of the extract was sustained throughout the treatment period. In fact a day after the fourteenth day of treatment, the behavioural effect of the extract was still maintained in the tail suspension test, one of the widely used and validated models of antidepressant.

Depression in humans has dramatic impact on cognition, including memory and learning (Troster *et al.*, 1995; Kuzis *et al.*, 1997; Dolan, 2002; Ravnkilde *et al.*, 2002; Uekermann *et al.*, 2003). Sun and Alkon (2004) have demonstrated that the induced depressive behaviour in rodents has a lasting impact (at least for days) on learning and memory and that the impairment is sensitive to antidepressant treatment. Findings in this study support this fact. The time taken to locate the hidden platform was higher (indicative of cognitive impairment) in untreated depressed mice but reduced with treatment. In extract treated mice, naïve unswum mice or fluoxetine treated mice latency to find the hidden platform decreased on daily basis but not in the depressed untreated mice or imipramine treated mice. The role of the serotonergic and the glycine/NMDA systems in memory has been well documented (Buhot *et al.*, 2000; Meneses, 2002; Williams *et al.*, 2002). Converging evidence indicates that activation of 5HT<sub>2A/2C</sub> and 5HT<sub>4</sub> receptor agonists prevent memory impairment and facilitate learning while their antagonists have opposite effect (Buhot *et al.*, 2000; Harvey, 2003). It is therefore not surprising that MOE should improve memory since it potentiated the 5-hydroxytryptophan-induced head twitch response, a model sensitive to 5HT<sub>2A</sub> receptor activation (Sun *et al.*, 2003; Fantegrossi *et al.*, 2010). Though some studies

suggest that NMDA antagonists impair memory (Hadj Tahar *et al.*, 2004; Rowland *et al.*, 2005), there have also been reports about their beneficial effects on memory and learning (Si *et al.*, 2004; Parsons *et al.*, 2007; Dashniani *et al.*, 2010; Lockrow *et al.*, 2011). For instance memantine, an NMDA antagonist have been shown to be value in conditions like Alzheimer's, dementia and Parkinson's disease where memory is impaired (Rogawski, 2000; Reisberg *et al.*, 2003; Ota and Godwin, 2006; Lockrow *et al.*, 2011). It is possible that the inhibitory effect of the extract against glycine/NMDA receptor complex or pathway also contributed to the memory improvement in the Morris water maze test.

Significant weight changes (increase or decrease) are found among many depressed patients (Polivy and Herman, 1976; Anderson *et al.*, 2006). This could be attributed to the condition and/or antidepressant-induced adverse effects (Weissenburger *et al.*, 1986; Fava, 2000; Dixon *et al.*, 2003; Roberts *et al.*, 2003; Zimmermann *et al.*, 2003). Serotonin is a neurotransmitter that has significant influence on appetite and therefore weight changes (Wurtman, 1993; Halford and Blundell, 2000; Finlayson *et al.*, 2007). Considering this fact and that MOE interact with serotonergic pathways, effect of extract on weight variation was investigated. Results indicated that the extract did not affect the weight of the mice significantly although slight weight reductions were observed at all dose levels.

## 5.5 CONCLUSION

This study provides support not only to the multifaceted aspects of depression as a syndrome but also to the fact that antidepressants can treat some of these comorbid states. It is also evident that the extract has a rapid and sustained antidepressant effect in the open space swim test without affecting the weight of the mice—the plant could be a possible source of bioactive compounds with faster onset of antidepressant action. Moreover the extract improved the spatial learning and memory in mice. MOE demonstrated its behavioural effects by interacting with serotonergic pathway (e.g. activating 5-HT<sub>2A</sub> receptors) as well as the glycine/NMDA receptor complex.



## *Chapter 6* **ANXIOLYTIC EFFECT**

### **6.1 INTRODUCTION**

Anxiety is a normal and adaptive state of autonomic arousal and behavioural defence in response to threat but can become pathological when it increases in scope, magnitude, or duration (Nesse, 1999). Anxiety disorders are the most common mental illness in the world (Rabbani *et al.*, 2008). Moreover, complaints of anxiety are common among healthy individuals and have been associated with numerous negative health consequences (Muller *et al.*, 2005; Balon, 2006; Scott *et al.*, 2007), absenteeism (Hoffman *et al.*, 2008), and decreased work productivity (Sanderson *et al.*, 2007).

Drug therapy for anxiety though effective is fraught with dependence, tolerance and side effect issues (Baldwin *et al.*, 2005; Nutt, 2005). It is evident that alternative therapies are needed and plants can be invaluable sources for compounds with anxiolytic effects.

The plant *Mallotus oppositifolius*, is commonly used in Ghana for epilepsy. Sleep enhancing effect of the extract and enhancement of GABAergic inhibitory neurotransmission was observed as some of the pharmacological effects of the extract. Based on these findings, possible anxiolytic effects of the extract were investigated.

### **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 Chemicals**

Diazepam was obtained from Phyto-Riker, Accra, Ghana.

#### **6.2.3 Elevated Plus-Maze test**

The method used was as described for rats (Pellow *et al.*, 1985) with some modifications. Mice were grouped into seven groups (n=5); group 1 was the control, groups 2 to 4 received the extract (10 - 100 mg kg<sup>-1</sup>, *p.o.*), and groups 5 to 7 received diazepam (0.1 - 1 mg kg<sup>-1</sup>, *i.p.*). Behavioural parameters were scored from the videotapes as follows: (1) number of closed and open arm entries (absolute value and percentage of the total number); (2) time spent in exploring the open and closed arms of the maze (absolute time and percentage of the total time of testing); (3) number of head-dips- protruding the head over



the edge of either an open (unprotected) or closed (protected) arm and down toward the floor; (4) number of stretch-attend postures- the mouse stretches forward and retracts to original position from a closed (protected) or an open (unprotected) arm. An arm entry was counted only when all four limbs of the mouse were within a given arm. Behavioural parameters for all the tests were scored from videotapes with the aid of the public domain software JWatcher Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia. Available at <http://www.jwatcher.ucla.edu/>)

#### 6.2.4 Light/Dark Box

This apparatus is based on the initial model described by (Belzung *et al.*, 1987; Belzung and Le Pape, 1994). Mice were grouped into seven groups (n=5); group 1 was the control, groups 2 to 4 received the extract (10 - 100 mg kg<sup>-1</sup>, *p.o.*), and groups 5 to 7 received diazepam (0.1 - 1 mg kg<sup>-1</sup>, *i.p.*). At the beginning of the experiment, mice were placed individually in the centre of the illuminated box, facing away the opening from the dark compartment. Behaviours of the animals were recorded for 5 minutes with a digital camera placed 1 m above the box. Videotapes were scored as mentioned above for the following parameters: (frequency of compartment entries, total time spent by mice in each compartment and the number of transitions).

#### 6.2.5 Open-field test

The test was based on that described previously by Kasture *et al.*, (2002). The animals were divided into seven groups of five animals each and received the extracts (10 - 100 mg kg<sup>-1</sup>, *p.o.*), the standard drug diazepam (0.1 - 1 mg kg<sup>-1</sup>, *i.p.*) and the vehicle. Thirty minutes after *i.p.* and 1 h after oral administration of the test compound, mice were placed individually in the centre of the open field and allowed to explore freely for 5 min. Each session was recorded by a video camera suspended approximately 100 cm above the arena. All animals were regularly handled before individual tests in order to minimize handling-related stress. Videotapes of the arena and the following variables were recorded: number of entries as well as the duration of stay in individual zones. Thereafter, behaviour in the open field was analyzed for 5 min with the aid of the public domain software JWatcher Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia. Available at <http://www.jwatcher.ucla.edu/>). Mean values  $\pm$  SEM were calculated for each and compared to vehicle-treated animals.

## 6.3 RESULTS

### 6.3.1 Effect of MOE and DZP on mice in the elevated plus-maze

In the EPM, extract showed similar anxiolytic effect as diazepam. Administration of extract significantly increased the open arm activity by increasing open arm entries ( $F_{3,16}=13.52$ ,  $P=0.0001$ ) (Figure 6.1a) and percentage open arm entries ( $F_{3,16}=19.29$ ,  $P<0.0001$ ) (Figure 6.1c). It also increased percentage time spent in the open arm ( $F_{3,16}=21.99$ ,  $P<0.0001$ ) (Figure 6.2c). The frequency and the duration of rearing were also significantly decreased by the extract. MOE decreased the number of protected stretch attend postures and protected head dips. See (Table 6.1 and Table 6.2).



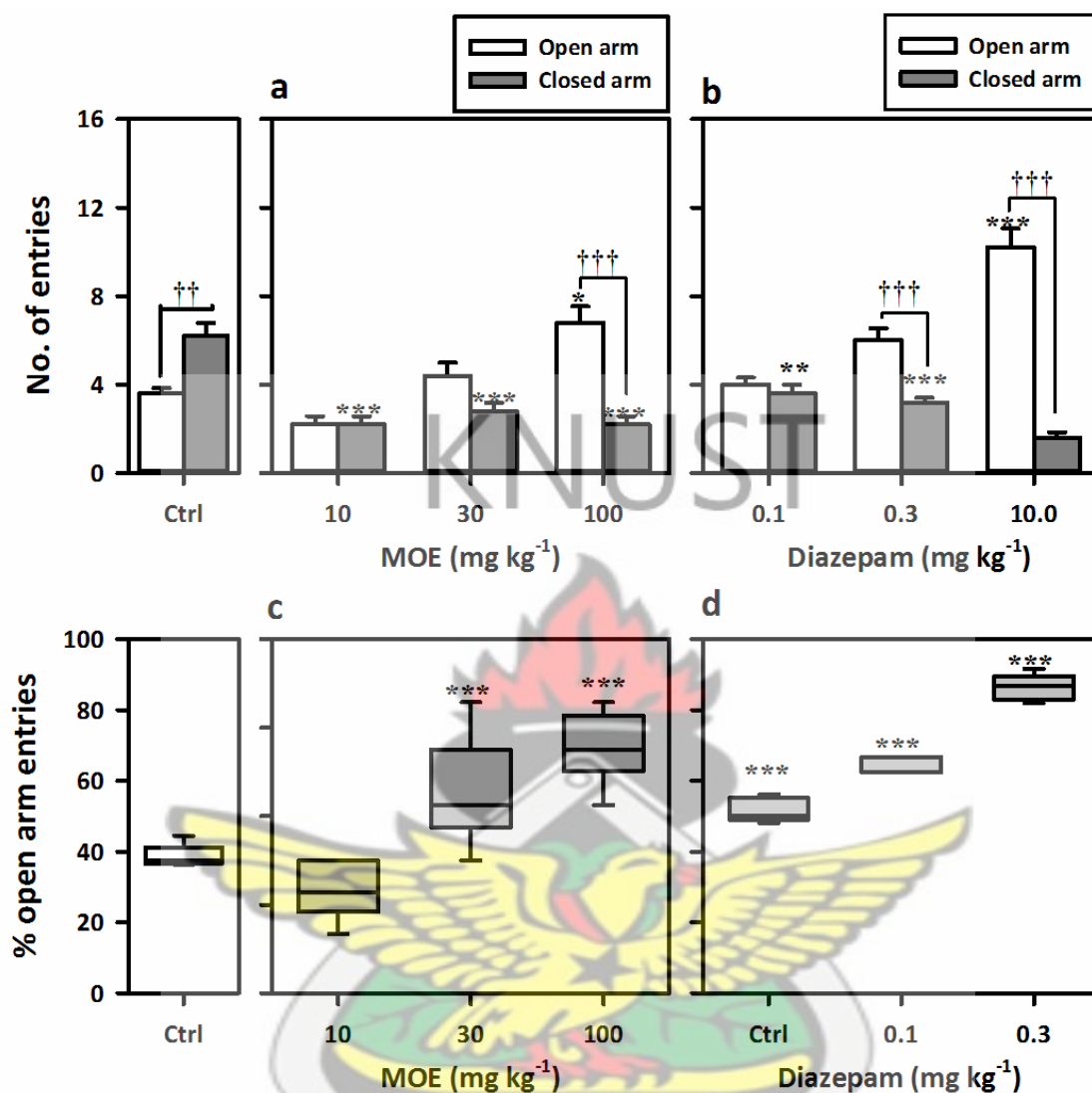


Figure 6.1 Effect of (a) the extract, MOE (10 - 100 mg kg<sup>-1</sup>) and (b) diazepam, DZP (0.1 - 1.0 mg kg<sup>-1</sup>) treatment on the open and closed arm entries; (c - d) Effect of the extract (MOE) and diazepam, treatment on % open arm entries respectively. The lower and upper margins of the boxes represent the 25th and 75th percentiles, while the extended arms represent the 10th and 90th percentiles respectively. \*\*\*P<0.001; \*\*P<0.01; compared to vehicle-treated group; (One-way ANOVA followed by Newman Keuls' test). †††P<0.001 comparison between open and closed arm entries (Two-way ANOVA followed by Bonferroni's test). Data are presented as group Means  $\pm$  SEM of 5 animals.

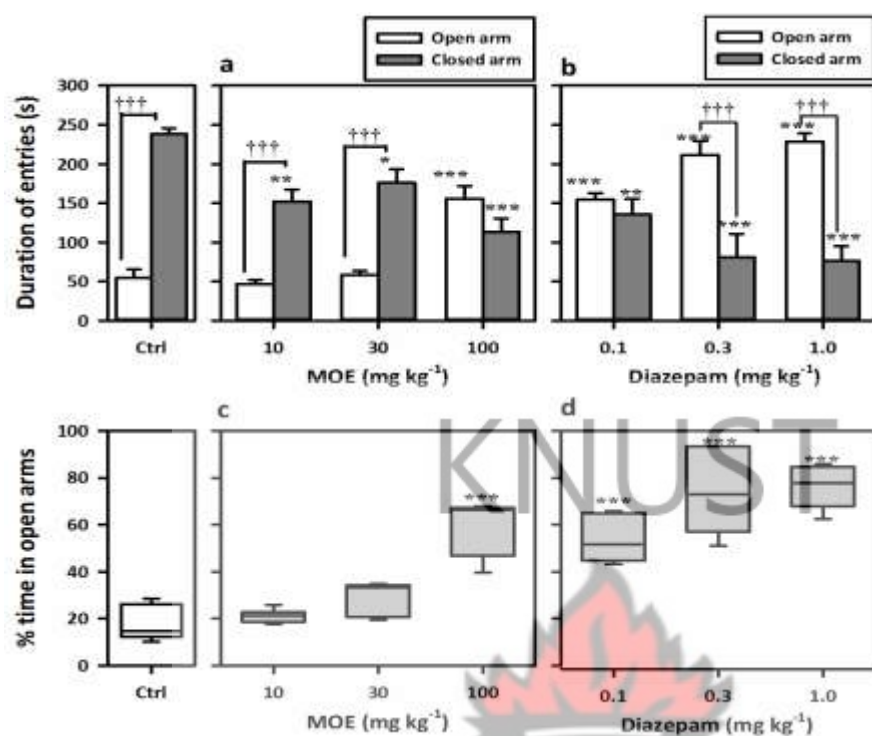


Figure 6.2 Effect of (a) the extract, MOE (10 - 100 mg kg<sup>-1</sup>) and (b) diazepam, DZP (0.1 – 1.0 mg kg<sup>-1</sup>) treatment on the time spent in the open and closed arm; (c - d) Effect of the extract, MOE, and diazepam, DZP treatment on % time spent in the open respectively. The lower and upper margins of the boxes represent the 25th and 75th percentiles, while the extended arms represent the 10th and 90th percentiles respectively. \*\*\**P*<0.001; \*\**P*<0.01; compared to vehicle-treated group; (One-way ANOVA followed by Newman Keuls' test). †††*P*<0.001 comparison between open and closed arm duration (Two-way ANOVA followed by Bonferroni's test). Data are presented as group Means ± SEM of 5 animals.

Table 6.1 Effect of Drug treatment on the frequency of some ethological parameters

Treatment	Frequency				
	Rearing	PHD	UPHD	PSAP	UPSAP
Ctrl	14.8±1.2	1.8±0.2	8.2±0.6	1.4±0.3	2.2±0.2
MOE					
10	9.0±0.6**	2.2±0.2	4.8±0.2***	1.2±0.2	2.2±0.2
30	5.6±0.7***	1.2±0.2***	2.2±0.2***	0.0±0.0***	0.6±0.3***
100	5.2±0.4***	0.6±0.3***	1.4±0.3***	0.0±0.0***	0.2±0.2***
DZP					
0.1	6.8±0.5***	0.1±0.1***	4.2±0.5***	0.4±0.3***	0.6±0.3***
0.3	3.8±0.5***	0.0±0.0***	4.2±0.2***	0.0±0.0***	0.4±0.3***
1.0	3.3±0.6***	0.0±0.0***	2.4±0.3***	0.0±0.0***	0.2±0.2***

Values are mean±S.E.M. (n=5). \*\*\**P*<0.001, \*\**P*<0.01 considered statistically significant from control. PHD: protected head dip; UPHD: unprotected head dip; PSAP: protected stretch attend posture; UPSAP: unprotected stretch attend posture.



Table 6.2 Effect of Drug treatment on the duration of some ethological parameters

Treatment	Duration				
	Rearing	PHD	UPHD	PSAP	UPSAP
Ctrl	14.3±1.7	0.9±0.1	4.6±2.3	1.1±0.2	1.6±0.6
MOE					
10	9.9±1.3*	3.8±0.9**	3.9±1.8	1.6±0.4***	1.4±0.7
30	9.4±0.7*	0.7±0.1	1.9±0.9	0.2±0.2***	0.9±0.4
100	7.2±0.2**	0.9±0.2	0.9±0.4	0.0±0.0***	0.2±0.1
DZP					
0.1	6.4±0.5***	0.0±0.0***	8.9±5.8	0.0±0.0***	0.4±0.3
0.3	4.5±0.3***	0.0±0.0***	5.9±1.3	0.0±0.0***	0.4±0.3
1.0	3.2±0.6***	0.1±0.0***	4.5±0.9	0.2±0.2***	0.0±0.0***

Values are mean±S.E.M. (n=5). \*\*\* $P<0.001$ , \*\* $P<0.01$  considered statistically significant from control. PHD: protected head dip; UPHD: unprotected head dip; PSAP: protected stretch attend posture; UPSAP: unprotected stretch attend posture.

### 6.3.2 Effect of MOE and DZP on mice in the light-dark test.

Extract treated mice exhibited anxiolytic effect in the light-dark box. MOE significantly increased the time spent by mice in the lit region ( $F_{3,16}=24.58$ ,  $P<0.0001$ ) while decreasing the time spent in the dark region (Figure 6.3a, c). There was also a significant increase in latency to leave the lit area into the dark area ( $F_{3,16}=24.32$ ,  $P<0.0001$ ) (Figure 6.3a). There was however a decrease in the number of transitions by extract treatment 100 mg kg<sup>-1</sup> ( $F_{3,16}=6.82$ ,  $P<0.036$ ) (Figure 6.3a). Diazepam (0.1, 0.3 and 1.0 mg kg<sup>-1</sup>), the reference anxiolytic, showed similar effects as the extract (Figure 6.3b, d).

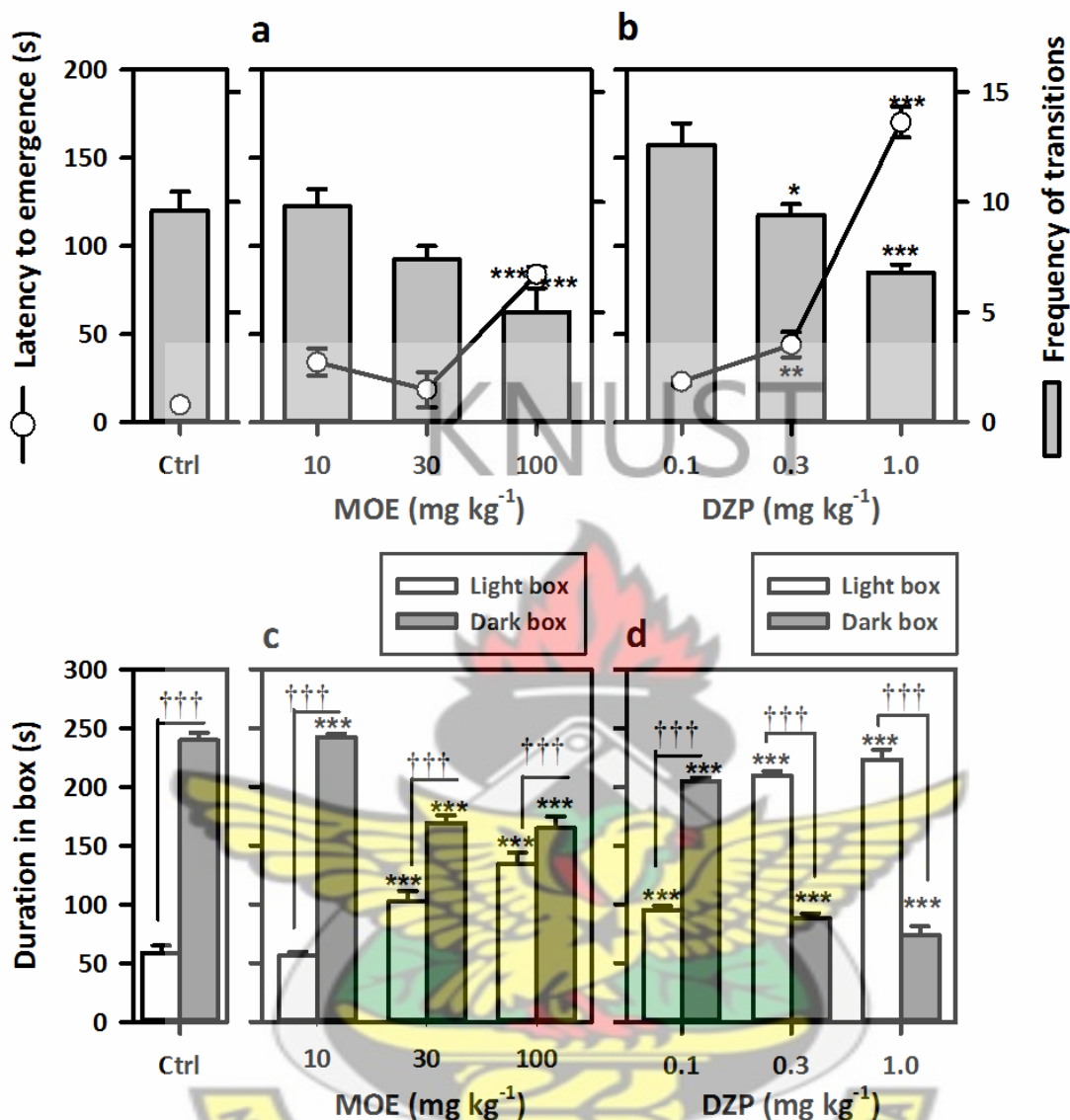


Figure 6.3 Effect of (a) the extract, MOE (10 - 100 mg kg<sup>-1</sup>) and (b) diazepam, DZP (0.1 - 1.0 mg kg<sup>-1</sup>) treatment on the latency to enter dark compartment and the frequency of transitions; (c - d) Effect of the extract, MOE, and diazepam, DZP treatment on time spent in the lit and dark compartments respectively. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; compared to vehicle-treated group; (One-way ANOVA followed by Newman Keuls' test). ††† $P < 0.001$  comparison between open and closed arm duration (Two-way ANOVA followed by Bonferroni's test). Data are presented as group Means  $\pm$  SEM of 5 animals.

### 6.3.3 Effect of MOE and DZP on mice in the open field test

In the open field test, all drug treated mice showed significant differences in both the number of entries into the various fields as well as the time spent in the various zone. *M. oppositifolius* treated mice exhibited anxiolytic activity similar to diazepam by significantly increasing the absolute number and percentage number of centre entries ( $F_{6,28}=49.17$ ,

$P<0.0001$ ) (Figure 6.4) and the percentage time spent in the centre of the open field ( $F_{3,15}=36.12$ ,  $P<0.001$ ) (Figure 6.5).

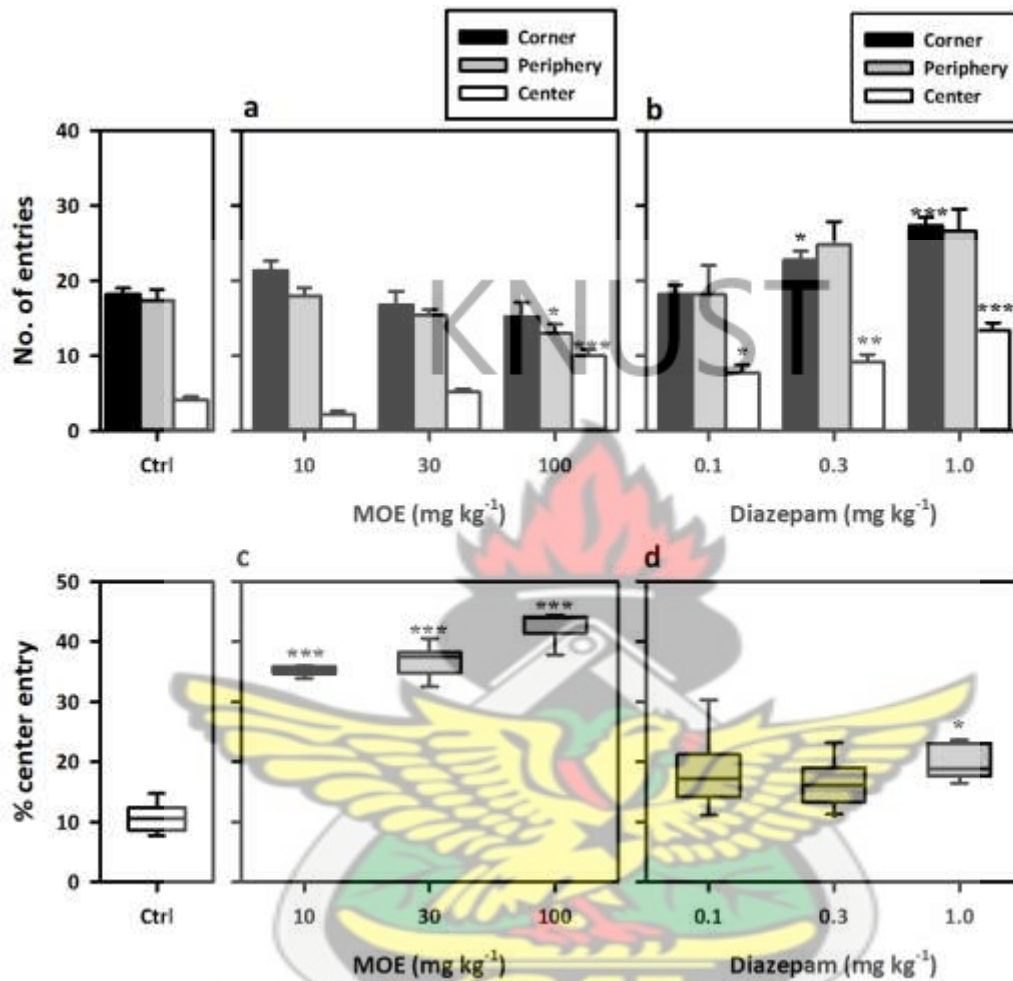


Figure 6.4 Effect of (a) the extract, MOE (10 - 100 mg kg<sup>-1</sup>) and (b) diazepam, DZP (0.1 – 1.0 mg kg<sup>-1</sup>) treatment on the frequency of entries into the corner, peripheral or central compartments; (c - d) Effect of the extract, MOE, and diazepam, DZP treatment on % centre entries respectively. \*\*\* $P<0.001$ ; \*\* $P<0.01$ ; compared to vehicle-treated group; (One-way ANOVA followed by Newman Keuls' test). Data are presented as group Means  $\pm$  SEM of 5 animals. 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.

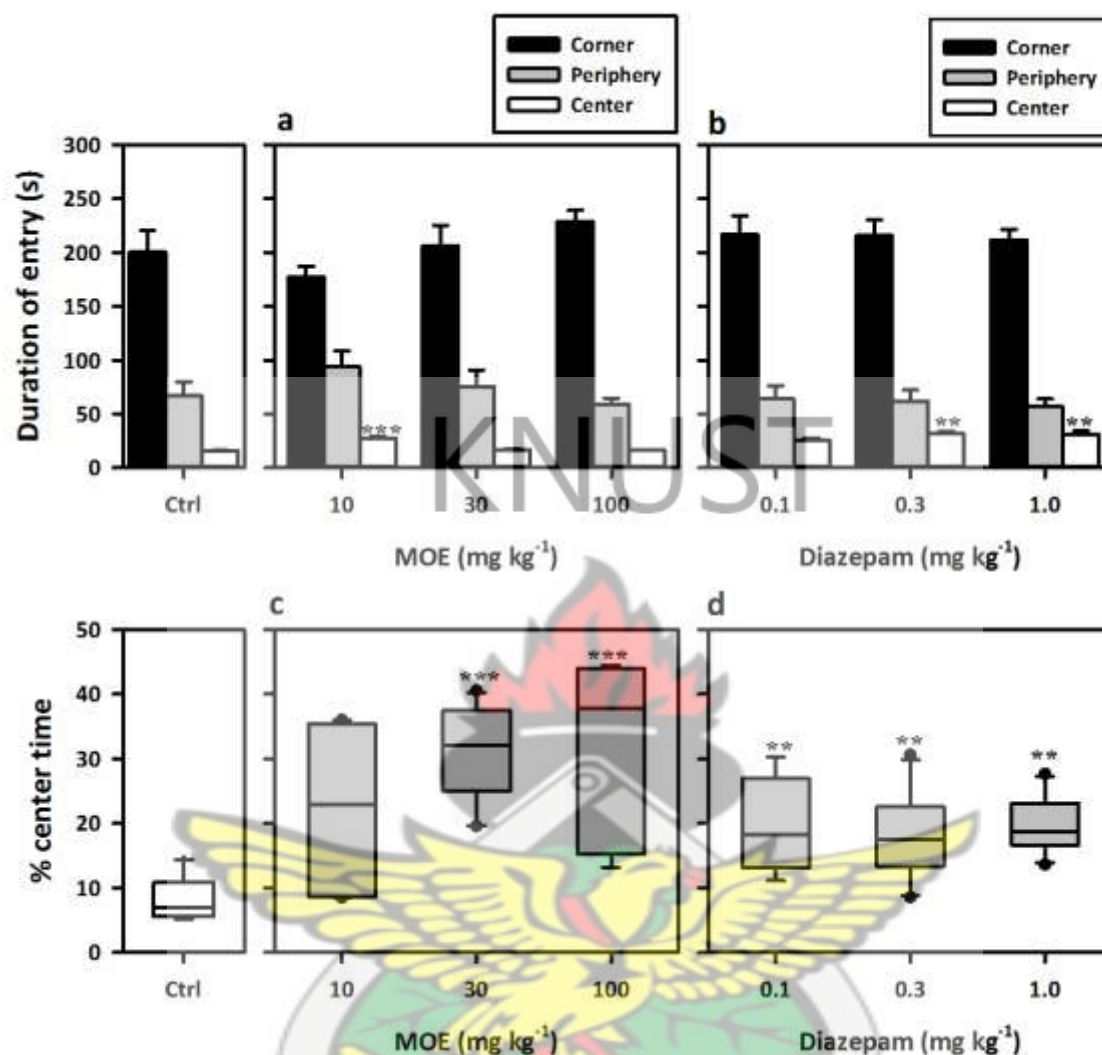


Figure 6.5 Effect of (a) the extract, MOE (10 - 100 mg kg<sup>-1</sup>) and (b) diazepam, DZP (0.1 – 1.0 mg kg<sup>-1</sup>) treatment on the time spent in the corner, peripheral or central compartments; (c - d) Effect of the extract, MOE, and diazepam, DZP treatment on % time spent in the centre respectively. 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. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; compared to vehicle-treated group; (One-way ANOVA followed by Newman Keuls' test). Data are presented as group Means  $\pm$  SEM of 5 animals.

## 6.4 DISCUSSION

The present study revealed that the hydroalcoholic extract of *M. oppositifolius* has anxiolytic effects. In all the models used the extract showed an attenuation of anxiety-like behaviours.

MOE caused statistically significant increase in percentage open arm entries and time spent there in the elevated plus-maze (EPM), one of the most widely used animal models for screening putative anxiolytics (Cole and Rodgers, 1994; Dawson and Tricklebank,



1995; Wei *et al.*, 2007). This is indicative of possible anxiolytic effect. Ethological measures of risk assessment, such as stretched-attend postures head-dipping and rearing, which have been validated and shown by factor analysis to be a more predictive determinant of anxiety were also used in addition to the spatio-temporal indices (frequency and duration of arm entries) of anxiety (Rodgers and Johnson, 1995; Rodgers and Dalvi, 1997). Extract treated mice demonstrated less protected stretch attend postures, protected head dips and rearing suggestive of anxiolysis. Rodents display enhanced risk assessment behaviours even in the closed arm of the maze, suggesting that this defensive pattern is more sensitive to anxiety modulating drugs than avoidance-related measures (Rodgers and Cole, 1994; Griebel *et al.*, 1997; Rodgers and Dalvi, 1997; Setem *et al.*, 1999). Thus the anxiolytic effects of the extract were demonstrated not only by the increased open arm entries and percentage of open arm time, but also by the decreased protected stretch-attend postures, protected head dips and rearing.

The light/dark box test exploits rodents' natural aversion to bright areas compared to dark areas and their innate exploratory behaviour (File *et al.*, 2004). Thus, drug-induced increase in behaviours in the lit region and increase in transitions without an increase in spontaneous locomotion is considered to reflect anxiolytic activity (Treit, 1990; Njunge and Handley, 1991; File, 1992; De Vry *et al.*, 1993). In the present study, the extract showed significant anxiolytic activity by delaying the time for the mice to leave the lit compartment and increasing the amount of time spent in the lit compartment. This affirms that the extract possibly contains compounds that have anxiolytic activity. Interestingly there was a decrease in transition at the dose of 30 mg kg<sup>-1</sup> though previous studies indicated that this dose does not cause sedation or motor impairment which could have accounted for the decrease in transitions (Crawley, 1981). It is worth mentioning that the measurement found to be most consistent and useful for assessing anxiolytic-like action is the time spent in the lit area and not frequency of transitions, because this parameter provides the most consistent dose–effect results with drugs (Hascoet and Bourin, 1998; Lopicard *et al.*, 2000). The decrease in transition therefore should not detract from the effects observed in both the EPM and the light/dark exploration test.

A third model, the open field test, was utilized to further clarify the possible effects of the extract. The open-field model examines anxiety-related behavior characterized by the normal aversion of the animal to an open, brightly lit area (Choleris *et al.*, 2001; Mechan *et al.*, 2002). Thus, animals removed from their acclimatized cage and placed in a novel environment express anxiety and fear, by showing decreases in ambulation and exploration time in the centre of the open field with thigmotaxis (Bhattacharya and Mitra, 1991). MOE

increased the percentage time spent in the central portion of the arena and reduction in peripheral movements just like diazepam. These results confirm the anxiolytic-like effect of the extract.

## 6.5 CONCLUSION

The present study indicates that the hydroalcoholic extract of *M. oppositifolius* has anxiolytic effect.



## *Chapter 7*

### **ACUTE AND SUBACUTE TOXICITY STUDIES**

#### **7.1 INTRODUCTION**

During the last decade, use of traditional medicine has expanded globally and has gained popularity not only developing countries, but also in countries where conventional medicine is predominant (Cupp, 1999; Marinac *et al.*, 2007). Herbal medicines are widely thought to confer health benefits and safer than their orthodox counterparts (Sheehan, 1998; Cupp, 1999). With the tremendous expansion in the use of traditional medicine worldwide, safety assessments of herbal medicines have become imperative because this notion that herbal preparations are natural and therefore must safe may be misleading in the absence of scientific facts (Stedman, 2002; Seeff, 2007). For instance, neurotoxicity, cardiac toxicity, pulmonary toxicity, hepatotoxicity, and nephrotoxicity have been traced to the use of some herbal medicines (Gertner *et al.*, 1995; Stedman, 2002; Su *et al.*, 2011).

Though *Mallotus oppositifolius* is used in managing many conditions traditionally, there is no scientific data on its toxic effect on critical organs like the brain, kidneys, heart, spleen, stomach and liver. The study was therefore conducted to investigate the toxic effects of *M. oppositifolius* in general and on these organs.

#### **7.2 METHOD**

##### **7.2.1 Acute toxicity**

Male Sprague-Dawley rats (200 - 230 g) were randomly divided into seven groups (n=5) and kept in the experimental environment for an acclimation period of 1 week. The animals were fasted overnight, but with access to water *ad libitum*, and then treated orally with *M. oppositifolius* extract in doses of 30 - 3000 mg kg<sup>-1</sup> of body weight. The control group received 10 ml kg<sup>-1</sup> *p.o.* of water. The rats were observed up to 24 hours for general changes in behaviour and physiological function as well as mortality. The assessment of behaviour and physiological function was carried out similar to the primary observation procedure (Irwin test) originally described by Irwin (1968). In accordance with the Irwin test, the rats were observed at 0, 15, 30, 60, 120 and 180 min, and 24 h after treatment for behaviours specifically related to neurotoxicity, such as convulsions and tremor, for behaviours related to CNS stimulation, such as excitation, Straub tail, jumping, hypersensitivity to external stimuli, stereotypies, and aggressive behaviour, and for behaviours related to CNS depression, such as sedation, rolling gait, loss of balance, loss of traction, motor incoordination, hyposensitivity to external stimuli, decreased muscle tone,

akinesia, catalepsy, and hypothermia. Effects on autonomic functions, such as respiration, body temperature, salivation, urination and defecation, were also noted.

### 7.2.2 Subacute toxicity

Male Sprague-Dawley rats (180 – 200 g); n=5, were treated orally with *M. oppositifolius* extract in doses of 30 - 3000 mg kg<sup>-1</sup> of body weight daily, for 14 consecutive days. Group A, the control, received 10 ml kg<sup>-1</sup> *p.o.* of water daily. Group B, C and D were treated with daily doses of extract i.e. 30 - 3000 mg kg<sup>-1</sup> *p.o.* respectively. The extract was prepared such that not more than 2 ml was given orally. The animals were monitored closely for signs of toxicity. Appearance and behaviour pattern were assessed daily and any abnormalities in food and water intake were noted.

#### 7.2.2.1 Preparation of serum and isolation of organs

The rats were sacrificed on the fifteenth day by cervical dislocation, the jugular vein was cut and blood flowed freely. About 1.5 ml of blood was collected into vacuum tubes containing 2.5 µg of ethylenediaminetetraacetic acid (EDTA) as an anticoagulant for haematological assay and 3.5 ml of the blood was collected into sample tubes without anticoagulant. The blood without the anticoagulant was allowed to clot before centrifugation (4000 rpm at 4 °C for 10 min) to obtain serum, which was collected and stored at -20°C until assayed for biochemical parameters the next day. After collecting blood, the rats were quickly dissected and the organs (spleen, liver, kidney, brain, heart and stomach) removed, freed of fat and connective tissue, blotted with clean tissue paper and then weighed on a balance.

#### 7.2.2.2 Effect of extract on haematological parameters

Haematological parameters including haemoglobin (HGB), red blood cells (RBC), white blood cells (WBC), haematocrit (HCT), platelets (PLT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were determined by an automatic analyzer (BC-3000 Plus Auto Haematology Analyzer, Shenzhen Mindray Bio-Medical Electronics Co. Ltd, China).

#### 7.2.2.3 Effect of extract on serum biochemical parameters

Biochemical analyses were performed on serum collected for the determination of the following parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (T-BIL), direct bilirubin (D-BIL), indirect bilirubin (I-BIL), total protein, albumin, urea and creatinine. All analyses were carried out



using an automated clinical chemistry analyser (Flexor Junior®, Vital Scientific, AC Dieren, The Netherlands).

#### **7.2.2.4 Effect of extract on body and organ weights**

Body weights of the rats were taken on days 0 and 15. The relative organ weight (ROW) of each organ was calculated as follows:

$$ROW = \frac{\text{Absolute organ weight (g)}}{\text{Rat body weight on sacrifice day (g)}} \times 100$$

#### **7.2.2.5 Histological examination**

Portions 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, Ottawa, Canada).

#### **7.2.3 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.

## 7.3 RESULTS

### 7.3.1 Acute toxicity

There was no mortality after 24 hour period—LD<sub>50</sub> is above 3000 mg kg<sup>-1</sup>. Rats did not show any signs of neurotoxicity such as convulsions and tremor. Behaviours related to central nervous system (CNS) stimulation, such as excitation, Straub tail, jumping, hypersensitivity to external stimuli, stereotypies, and aggressions were absent. In contrast there were signs of CNS depression such as sedation and motor incoordination at 1000 and 3000 mg kg<sup>-1</sup>. There were no visible signs on autonomic functions such as respiration, pupil diameter, body temperature, salivation and defecation.

Table 7.1 Observations in the acute toxicity test after oral administration of *M. oppositifolius* in rats.

Dose	Mortality		Toxicity
	D/T	Latency (h)	
0	0/5	-	-
30	0/5	-	-
100	0/5	-	-
300	0/5	-	-
1000	0/5	-	Sedation, motor incoordination
3000	0/5	-	Sedation, motor incoordination

The hydroalcoholic extract of *M. oppositifolius*, was administered orally; each dose was administered to groups of 5 rats. D/T: dead/treated rats; -: no toxic symptoms were seen during the observation period; latency: time to death (in hours) after the dose.

### 7.3.2 Subacute toxicity

There was no death after 14 days. Behavioural signs related to toxicity were absent except for sedation at the dose of 1000 - 3000 mg kg<sup>-1</sup> which lasted for the first week but receded afterwards.

#### 7.3.2.1 Effect of extract on body weight and organ weight

At lower doses (30 – 100 mg kg<sup>-1</sup>) there was a decline in the percentage change in weight during the 14 days of extract treatment. This declined was reversed from 300 to 3000 mg kg<sup>-1</sup>. The extract significantly increased the weight of the rats at 3000 mg kg<sup>-1</sup>, when compared to control (Figure 7.1). There was no statistical difference in the relative organ weight of control and extract-treated groups.

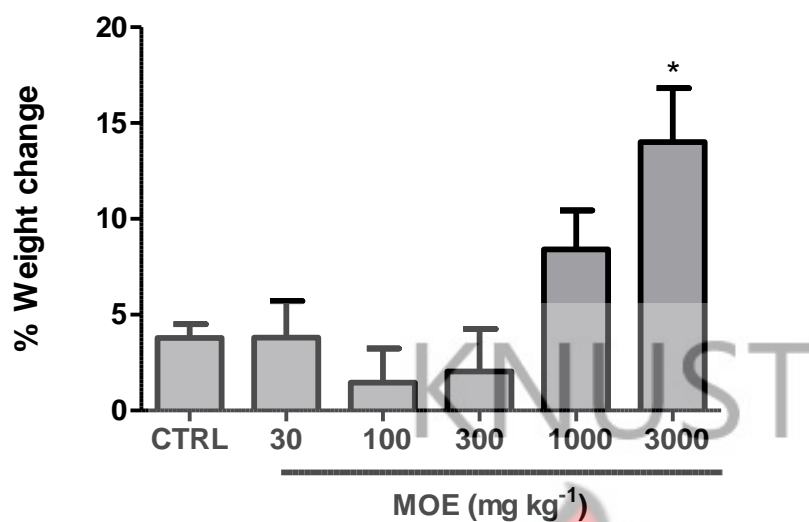


Figure 7.1 Effect of oral administration of *Mallotus oppositifolius* extract (MOE), on the % change in body weights of rats in the sub-acute toxicity test. Data are expressed as mean±S.E.M. (n=5). Treated groups were compared to controls with a one-way ANOVA followed by Newman Keuls' test.



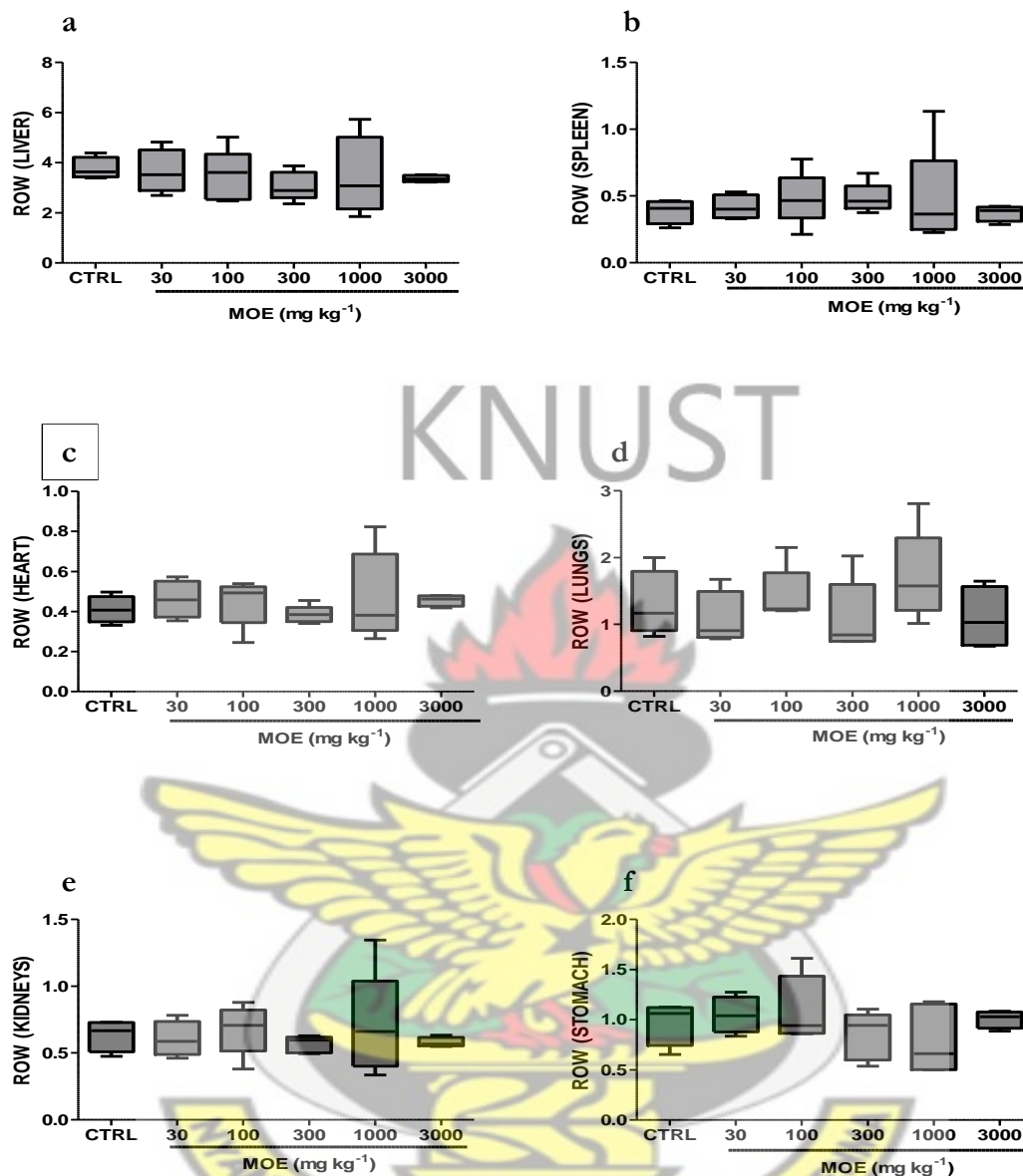


Figure 7.2 Effect of oral administration of *Mallotus oppositifolius* extract (MOE), on the relative organ weights (ROW) of rats in the sub-acute toxicity test. Data are expressed as mean±S.E.M. (n=5). Treated groups were compared to controls with a one-way ANOVA followed by Newman Keuls' test. The lower and upper margins of the boxes represent the 25th and 75th percentiles, while the extended arms represent the 10th and 90th percentiles respectively.

### 7.3.2.2 Effect of extract on haematological parameters

Haematological parameters did not reveal any extract induced toxicity on (Table 7.2).



### **7.3.2.3 Effect of extract on serum biochemical parameters**

Clinical biochemical parameters indicated that the extract did not affect the synthetic function of the liver or the integrity of the hepatocytes. The levels of albumin and globulin, indicative of the liver synthetic function did not differ significantly from the control although there were slight increases. Alanine transaminase (ALT), aspartate transferase (AST) and alkaline phosphatase (ALP) levels were normal compared to control. Bilirubin levels were normal except at 300 and 3000 mg kg<sup>-1</sup> where there were significant increases ( $P=0.0093$ ). Urea and creatinine levels increased at 30 mg kg<sup>-1</sup> but only urea increased at 100 mg kg<sup>-1</sup>. The values decreased however at higher doses (300 – 3000 mg kg<sup>-1</sup>) although not statistically significant. The liver profile showed general decrease in the total cholesterol, very low density lipoprotein (VLDL), and triglycerides but higher levels of high density lipoproteins (HDL). The differences were however not statistically significant compared to control. The coronary risk also decreased with extract treatment although not significantly. See Table 7.3.

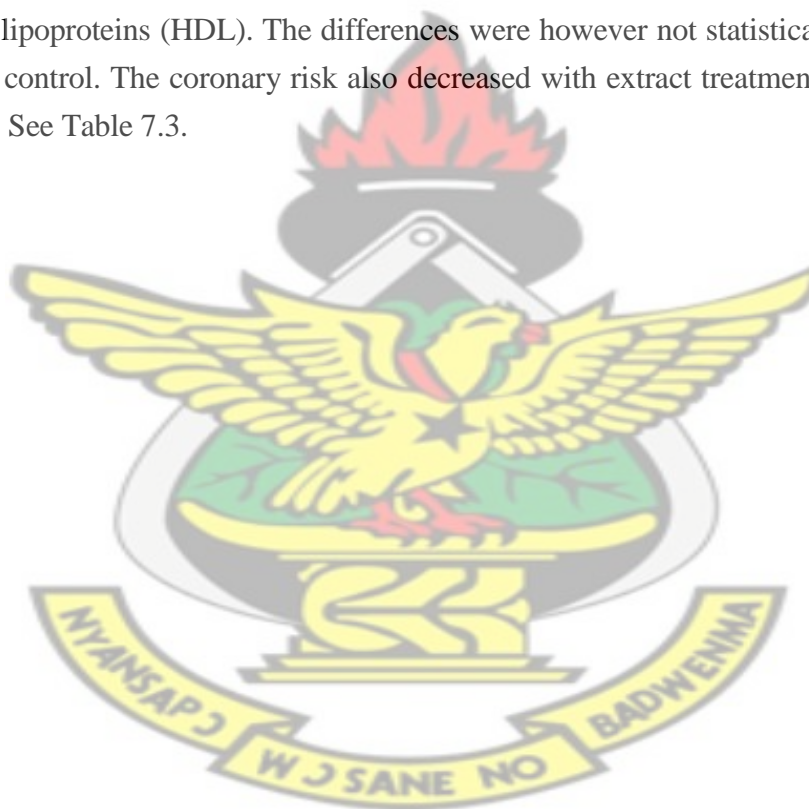


Table 7.2 Haematological values of control and rats treated with *M. oppositifolius* for 14 days.

Parameters	<i>Mallotus oppositifolius</i> extract (mg kg <sup>-1</sup> )						<i>F</i>	<i>P</i> Value
	0	30	100	300	1000	3000		
WBC (10 <sup>9</sup> /L)	12.03±1.01	10.15±2.06	12.64±0.93	8.440±0.60	13.90±2.43	13.98±1.09	<i>F</i> <sub>5,21</sub> =2.144	0.0998
LYM (%)	59.40±2.09	63.73±4.14	67.04±5.71	79.70±5.32	60.04±4.89	71.30±5.58	<i>F</i> <sub>5,21</sub> =2.521	0.0615
RBC (10 <sup>12</sup> /L)	7.19±0.21	7.32±0.53	8.23±0.28	7.36±0.53	7.61±0.66	7.39±0.17	<i>F</i> <sub>5,21</sub> =0.701	0.6289
HGB (g/dL)	12.73±0.47	13.40±0.57	14.24±0.27	13.08±0.48	13.92±1.16	13.83±0.45	<i>F</i> <sub>5,21</sub> =0.725	0.6126
HCT (%)	41.68±1.43	42.48±3.02	44.96±1.29	45.78±1.89	43.42±4.11	42.83±1.08	<i>F</i> <sub>5,21</sub> =0.393	0.8482
MCV (fL)	57.98±1.16	58.73±0.49	56.84±0.65	62.84±3.00	56.92±0.99	57.95±0.49	<i>F</i> <sub>5,21</sub> =2.235	0.0887
MCH (pg)	17.72±0.38	18.65±0.53	18.02±0.36	18.08±1.25	18.38±0.58	18.70±0.36	<i>F</i> <sub>5,21</sub> =363.4	0.9246
MCHC (g/dL)	30.53±0.16	31.75±1.12	31.70±0.35	28.68±1.15	32.28±0.79	32.30±0.87	<i>F</i> <sub>5,21</sub> =2.170	0.0351
RDW_CV (%)	17.50±1.36	14.00±1.18	14.00±0.08	16.36±1.72	13.70±1.30	12.83±0.23	<i>F</i> <sub>5,21</sub> =02.167	0.0968
RDW_SD (fL)	35.58±1.72	32.23±1.84	30.64±0.42	38.34±4.61	30.38±1.21	29.88±0.44	<i>F</i> <sub>5,21</sub> =2.135	0.1009
MPV (fL)	7.43±0.12	7.50±0.40	7.16±0.11	7.28±0.11	7.42±0.13	7.33±0.13	<i>F</i> <sub>5,21</sub> =0.465	0.7977
PDW (fL)	9.03±0.18	9.55±0.69	8.80±0.23	8.84±0.22	9.02±0.19	9.05±0.13	<i>F</i> <sub>5,21</sub> =0.714	0.6196
Platelets (10 <sup>9</sup> /L)	841.0±132.7	734.5±107.3	698.0±65.09	781.4±165.2	788.2±102.5	642.0±131.5	<i>F</i> <sub>5,21</sub> =0.319	0.8954

Values are mean±S.E.M. (n=5). \**P*<0.05 considered statistically significant from control.

Table 7.3 Clinical biochemistry values of control and rats treated with *M. oppositifolius* for 14 days.

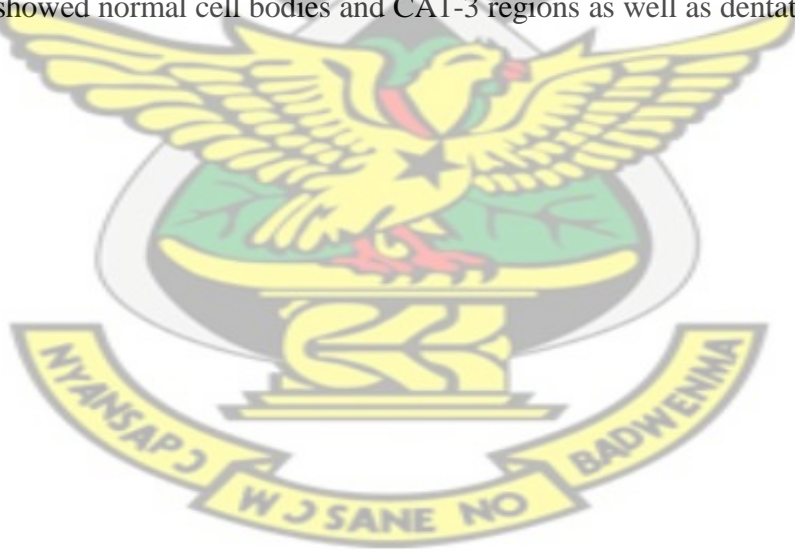
Parameters	<i>Mallotus oppositifolius</i> extract (mg kg <sup>-1</sup> )						<i>F</i>	<i>P</i> Value
	0	30	100	300	1000	3000		
Albumin (g/L)	30.68±0.72	33.75±1.14	32.94±2.27	34.44±0.95	31.12±1.20	31.68±0.72	1.153	0.3646
Globulin (g/L)	36.16±2.59	28.77±2.23	31.69±2.24	28.65±0.72	33.09±1.32	29.68±1.16	2.293	0.0823
Total Prot (g/L)	66.83±3.04	62.53±2.89	64.62±1.98	63.10±1.42	64.22±1.33	61.35±0.88	0.696	0.6323
A/G Ratio	0.85±0.06	1.17±0.05	1.04±0.12	1.20±0.04	0.95±0.07	1.076±0.06	2.815	0.0425
D-Bil (µmol/L)	0.48±0.02	0.37±0.05	0.36±0.16	0.26±0.08	0.38±0.13	0.68±0.24	1.183	0.3508
I-Bil (µmol/L)	0.76±0.08	1.11±0.05	1.48±0.19	1.89±0.20**	1.20±0.27	1.29±0.17	4.117	0.0092
T-Bil (µmol/L)	1.20±0.07	1.48±0.04	1.86±9.10	2.14±0.24*	1.56±0.18	1.98±0.09*	4.106	0.0093
ALT (U/L)	128.60±17.11	105.63±8.95	119.28±14.16	100.36±10.54	83.3±9.12	95.98±3.37	2.169	0.0966
AST (U/L)	183.95±16.96	162.68±16.45	174.64±1.46	164.30±17.09	129.68±13.16	150.45±4.24	1.573	0.2110
ALP (U/L)	3.25±0.74	11.50±6.50	3.20±0.41	9.80±2.78	5.6±2.69	5.00±0.35	1.744	0.1685
Urea (mmol/L)	3.78±0.40	8.36±1.99*	7.91±2.38*	6.12±0.38	6.668±0.42	4.32±0.76	3.538	0.0178
Creat. (µmol/L)	66.83±3.40	69.93±4.56*	66.56±1.30	63.80±4.72	57.42±3.58	51.98±1.49	2.932	0.0368
Chol (mmol/L)	1.84±0.15	2.24±0.10	3.57±0.30	2.03±0.15	1.736±0.05	2.05±0.11	1.345	0.2846
TG (mmol/L)	3.13±0.40	3.69±0.90	2.73±0.20	3.21±0.42	2.724±0.23	2.73±0.10	0.633	0.6766
HDL (mmol/L)	0.67±0.04	0.84±0.03	0.84±0.04	0.75±0.05	0.658±0.06	0.75±0.03	2.436	0.0685
VLDL (mmol/L)	1.43±0.18	1.68±0.41	1.24±0.09	1.46±0.19	1.236±0.10	1.24±0.042	0.644	0.6688
Coronary Risk	3.84±0.33	3.89±0.09	3.77±0.12	3.71±0.23	3.75±0.25	3.79±0.10	0.079	0.9947

Values are mean±S.E.M. (n=5). \*\**P*<0.01; \**P*<0.05 compared to vehicle-treated group (one-way ANOVA with Newman Keuls' *post hoc* test).

#### **7.3.2.4 Histopathological changes**

Histopathological evaluation of the organs isolated from rats sacrificed at the end of the sub-acute toxicity study revealed no significant extract-related changes compared to the control animals except for some changes in the kidney.

Comparison of liver morphological structure in extract-treated rats to control (Plate 7.1) showed no remarkable abnormalities. There was no tissue necrosis or cellular degeneration. The morphology of the capsule and hepatic lobules were normal. Furthermore there was no fatty infiltration (steatosis) in the hepatocytes neither was there infiltration of inflammatory cell. Extract treatment did not affect the histomorphology of the spleen (Plate 7.2). Splenic congestion or haemorrhage, which are considered important features of toxicity in the spleen were absent. Cardiac myocytes did not suffer any injury after 14 days of extract treatment (Plate 7.3). All stomach samples showed normal zymogenic cells, parietal cells and normal grooves in the mucosa with neither atrophy nor inflammatory cell infiltration (Plate 7.4). At 30 mg kg<sup>-1</sup> there were some changes in the histomorphology of the kidney but this was not apparent in doses higher than 30 mg kg<sup>-1</sup> (Plate 7.5). The histology of the hippocampus showed normal cell bodies and CA1-3 regions as well as dentate gyrus (Plate 7.6).





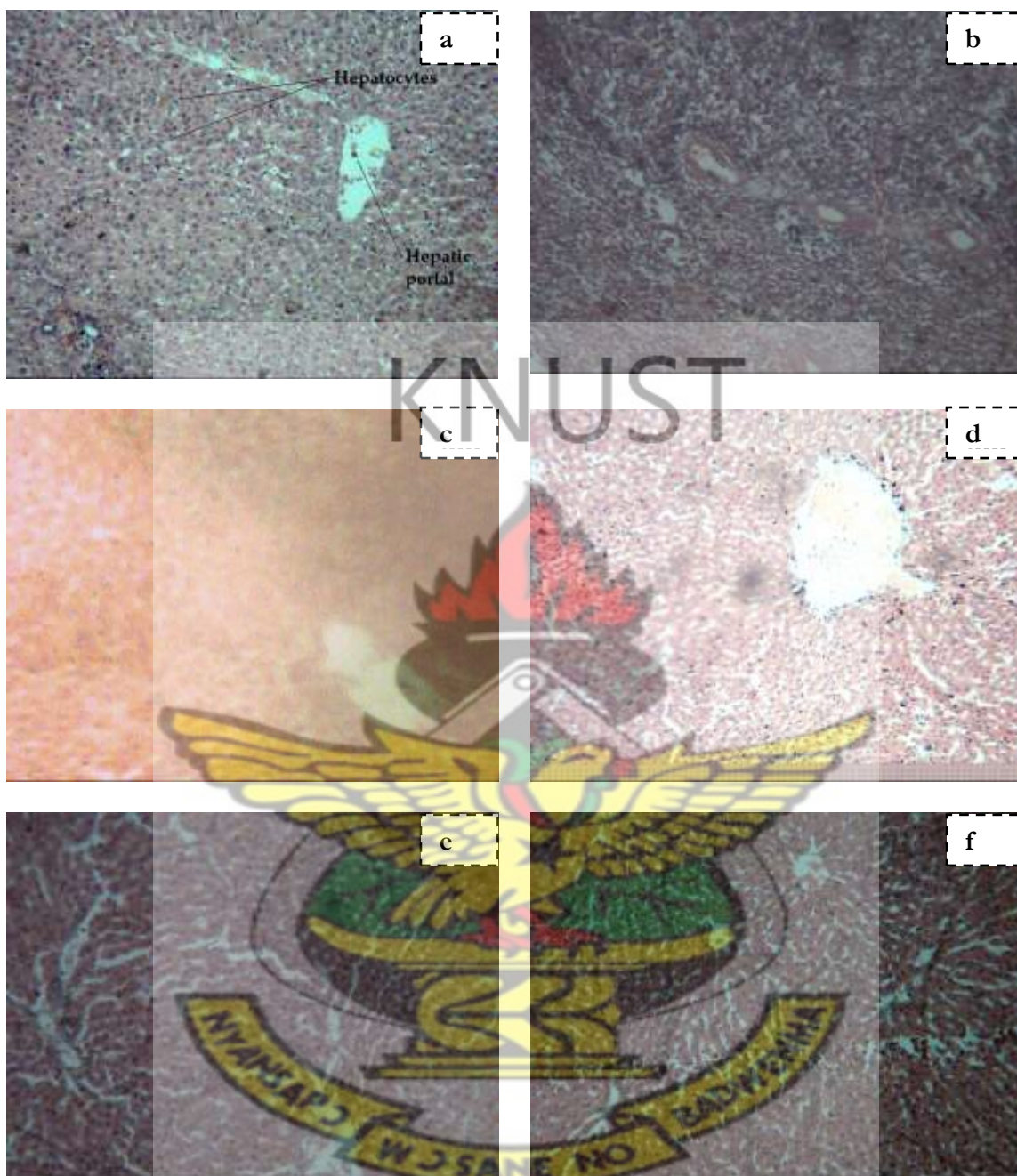


Plate 7.1 Photomicrograph of the sections of (a) the liver in control rats, and (b-f) rats treated orally with 30 - 3000 mg kg<sup>-1</sup> of the extract for 14 days in the sub-acute toxicity study (H & E,  $\times 400$ ).



Plate 7.2 Photomicrograph of the sections of (a) the spleen in control rats, and (b-f) rats treated orally with 30 - 3000 mg kg<sup>-1</sup> of the extract for 14 days in the sub-acute toxicity study (H & E,  $\times 400$ )



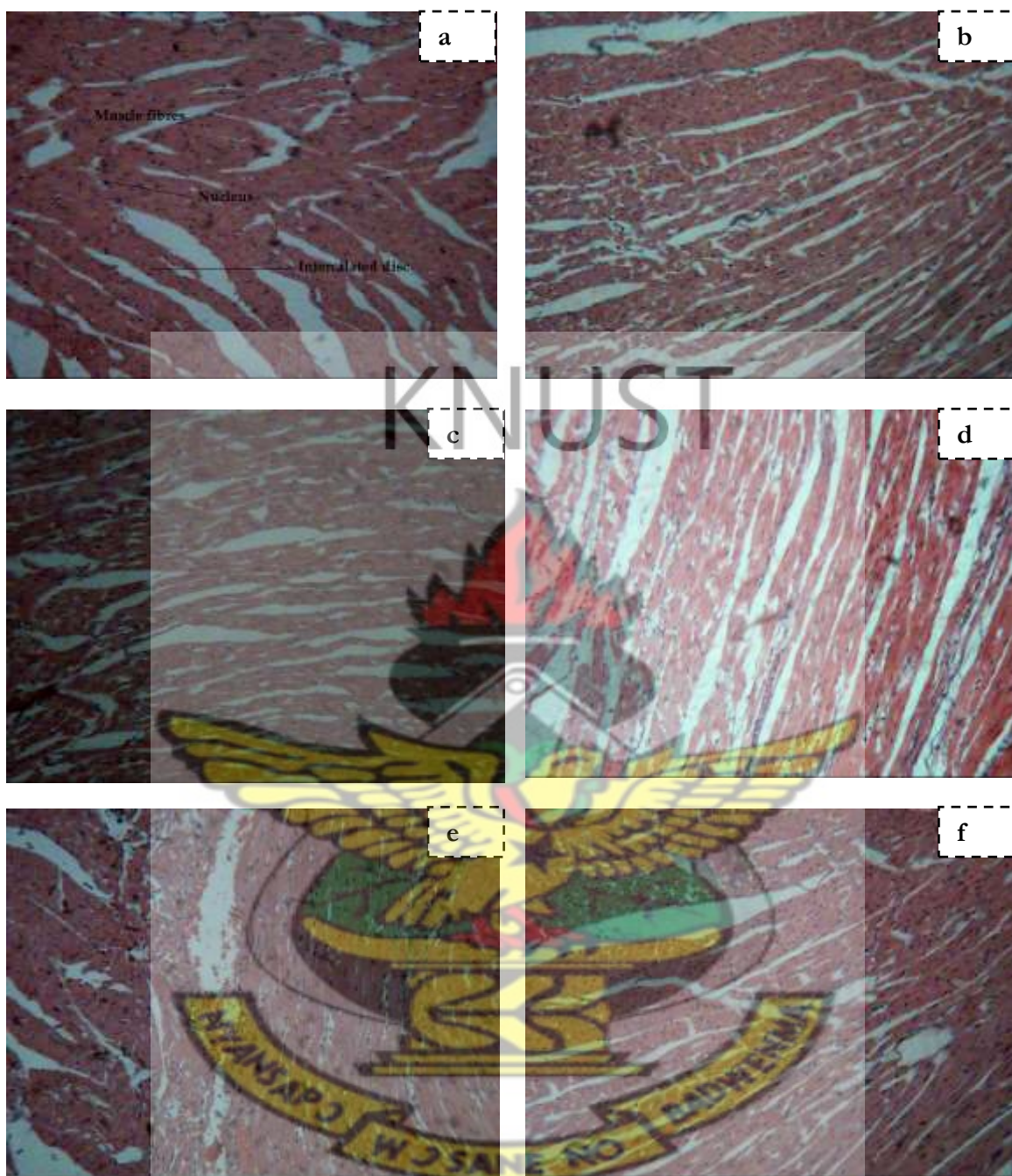


Plate 7.3 Photomicrograph of the sections of (a) the heart in control rats, and (b-f) rats treated orally with 30 - 3000 mg kg<sup>-1</sup> of the extract for 14 days in the sub-acute toxicity study (H & E,  $\times 400$ )

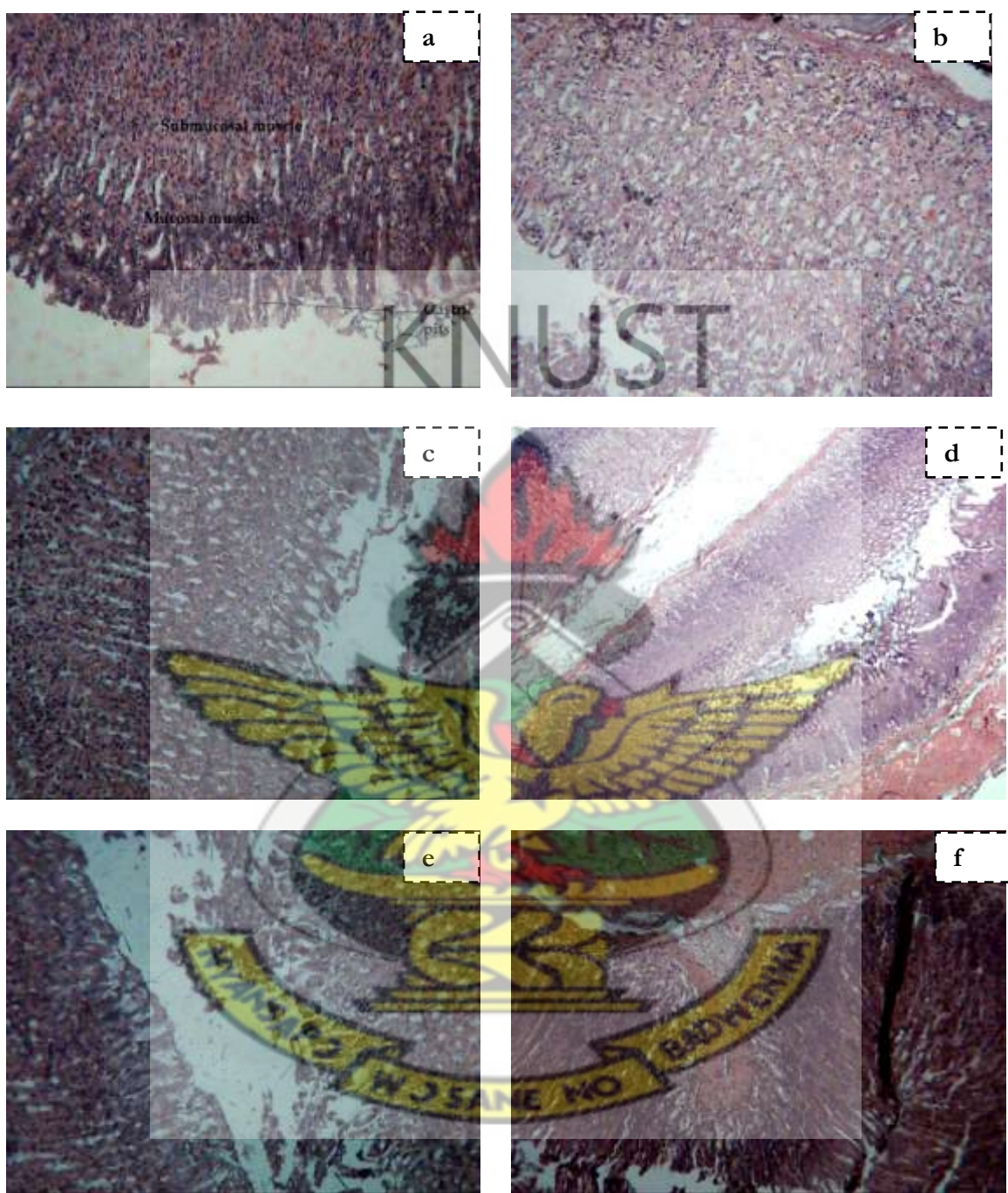


Plate 7.4 Photomicrograph of the sections of (a) the stomach in control rats, and (b-f) rats treated orally with 30 - 3000 mg kg<sup>-1</sup> of the extract for 14 days in the sub-acute toxicity study (H & E, ×400)



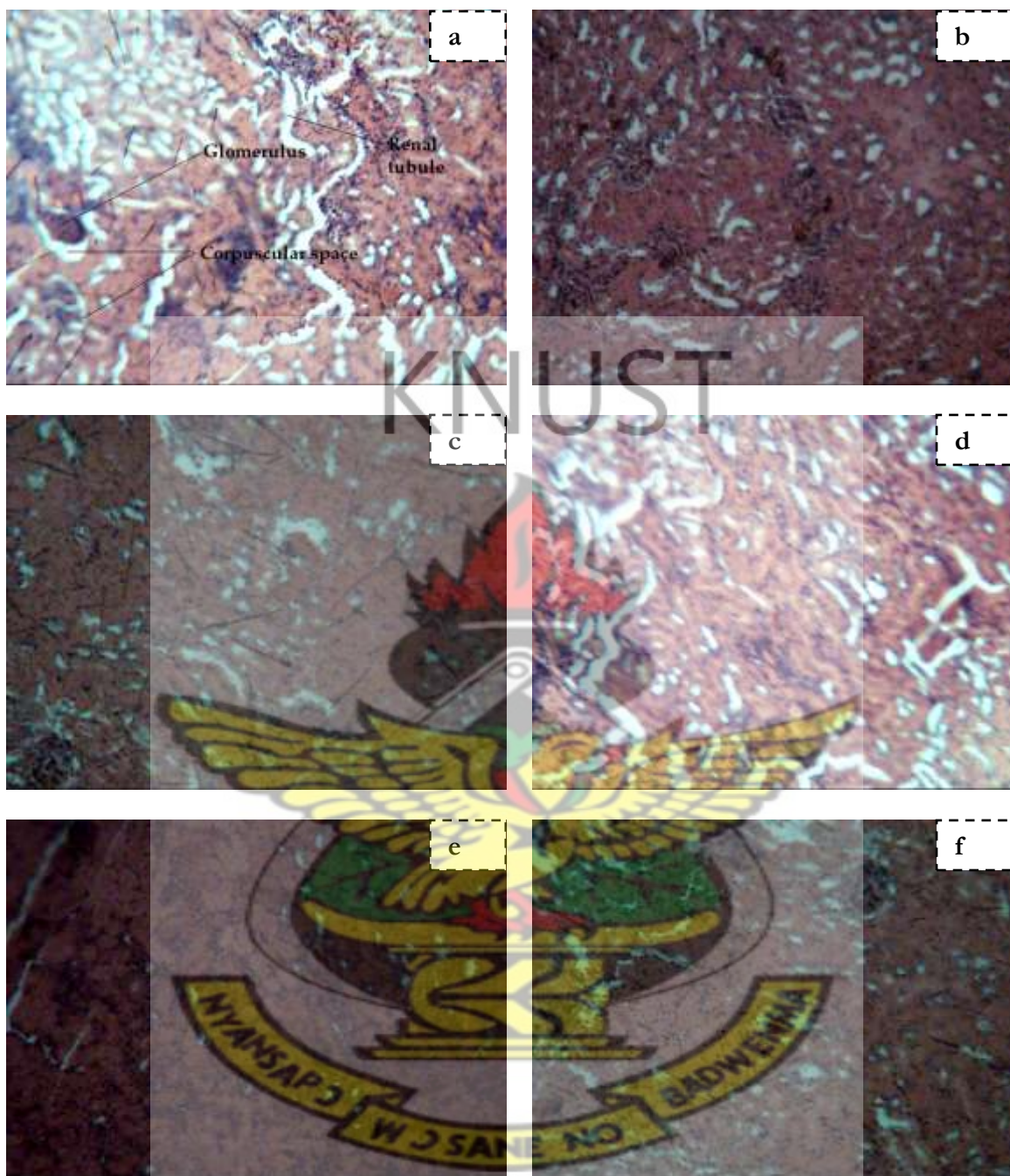


Plate 7.5 Photomicrograph of the sections of (a) the kidney in control rats, and (b-f) rats treated orally with 30 - 3000 mg kg<sup>-1</sup> of the extract for 14 days in the sub-acute toxicity study (H & E, ×400)

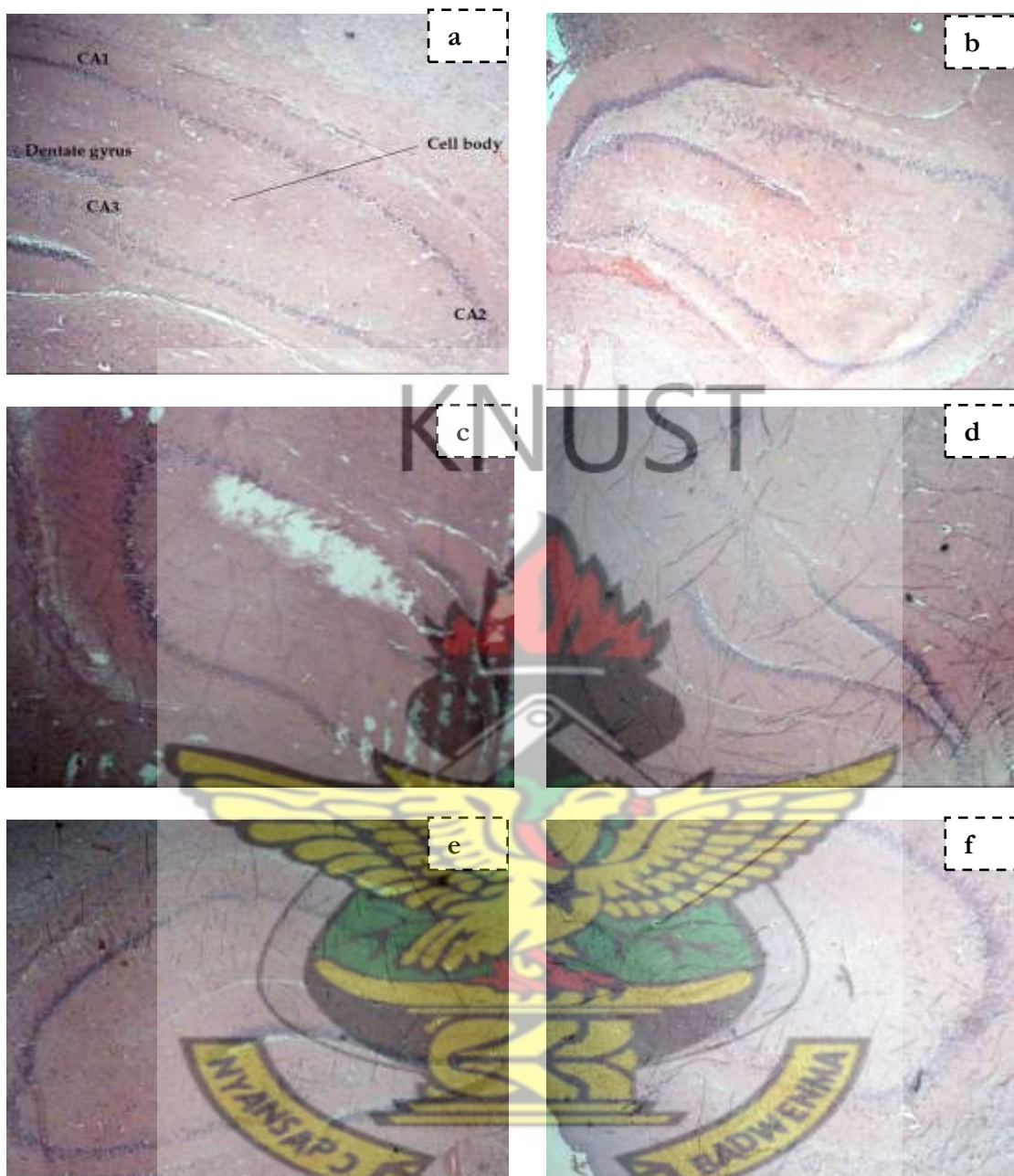


Plate 7.6 Photomicrograph of the sections of (a) the brain showing regions of the hippocampus in control rats, and (b-f) rats treated orally with 30 - 3000 mg kg<sup>-1</sup> of the extract for 14 days in the sub-acute toxicity study (H & E,  $\times 400$ )



## 7.4 DISCUSSION

Just as the therapeutic potential of plants can be rapid or delayed, the toxic effect can also be rapid or delayed (Anderson *et al.*, 1996; Haller *et al.*, 2002). It is therefore necessary to subject medicinal plants to safety assessment procedures to ensure their safety before they are recommended for human use.

Acute toxicity studies revealed that the extract of *M. oppositifolius* leaves was not lethal to the rats after 24 hours, indicating that the LD<sub>50</sub> is above 3000 mg kg<sup>-1</sup>. This suggests that the extract is relatively safe in rodents. At all doses, the extract did not show any toxic signs except sedation and motor incoordination at doses ranging between 1000 and 3000 mg kg<sup>-1</sup> in the rats suggesting CNS depressant activity.

Even at low doses, cumulative toxic effects can occur with repeated dosing (Chitturi and Farrell, 2000; Niggemann and Gruber, 2003). Therefore subacute toxicity studies were carried out for 14 days to ascertain whether the extract will be toxic when administered repeatedly. All the rats survived after the fourteen days of treatment. Rats did not show any toxic signs during this period except for sedations and motor incoordination at high doses (1000 and 3000 mg kg<sup>-1</sup>) but these waned after some days. Water and food consumption was normal. In addition to these parameters, changes in body weight and relative organ weights were evaluated. Changes in body weight and relative organ weights provide a simple and sensitive index of toxicity after exposure to toxic substances (Teo *et al.*, 2002; Obici *et al.*, 2008). The extract treated rats did not show any significant difference in these parameters when compared to the control.

The blood is a very important tissue as far as drug action and toxicity is concerned— it is vital in the transportation and bioavailability of drug molecules. The white blood cells also fight against infection while the platelets regulate blood clotting (Heiser *et al.*, 2000; Misra *et al.*, 2003). Owing to these functions among many others, any haematological toxicity in the course of treatment may be deleterious. Thus an evaluation of the effect of the extract on some haematological parameters (Table 8.1) was conducted. MOE did not have any toxic effects on the haematological parameters measured. Histological sections of the cardiomyocytes did not show any toxic effects indicating any lack of cardiotoxic effect.

Integrity of the synthetic function of the liver was assessed by measuring total protein, albumin, globulin and albumin to globulin ratio (Rothschild *et al.*, 1988; Johnston, 1999). The extract had no toxic effect on these parameters. Levels of ALT, AST and ALP, which are used as markers of liver function, were measured (Johnston, 1999). Liver damaged is suspected when ALT and AST levels increase significantly though ALT is considered a

more sensitive marker because it is invariably found in hepatocytes (Beckingham and Ryder, 2001). Increases in ALP levels reveal possible biliary duct obstruction (Whitfield *et al.*, 1972). The levels of these markers in the extract treated rats did not differ from the control. Another test for liver function is the estimation of serum bilirubin levels (Beckingham, 2001; Beckingham and Ryder, 2001). Results from these estimates indicate the liver's ability to take up, process, and secrete bilirubin into the bile (Beckingham, 2001; Beckingham and Ryder, 2001; ). There were no increases in the direct, indirect and total bilirubin levels after extract treatment except an increase in indirect and total bilirubin at 300 mg kg<sup>-1</sup>. An increase in indirect and total bilirubin without commensurate increase in direct bilirubin may be an indication of prehepatic or hepatic disorder such as excessive haemolysis, cirrhosis or viral hepatitis (Ryder and Beckingham, 2001). However haematological indices did not indicate any haematological toxicity at this dose and the lack of hepatotoxicity of the extract was further confirmed by microscopic examination of the liver histology. The examinations did not reveal any signs of steatosis or necrosis. Increased bilirubin levels at 300 mg kg<sup>-1</sup> may therefore be artefactual.

Kidneys play very important function in the pharmacokinetic effects of drugs especially metabolism and excretion of metabolic wastes (Lin *et al.*, 2003; Fagerholm, 2007). Aside these, the kidneys regulate blood pressure, water and electrolyte balance (Lotspeich, 1958; Finsterer *et al.*, 1988). The effect of MOE on kidney function was therefore investigated since any toxic effect on it can be detrimental or fatal. Results indicated that the extract at (30 and 100 mg kg<sup>-1</sup>) increased the urea levels while only the 30 mg kg<sup>-1</sup> significantly increased the serum creatinine levels compared to the control. This was however within the normal range (60 – 120 µmol/L). At higher doses however the increases of these parameters were reversed. Histological specimens did not reveal any extract induced injuries except a slight change in morphology at 30 mg kg<sup>-1</sup>. This may suggest that the extract may not have any destructive effect on the kidney.

Plasma lipids such as cholesterol and triglycerides are essential for formation of cell membrane, synthesis of hormones and free fatty acids but can pose health challenges when their levels increase abnormally (Dietschy, 1998; Feroz *et al.*, 2011). Serum biochemical analysis revealed a general pattern of reduction in the levels of total cholesterol, triglycerides and very low density lipoproteins (VLDL) but increase in high density lipoproteins (HDL) though not significant statistically. The low density lipoproteins are considered 'bad cholesterol' because they carry a higher risk for cardiovascular incidence while the high density ones are the 'good cholesterol'. Human studies have demonstrated that the risk for atherosclerotic disease, which can affect critical organs such as the heart,

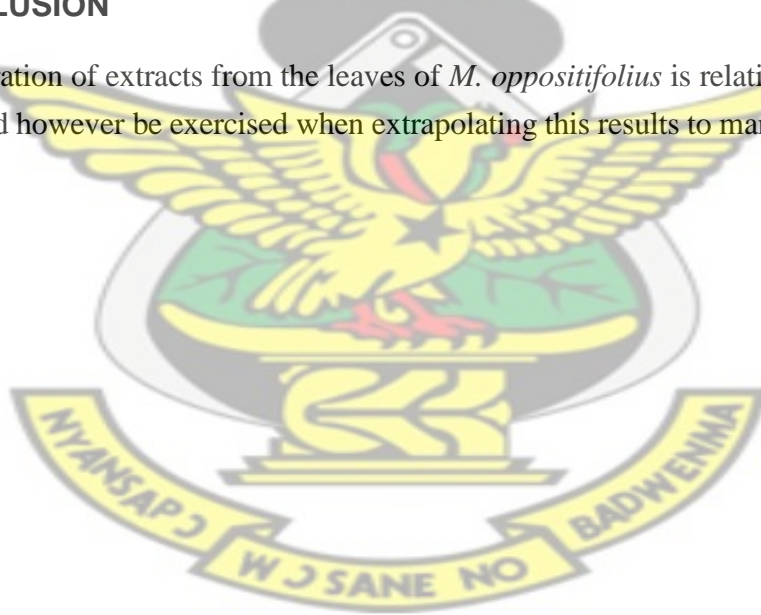


brain and kidneys, is inversely related to blood levels of HDL (Miller and Miller, 1975; Toth, 2005). It is therefore not surprising that the coronary risk decreased since the very low density lipoproteins depreciated while the high density lipoproteins increased. This may also explain why no fatty infiltration was found in the liver or the heart histology.

The brain especially the hippocampus plays a very critical role with regards to disease conditions like epilepsy, depression and anxiety (Sahay and Hen, 2007). In these conditions research has shown morphological derangements in the hippocampus formation while some effective therapies may lead to reversal or worsening in these derangements. Thus the histology investigated possible extract induced changes in the hippocampal formation (CA1-3 and dentate gyrus). From the results it was apparent that the extract did not have any toxic effect on the brain. Visually the intensity of the cells in the hippocampus seems to be greater compared to the control. This could be possibly due to neurogenic activity in this region and may explain the effectiveness of the extract in memory improvement, depression as well as epilepsy (Duman and Monteggia, 2006; Sahay and Hen, 2007).

## 7.5 CONCLUSION

Oral administration of extracts from the leaves of *M. oppositifolius* is relatively safe in rats. Caution should however be exercised when extrapolating this results to man.



## *Chapter 8*

### **GENERAL DISCUSSION**

#### **8.1 SUMMARY**

Epilepsy, major depression and anxiety remain major health menace in the world because the burden of these non-communicable diseases is on the ascendancy (Lambert and Robertson, 1999; Jackson and Turkington, 2005). There is an overwhelming percentage of the population with conditions that are refractory to the current medications; the onset of action of current medication is slow; adverse effects and toxicity are rife (Lambert and Robertson, 1999; Jackson and Turkington, 2005). These reasons accentuate the need for alternative medications that are effective against the refractory conditions, safe, have rapid onset of action and can improve patient quality of life.

The therapeutic potential of plants in managing psychiatric and affective disorders on the other hand has been gaining credibility not only in developing nations but also the developed ones (Firenzuoli and Gori, 2007). Hence researching into the ability of medicinal plants to treat epilepsy, depression and anxiety is justified. *Mallotus oppositifolius*, a common shrub used for epilepsy and other psychiatric disorders in Ghana was effective as an anticonvulsant, antidepressant and anxiolytic in their respective models.

Central to every epileptic seizure is the imbalance between the equilibrium existing between excitation and inhibition—both enhancement of excitatory and impairment of inhibitory mechanisms can disturb this equilibrium leading to epileptic discharges (Dudek and Staley, 2007; Talathi *et al.*, 2009). Two basic mechanisms underlie the electrophysiological excitability of and the communication between neurons—axonal conduction, which is mediated by action potentials and signal transduction from cell to cell by synaptic transmission and ion channels provide the basis for the occurrence of these processes (Talathi *et al.*, 2009). Hence alterations in the function of ion channels may give rise to seizures and most of the currents antiepileptics alter ion channel processes to some extent (Mody, 1998; Jung *et al.*, 2007). Potassium channel activation or increase in potassium conductance may be a plausible mechanism of action of MOE because it was effective against 4-aminopyridine-induced seizures. When 4-aminopyridine is administered to rodents there is blockade of potassium channels which leads to increase in excitation through the release of glutamate and calcium and this forms the bedrock for the convulsant and neurodegenerative effect of 4-aminopyridine (Pena and Tapia, 2000; Sitges *et al.*, 2011). Owing to the fact that potassium channels play a major role in controlling all aspects of neuronal excitability and neurotransmitter release, any substance that activates these

channels may possess broad spectrum antiepileptic effect or be effective in painful conditions. For instance retigabine, a potassium channel activator, prevents epileptiform activity induced by 4-aminopyridine, bicuculline, low magnesium ( $Mg^{2+}$ ) and low calcium ( $Ca^{2+}$ ) in hippocampal slices and seizures induced by PTZ, maximal electroshock, kainate, penicillin, picrotoxin and NMDA in rodents (Rostock *et al.*, 1996; Armand *et al.*, 1999; Dost and Rundfeldt, 2000). Retigabine is also effective against audiogenic seizures in DBA/2J mice, against seizures in epilepsy-prone rats and against seizures in an amygdala-kindling model (Dailey *et al.*, 1995; Rostock *et al.*, 1996; Tober *et al.*, 1996). Possibly the enhancement of potassium conductance by the extract may explain its broad spectrum anticonvulsant effect against 4-aminopyridine-, pentylenetetrazole-, picrotoxin-, strychnine and pilocarpine -induced seizures. It may also explain its analgesic effect since hyperpolarization as a result of activating potassium channels may lead to analgesic effect. MOE, by activating GABAergic mechanisms in the PTZ seizure test will increase chloride conductance resulting in hyperpolarization and subsequent attenuation of seizures (Harris and Allan, 1985; Treiman, 2001). The extract may also increase chloride conductance by enhancing glycinergic activity since it was effective against seizures induced by strychnine, a glycine receptor antagonist.

That the extract was effective against 4-aminopyridine induced seizures might explain its possible inhibition of glutamatergic neurotransmission in the pilocarpine induced *status epilepticus* since 4-aminopyridine induces the release of glutamate (Sitges *et al.*, 2011). Pilocarpine induced *status epilepticus* in rodents represent a model of human temporal lobe epilepsy (Sanabria *et al.*, 2002; Curia *et al.*, 2008). Temporal lobe epilepsy is a partial seizure representing 70% of all drug-refractory epilepsies (Pitkanen, 2002). With 30% of all epilepsy cases being refractory to drug treatment, the efficacy of the extract against *status epilepticus* should be received with great glee and this information should help in the quest for isolating compounds that will treat drug-refractory epilepsies.

Clinical efficacy of an antiepileptic drug is defined as the ability of the drug to decrease the frequency of seizures by  $\geq 50\%$  (Cramer *et al.*, 1999; Glauser *et al.*, 2006). Since the extract demonstrated greater than 50% reduction in seizure frequency, it may be inferred that the extract will be promising as an anticonvulsant. It can also be deduced that the extract may be effective against temporal lobe epilepsy since it demonstrated activity against pilocarpine-induced *status epilepticus* and against generalized seizures because of its effectiveness in the MES, PTZ and picrotoxin-induced seizures. These effects observed attaches credibility to the traditional use of the plant in epilepsy.

Isobolographic analysis of low doses of fractions of extract and diazepam combination showed synergistic effect. However the mode of action of this combination may not be dependent on GABAergic mechanisms since flumazenil pretreatment could not inhibit the anticonvulsant effect of the combined extract and diazepam effect.

Aside its anticonvulsant effect, the extract demonstrated significant antidepressant effect in both acute and chronic models of depression. It is interesting to note that the extract exhibited a more rapid onset of action than fluoxetine and imipramine in the open space swim test, the chronic model used—effect started on the first day of drug treatment. Thus MOE may be a good source of antidepressant compounds with a more rapid onset of action than the conventional ones. Not only did the extract show a rapid antidepressant effect but it also improved spatial learning and memory in depressed mice that received treatment. Scientific evidence suggests that the incidence of suicides and hence mortality is positively correlated with the delay in symptom improvement among the depressed (Lucas *et al.*, 2007). It is possible that MOE may be a potential source of potent compounds with rapid onset of action that can help improve the quality of life of depressed patients and thus a reduction in the rate of suicides. One of the major goals of depression therapy is to manage depression-induced memory and cognitive deficits (Parikh and Lam, 2001; Reesal and Lam, 2001). Some antidepressants however can also induce memory impairment—this may confound assessment of disease improvement (Moore and O'Keeffe, 1999; Gallassi *et al.*, 2006). That the extract improves memory while demonstrating a rapid onset of action is a finding that must be exploited, especially in those at risk of cognitive dysfunction like the elderly.

Central serotonergic mechanisms with weak opioidergic activity, devoid of central adrenergic mechanisms are responsible for the antidepressant effect observed in the acute depression models used. MOE's dependence on serotonergic enhancement was demonstrated through the extract's ability to increase swimming score and subsequent inhibition of this behavioural effect by para-chlorophenylalanine, a 5-HTP hydroxylase inhibitor (Park *et al.*, 1994; Carrillo-Vico *et al.*, 2005). The potentiation of 5-hydroxytryptophan induced head twitch further supported the serotonergic enhancement by the extract. By slightly increasing curling score in the tail suspension test and this effect being potentiated by D-cycloserine, a possible opioidergic activity by the extract is likely (Berrocso *et al.*, 2012). The opioidergic activity demonstrated in the tail suspension test may also explain the antinociceptive effect of the extract in the tail immersion test, a model sensitive to morphine-like compounds or opioids (Vonvoigtlander *et al.*, 1983; Romberg *et*



*al.*, 2003). The antinociceptive effect of the extract supports the traditional use of the leaves in the management of pain.

The antidepressant effect of MOE in both the tail suspension and modified forced swim test could be due to its antagonistic effect on the glycine/NMDA receptor complex or pathway. Antagonists of the glycine/NMDA receptor complex or pathway have demonstrated fewer side effects in comparison to their counterparts which are competitive or non-competitive antagonists (Poleszak *et al.*, 2011). Also some of these compounds have demonstrated rapid antidepressant effects in chronic depressions models and are used for conditions where memory is impaired (Poleszak *et al.*, 2011; Howard *et al.*, 2012). It can therefore be inferred that the rapid onset of action and the memory improvement may be attributable to the antagonistic effect of the extract on this pathway. This antagonistic effect may also be responsible for the anticonvulsant effect of the extract in the pilocarpine-induced *status epilepticus* and partly PTZ seizures (Eloqayli *et al.*, 2003; Costa *et al.*, 2004).

Anxiolytic effect was demonstrated by the extract in the elevated plus, light dark box and open field tests. Anxiety is one of the commonest psychiatric disorders in epileptic patients (Beyenburg and Schmidt, 2005; Caplan *et al.*, 2005). Finding a compound with combined anxiolytic and antiepileptic effect would be very important. Though examples like the benzodiazepines exist, tolerance and dependence limit their long term use (Bateson, 2002; Ashton, 2005). It will be important to further explore the anxiolytic effects of the extract. Most compounds that enhance GABA neurotransmission or cause sedation seem to demonstrate anxiolytic effect (Bateson, 2002; Donaldson *et al.*, 2007). It is therefore not surprising that the extract showed anxiolysis in rodents since it enhanced GABA neurotransmission and caused sedation.

Potential for drug interaction by the extract with other drugs must be borne in mind since it was metabolized by hepatic enzymes and demonstrated significant induction of liver enzymes. Drugs that are metabolized by the liver may have reduced therapeutic effect or reduced toxic effect—hence there may be positive or negative effects to it (Lynch and Price, 2007; Bibi, 2008).

The extract was relatively safe in mice and rats. In mice the LD<sub>50</sub> was approximately 6000 mg kg<sup>-1</sup> while it was above 3000 mg kg<sup>-1</sup> in rats. It also did not induce serious tissue or organ related damages in the rats.

The possible mechanism(s) by which the extract may be acting is shown below (Figure 8.1).

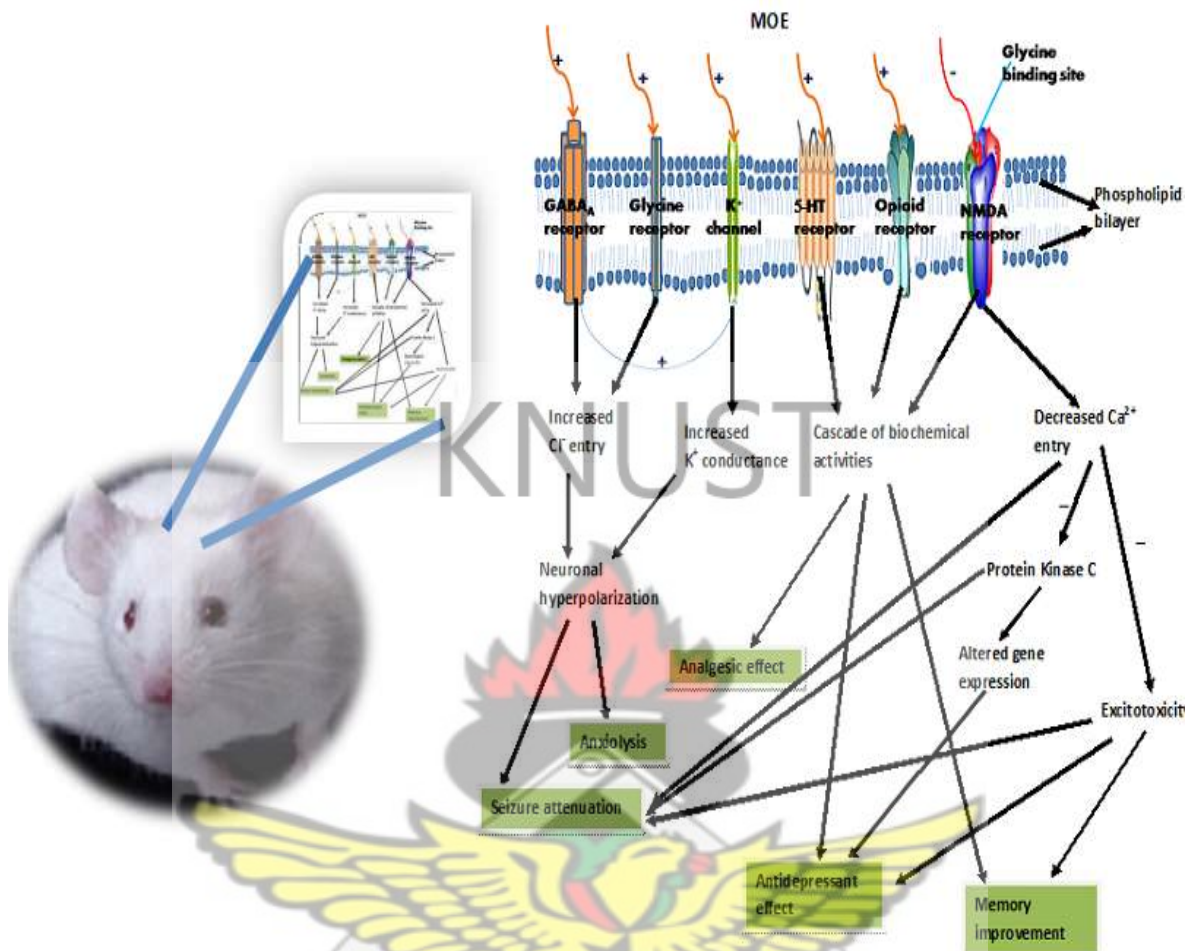


Figure 8.1 A simplified representation of the proposed mechanisms of action of the hydroalcoholic extract of the leaves of *Mallotus oppositifolius* (MOE). MOE activates GABA<sub>A</sub> and glycinergic neurotransmission causing an increase in chloride (Cl<sup>-</sup>) conductance and consequently neuronal hyperpolarization. The hyperpolarization results in seizure attenuation and anxiolysis. MOE activates K<sup>+</sup> channels leading to increased potassium conductance, hyperpolarization and decreased neuronal excitation (decreased excitotoxicity). This also contributes to attenuation of seizures. GABA activation can result in increased potassium conductance leading to the anticonvulsant effect observed. MOE also antagonises NMDA receptors or pathway leading to a decrease in Ca<sup>2+</sup> entry. Decreased Ca<sup>2+</sup> entry inhibits calcium dependent excitotoxicity which can also contribute to the anticonvulsant, antidepressant effects as well as improvement in memory. The decreased Ca<sup>2+</sup> entry can also lead to altered gene expressions resulting in antidepressant effect. The interaction of MOE with the 5-HT-opioid-glycine/NMDA pathways leads to a cascade of signal transduction mechanisms which are responsible for the observed antidepressant effect, analgesic and memory improvement.

## 8.2 CONCLUSION

It is evident from the research conducted that the hydroalcoholic leaf extract of *Mallotus oppositifolius* has anticonvulsant, anxiolytic, and rapid sustained antidepressant properties. The extract also improved spatial learning and memory and showed analgesic effect. The extract is an inducer of liver metabolising enzymes and serves as a substrate to these enzymes.

- Enhancement of GABAergic and glycinergic neurotransmission as well as increase in potassium conductance and inhibition of glutamatergic activity was responsible for the anticonvulsant effect observed.
- Interaction with central serotonergic-opioid-glycine/NMDA pathways accounted for the rapid antidepressant effect observed. These mechanisms may also explain the analgesic properties of the extract in the tail immersion test. Enhancement of 5HT<sub>2A</sub> and inhibitory interaction with the strychnine insensitive glycine site of the NMDA receptor may be responsible for the memory improvement properties of the extract.
- Interplay between enhancement of GABAergic and serotonergic neurotransmission may support the role of the extract in anxiety states.

### 8.3 RECOMMENDATIONS

- The extract should be fractionated to isolate the compounds responsible for the observed pharmacological effects especially the compound(s) responsible for the rapid and sustained antidepressant effect since there is so far no drug approved with such effect. Also animal models such as genetic absence epilepsy rats from Strasbourg (GAERS) or lethargic mice should be used to further confirm the possible anti-absence properties of the extract observed in the PTZ seizure test.
- Chronic toxicity should be carried out to establish the safety of the extract.
- This work should be repeated in non-human primates in order to assess and/or confirm the scientific information gathered or discover those that were not apparent in this study.
- Further isobolographic analysis of the extract and diazepam should be carried out to elucidate the exact mechanism by which their combination demonstrated synergism.
- Other pain models should be used to fully characterize the antinociceptive properties of the extract and its mode of action`



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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	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Convulsions	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Tremor	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Straub	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Sedation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	++	++	++	++	++	0
Excitation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Abnormal gait(rolling)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Abnormal gait(tiptoe)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Jumps	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Motor incoordination	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	+	+	+	++	+	0
Loss of balance	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Fore-paw treading	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Writhes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Piloerection	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Stereotypies(sniffing)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Stereotypies(chewing)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Stereotypies(head movements)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Head twitches	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Scratching	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Respiration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Aggressiveness	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Fear	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	
Reactivity to touch	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	

Muscle tone	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Loss of writhing reflex	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Ptosis	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Exophthalmos	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Loss of grasping	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Akinesia	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Catalepsy	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Loss of traction	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Loss of corneal reflex	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Analgesia	#	0	0	0	0	0	0	#	+	++	++	++	++	0	#	+	++	++	++	++	++	0	#	++	++	++	++	++
Defaecation	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Salivation	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Lacrimation	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Urination	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Change in Rectal temperature	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0

Dose (mg/kg)	1000							3000						
Time(min)	0-15	15	30	60	120	180	24h	0-15	15	30	60	120	180	24h
Lethality	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Convulsions	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tremor	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Straub	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sedation	+	++	++	++	++	++	0	+	++	++	++	++	++	0
Excitation	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Abnormal gait(rolling)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Abnormal gait(tiptoe)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Jumps	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Motor incoordination	#	++	++	++	++	++	0	#	++	++	++	++	++	0
Loss of balance	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fore-paw treading	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Writhes	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Piloerection	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Stereotypies(sniffing)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Stereotypies(chewin	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Stereotypies(head movements)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Head twitches	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Scratching	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Respiration	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aggressiveness	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fear	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Reactivity to touch	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Muscle tone	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Loss of writhing reflex	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Ptosis	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Exophthalmos	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Loss of grasping	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Akinesia	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Catalepsy	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Loss of traction	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Loss of corneal refle	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Analgesia	#	++	+++	+++	++	++	0	#	++	++	++	+++	+++	0
Defaecation	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Salivation	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Lacrimation	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Urination	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Change in Rectal temperature	#	0	0	0	0	0	0	#	0	0	0	0	0	0

MOE was administered at 30, 100, 300, 1000 and 3000 mg kg<sup>-1</sup>, *p.o.*; 5 rats per group were used. Data is presented as the number of animals showing symptoms during the test, with an indication of intensity for sedation, analgesia, fear, reactivity to touch, defaecation, urination, salivation and lacrimation ( + = slight increase, ++ = moderate increase, +++=marked increase). Observations were performed at 15, 30, 60, 120, 180 min and 24 hr after administration. The symptoms that did not necessitate handling were also observed up to 15 min immediately following administration. # Parameters not measured.