

**EVALUATION OF ANTICONVULSANT, SEDATIVE AND ANXIOLYTIC ACTIONS  
OF *JATROPHA CURCAS* AND *ALLIUM SATIVUM* IN MICE.**

**OSASAN, JOSEPHINE YETUNDE**

**2014**

**EVALUATION OF ANTICONVULSANT, SEDATIVE AND ANXIOLYTIC ACTIONS  
OF *JATROPHA CURCAS* AND *ALLIUM SATIVUM* IN MICE.**

**OSASAN, JOSEPHINE YETUNDE**

**B.PHARM., M.SC. (PHARMACOLOGY), (IFE)**

**(PHP/08/09/H/1859)**

**A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE  
AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY (PHARMACOLOGY)**

**IN THE**

**DEPARTMENT OF PHARMACOLOGY**

**FACULTY OF PHARMACY**

**OBAFEMI AWOLOWO UNIVERSITY,**

**ILE-IFE, NIGERIA.**

**2014**

**OBAFEMI AWOLOWO UNIVERSITY****HEZEKIAH OLUWASANMI LIBRARY****POSTGRADUATE THESIS****AUTHORIZATION COPY****AUTHOR:** OSASAN, JOSEPHINE YETUNDE**TITLE:** Evaluation of Anticonvulsant, Sedative and Anxiolytic Actions of  
*Jatropha curcas* and *Allium sativum* in mice.**DEGREE:** Ph. D (Pharmacology)**YEAR:** 2014

I, OSASAN, J.Y., hereby authorize the Hezekiah Oluwasanmi Library to copy my thesis, in whole or in part, in response to request from individual researchers or organization for the purpose of private study or research.

.....

Signature

.....

Date

### DEDICATION

This dissertation is dedicated to the Almighty God who made all things possible, my husband and our children Jesutofunmi and Oluwabukunmi.

OBAFEMI AWOLOWO UNIVERSITY

### CERTIFICATION

We hereby certify that this work titled, ‘’ Evaluation of Anticonvulsant, Sedative and Anxiolytic actions of *Jatropha curcas* and *Allium sativum* in mice’’ was carried out in the Department of Pharmacology, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria by OSASAN, Josephine Yetunde under our supervision:

-----  
Dr. M.A. Akanmu

(Supervisor)

Department of Pharmacology

Faculty of Pharmacy,

Obafemi Awolowo University, Ile-Ife

-----  
Prof. A.A. Elujoba

(Co-Supervisor)

Department of Pharmacognosy

Faculty of Pharmacy,

Obafemi Awolowo University, Ile-Ife

## ACKNOWLEDGEMENTS

I wish to express my deep appreciation to the Almighty God for giving me the privilege to start and complete this programme.

My appreciation goes to my supervisor Dr. M.A. Akanmu, for his various invaluable and selfless supervision and contributions, which made this dissertation a reality. I am grateful for your understanding and untiring efforts towards achieving success. Special thanks to my co-supervisor, Prof. A.A. Elujoba for his fatherly care and wonderful input to this work and his constant and useful advice during this period.

My sincere gratitude also goes to all my Lecturers and colleagues in the Department namely Drs. O.R Ilesanmi, E.O. Iwalewa, I.A. Oyemitan, O.I. Adeyemi, N.O. Omisore, G. Olayiwola, M.O. Daniyan, Mr. J.A. Akingbasote, and Miss O.J. Olanipekun for their timely support and contributions. To the Technical and Administrative divide of the Department Mr. E.A. Adeyemi, Mrs. J.O. Omotayo, Mr. S.L. Ajibike, Mr. A.O. Olayioye, Mr A.O. Owolabi, Mrs. J.A. Oni, Miss G.B. okunlola, Mr. B.C. Onifade, Mr. O.R. Akinwale, Mr. I.O. Ilesanmi, Mrs. O. Adeoye, Mrs. O.E. Ayoade Mr. S.A. Okunade and Miss. Alaba. I say a big thank you.

I want to also express my gratitude to all the Ph.D students in the Department especially those in the laboratory when I was carrying out this research, Mr. A.T. Adegbuyi, Dr. Nwonu, Miss R.M. Sunday, Mr. J.K. Olaonipekun, Mr. S.S. Agboola and Mr. L.A. Akinpelu.

To my Spiritual father and mentor Dr. J. B. Alla (Daddy Joe) and the entire Charismatic Renewal Ministries Inc. Rehoboth Family, thank you for your ceaseless prayers and

encouragements. To all the people that I cannot mention their names one by one but have supported me during this period, I say thank you and God bless you all.

To my parents, Late Mr. E. A. & Mrs. V. A. Adeniyi and Mr. R. A. & Mrs. F. M. Osasan, I want to bless God for you, for your lives and the rarity of your species, I am grateful for having you. To my siblings and their families Mr & Mrs Jide Adeniyi, Auntie Sade, Mr. & Mrs. Wale Adeniyi, Pastor and Mrs Tayo Ibiyeye, Mr. & Mrs. Bode Adeniyi, Dr. & Mrs. Olugbenga Alfred and Mr. Kunle Osasan, I say you are the best.

Finally, I will like to express my unending gratitude and appreciation to my sweetheart, friend and mentor Dr. S. A. Osasan for his unflinching support, patience, love and understanding right from the outset. I am grateful for having you as my better half and to my children Jesutofunmi and Oluwabukunmi for their understanding throughout the period of this work. God bless you all.

## TABLE OF CONTENTS

### Pages

Title

i

Authorization

ii

Dedication

iii

Certification

iv

Acknowledgement

v

Table

of

Contents

vii

List

of

Tables

xx



List	of	Figures
xxiii		

List	of	Abbreviations
xxvi		

## Abstract

xxviii

## CHAPTER ONE

1.0	Introduction
-----	--------------

1

1.1

Literature Review	3
-------------------	---

1.1.1

Seizures	3
----------	---

1.1.1.1

Definition of Seizures	3
------------------------	---

1.1.1.2

Epidemiology of Seizures	3
--------------------------	---

1.1.1.3

Etiology of Seizures	4
----------------------	---

1.1.1.4

Classification of Seizures	5
----------------------------	---

1.1.1.4.1

Partial seizures	5
------------------	---

1.1.1.4.1.a	
Simple partial seizures	6
1.1.1.4.1.b	
Complex partial seizures	7
1.1.1.4.1.c	
Secondary generalized seizures	7
1.1.1.4.2	
Generalized seizures	7
1.1.1.4.2.a	
Tonic-Clonic seizures (grand mal)	8
1.1.1.4.2.b	
Absence seizure (Petit mal)	8
1.1.1.4.2.c	
Myoclonic seizure	9
1.1.1.4.2.d	
Atonic seizures	9
1.1.1.4.2.e	
Status epilepticus seizure	9
1.1.1.5	
Diagnostic tests for seizures	9
1.1.1.5.a	
Functional magnet resonance imaging (fMRI)	10
1.1.1.5.b	



Positron emissions tomography (PET)	11
1.1.1.5.c	
Ictal SPECT	11
1.1.1.5.d	
Magnetoencephalography (MEG)	11
1.1.1.5.e	
Wada test	12
1.1.1.6	
Treatment of seizures	12
1.1.1.6.a	
Non Pharmacological Management	12
1.1.1.6.b	
Pharmacological treatment	13
1.1.1.7	
Mechanism of action of antiepileptic drug (AED)	13
1.1.1.8.	
Classification of AEDS	15
1.1.1.8.a	
Classification of AEDS based on chemical group	15
1.1.1.8.b	
Classification of AEDS based on therapeutic uses	16
1.1.1.9	
Vagal nerve stimulation	16

1.1.1.10	
Adverse Effects of Antiepileptic Drugs	16
1.1.1.11	
Experimental Animal Models of Seizures	19
1.1.1.12	
Mechanism of Action of Convulsing Agents	19
1.1.1.12.a	Pentylentetrazol (PTZ)
19	
1.1.1.12.b	
Picrotoxin (PTX)	21
1.1.1.12.c	
Strychnine (STR)	23
1.1.1.12.d	
Pilocarpine	26
1.1.1.12.e	
Maximum Electroshock (MES)	28
1.1.2	
Sleep	29
1.1.2.1	
The order of sleep	31
1.1.2.2	
Homeostatic and Circadian regulation of sleep	32
1.1.2.3	

Functions of Sleep	33
1.1.2.3.a	
Restorative function of sleep	33
1.1.2.3.b	
Function of sleep in memory	34
1.1.2.3.c	
Thermoregulation	34
1.1.2.4	
Neurotransmitters and neurons involved in the process of sleep	34
1.1.2.5	
Effect of drugs on sleep	36
1.1.2.6	
Role of hypothalamus in sleep	37
1.1.2.7	
Sleep deprivation	38
1.1.2.8	
Epidemiology of sleep disorder	39
1.1.2.9	
Types of sleep disorders	39
1.1.2.10	
Treatment of sleep disorders	43
1.1.2.11	
Sodium pentobarbitone – induced sleep	44

1.1.3	
Anxiety	45
1.1.3.1	
Definition of anxiety	45
1.1.3.2	
Prevalence of anxiety disorder	45
1.1.3.3	
Causes of anxiety	46
1.1.3.4	
Symptoms and types of anxiety	46
1.1.3.5	
Treatment and management of anxiety disorders	49
1.1.3.5.a	
Antianxiety drugs	50
1.1.3.5.b	
Psychotherapy	52
1.1.3.6	
Experimental animal models of anxiety	52
1.1.4	
<i>JATROPHA CURCAS</i> LINN (EUPHORBIACEAE)	
	56
1.1.4.1	
Taxonomy of <i>Jatropha curcas</i> .	
	56
1.1.4.2	



Botanical features	56
1.1.4.3	
Ethnomedicinal uses	58
1.1.4.4	
Previous studies on <i>J. curcas</i>	59
1.1.4.5 Mineral and nutritive value of <i>J. curcas</i>	
61	
1.1.4.6	
Neuropharmacological Profile of <i>J. podagrica</i>	61
1.1.5	
<i>Allium sativum</i> Linn (Lilliaceae)	61
1.1.5.1	
Taxonomy of <i>A. Sativum</i> Linn.	61
1.1.5.2	
Mineral and Nutritive Value of <i>A. sativum</i>	62
1.1.5.3	
Ethno medicinal uses of <i>A. sativum</i>	62
1.1.5.4	
Previous studies on <i>A. sativum</i>	64
1.1.5.5 Adverse effects of <i>A. sativum</i>	
66	
1.1.6	
Objectives of the study	66

## 1.1.7

The significance of the studies	67
---------------------------------	----

**CHAPTER TWO**

## 2.0

Materials and Methods	68
-----------------------	----

## 2.1

Collection of plant materials and identification	68
--	----

## 2.2

Extraction	68
------------	----

## 2.3

Animals	68
---------	----

## 2.4

Drugs	69
-------	----

## 2.5

Laboratory equipment and reagents	69
-----------------------------------	----

## 2.6

Acute toxicity test	69
---------------------	----

## 2.7

Evaluation of anxiolytic activity	70
-----------------------------------	----

## 2.7.1

Open field test	70
-----------------	----

## 2.7.1.a





Anxiolytic Effect of <i>J. curcas</i> extractive in OFT	71
2.7.1.b	
Anxiolytic effect of <i>A. sativum</i> extractive in OFT	71
2.7.2. Elevated	
plus-maze test (EPM)	71
2.7.2.a	
Anxiolytic effect of <i>J. curcas</i> in EPM	72
2.7.2.b	
Anxiolytic effect of <i>A. sativum</i> in EPM	72
2.7.3	
Hole- board test (HB)	73
2.7.3.a	
Anxiolytic effect of <i>J. curcas</i> extractive in HB	73
2.7.3.b	
Anxiolytic effect of <i>A. sativum</i> extractive in HB	73
2.8	
Hypnotic activity	74
2.8.1	
Sodium pentobarbitone - induced sleeping time	74
2.8.1.a	



Hypnotic effect of *J. curcas* extractive

74

2.8.1.b

Hypnotic effect of *A. sativum* extractive

74

2.9

Assessment of anticonvulsant activity

75

2.9.1

Effect of *J. curcas* extractive on strychnine - induced seizure

75

2.9.2

Effect of *J. curcas* on pentylenetetrazol - induced seizures

75

2.9.3

Effect of *J. curcas* on picrotoxin - induced

76

2.9.4

Effect of *J. curcas* on pilocarpine - induced seizures

76

2.9.5

Effect of *J. curcas* on MES - induced seizures

77

2.9.6

Effect of *A. sativum* extractive on Strychnine - induced seizure

77

2.9.7

Effect of *A. sativum* on pentylenetetrazol - induced seizures

78

2.9.8

Effect of *A. sativum* on picrotoxin - induced seizures

78

2.9.9



Effect of <i>A. sativum</i> on pilocarpine - induced seizures	79
2.9.10	
Effect of <i>A. sativum</i> on MES - induced seizures	79
2.10	
Fractionation of <i>J. curcas</i> extractive	80
2.11	
Anxiolytic activities of the various fractions	80
2.11.1.	
Open Field Test	80
2.11.1.a	
Anxiolytic effect of n-hexane fraction of <i>J. curcas</i> in OFT	80
2.11.1.b	
Anxiolytic effect of ethyl acetate fraction of <i>J. curcas</i> in OFT	81
2.11.1.c	
Anxiolytic effect of butanol fraction of <i>J. curcas</i> using OFT	81
2.11.2	
Elevated plus-maze test	82
2.11.2.a	
Anxiolytic Effect of Hexane fraction of <i>J. curcas</i> in EPM	82
2.11.2.b	

Anxiolytic Effect of Ethyl acetate fraction of <i>J. curcas</i> in EPM	82
2.11.2.c	
Anxiolytic Effect of Butanol fraction of <i>J. curcas</i> in EPM	83
2.11.3	
Hole-board test	83
2.11.3.a	
Anxiolytic effect of n-hexane fraction of <i>J. curcas</i> in HB	83
2.11.3.b	
Anxiolytic effect of ethyl acetate fraction of <i>J. curcas</i> in HB	83
2.11.3.c	Anxiolytic effect of butanol fraction of <i>J. curcas</i> in HB
84	
2.12	Hypnotic activity
84	
2.12.1	Sodium pentobarbitone - induced sleeping time
84	
2.12.1.a	Hypnotic effect of n-hexane fraction of <i>J. curcas</i>
84	
2.12.1.b	Hypnotic effect of ethyl acetate fraction of <i>J. curcas</i>
85	



2.12.1.c	Hypnotic Effect of Butanol fraction of <i>J. curcas</i>	85
2.13	Assessment of anticonvulsant activity of the various fractions	85
2.13.1.	Effect of n-hexane fraction of <i>J. curcas</i> on PTZ - induced seizures	85
2.13.2	Effect of ethyl acetate fraction of <i>J. curcas</i> on PTZ - induced seizures	86
2.13.3	Effect of butanol fraction of <i>J. curcas</i> on PTZ - induced seizures	86
2.13.4	Effect of n-hexane fraction of <i>J. curcas</i> on PTX - induced seizures	86
2.13.5	Effect of ethyl acetate fraction of <i>J. curcas</i> on PTX - induced seizures	87
2.13.6	Effect of butanol fraction of <i>J. curcas</i> on PTX - induced seizures	87
2.13.7	Effect of n-hexane fraction of <i>J. curcas</i> on PILO - induced seizures	87
2.13.8	Effect of ethyl acetate fraction of <i>J. curcas</i> on PILO - induced seizures	88
2.13.9	Effect of butanol fraction of <i>J. curcas</i> on PILO - induced seizures	88

2.14	Mechanism of action of ethyl acetate fraction of <i>J. curcas</i>
88	

2.14.1	Effect of flumazenil on the anticonvulsant activity of Ethylacetate fraction of <i>J. curca</i>
88	

2.15	Statistical analysis
89	

### CHAPTER THREE

3.0	Results
90	

3.1	Acute Toxicity Test
90	

3.2	Anxiolytic Activity of crude methanolic extracts of <i>J. curcas</i> and <i>A. sativum</i>
90	

3.2.1	Open Field Test
90	

3.2.1.a	Anxiolytic effect of oral administration of <i>J. curcas</i> on Locomotor activity in OFT in mice.
90	

3.2.1.b	Anxiolytic effect of oral administration of <i>J. curcas</i> on Rearing activity in OFT in mice.
91	

3.2.1.c	Anxiolytic effect of oral administration of <i>J. curcas</i> on Grooming activity in OFT in mice.
91	

3.2.1.d	Anxiolytic effect of i.p. administration of <i>J. curcas</i> on Locomotor activity in OFT in mice.
91	



- 3.2.1.e Anxiolytic effect of i.p. administration of *J. curcas* on Rearing activity in OFT in mice 91
- 3.2.1.f Anxiolytic effect of i.p. administration of *J. curcas* on Grooming activity in OFT in mice. 92
- 3.2.1.g Anxiolytic effect of oral administration of *A. sativum* on Locomotor activity in OFT in mice 92
- 3.2.1.h Anxiolytic effect of oral administration of *A. sativum* on rearing activity in OFT in mice. 92
- 3.2.1.i Anxiolytic effect of oral administration of *A. sativum* on grooming activity in OFT in mice 92
- 3.2.1.j Anxiolytic effect of i.p. administration of *A. sativum* on Locomotor activity in OFT in mice 93
- 3.2.1.k Anxiolytic effect of i.p. administration of *A. sativum* on Rearing activity in OFT in mice. 93
- 3.2.1.l Anxiolytic effect of i.p. administration of *A. sativum* on Grooming activity in OFT in mice. 93
- 3.2.2 Anxiolytic Test on the Elevated Plus Maze (EPM) 98
- 3.2.2.a Effects of oral administration of *J. curcas* on Number of Open Arms Entries in EPM in mice 98
- 3.2.2.b Effects of oral administration of *J. curcas* on Duration in the open arms on the EPM in mice. 98



- 3.2.2.c Effects of oral administration of *J. curcas* on index of open arm avoidance on the EPM in mice. 98
- 3.2.2.d Effects of i.p. administration of *J. curcas* on Number of open Arms Entries in EPM in mice. 100
- 3.2.2.e Effects of i.p. administration of *J. curcas* on Duration in the open arms on the EPM in mice. 100
- 3.2.2.f Effects of i.p. administration of *J. curcas* on index of open arm avoidance on the EPM in mice. 100
- 3.2.2.g Effects of oral administration of *A. sativum* on Number of Open Arms Entries in EPM in Mice. 102
- 3.2.2.h Effects of oral administration of *A. sativum* on Duration in the open arms on the EPM in mice. 102
- 3.2.2.i Effects of oral administration of *A. sativum* on index of open arm avoidance on the EPM in mice. 102
- 3.2.2.j Effects of i.p. administration of *A. sativum* on Number of Open Arms Entries in EPM in mice. 104
- 3.2.2.k Effects of i.p. administration of *A. sativum* on Duration in the Open Arms on the EPM in mice. 104
- 3.2.2.l Effects of i.p. administration of *A. sativum* on index of open arm avoidance on the EPM in mice. 104
- 3.2.3. Holeboard (HB)  
106





- 3.2.3.a Effect of oral administration of *J. curcas* on number of head dips in HB in mice.  
106
- 3.2.3.b Effect of intraperitoneal administration of *J. curcas* on number of head dips in HB in mice 106
- 3.2.3.c Effect of oral administration of *A. sativum* on number of head dips in HB in mice  
106
- 3.2.3.d Effect of intraperitoneal administration of *A. sativum* on number of head dips in HB in mice 106
- 3.3. Hypnotic Activity  
111
- 3.3.a Effect of oral administration of *J. curcas* on sleep latency and duration of sleep in sodium pentobarbitone - induced sleeping time in mice.  
111
- 3.3.b Effect of intraperitoneal administration of *J. curcas* on sleep latency and duration of sleep in sodium pentobarbitone - induced sleeping time in mice  
111
- 3.3.c Effect of oral administration of *A. sativum* on sleep latency and duration of sleep in sodium pentobarbitone - induced sleeping time in mice  
114
- 3.3.d Effect of i.p. administration of *A. sativum* on sleep latency and duration of sleep in sodium pentobarbitone - induced sleeping time in mice  
114



3.4	Anticonvulsant effects
117	
3.4.1.1.a	Effects of oral administration of <i>J. cursas</i> on clonic and tonic seizures following strychnine (STR) (4 mg/kg) induction in mice
117	
3.4.1.1.b	Effects of intraperitoneal administration of <i>J. cursas</i> on on clonic and tonic seizures following strychnine (STR) (4 mg/kg) induction in mice
117	
3.4.1.2.a	Effects of oral administration of <i>J. cursas</i> on clonic and tonic seizures following pentylenetetrazole (PTZ) (85 mg/kg) induction in mice
117	
3.4.1.2.b	Effects of intraperitoneal administration of <i>J. cursas</i> on clonic and tonic seizures following pentylenetetrazole (PTZ) (85 mg/kg) induction in mice
118	
3.4.1.3.a	Effects of oral administration of <i>J. cursas</i> on clonic and tonic seizures following picrotoxin (PTX) (10 mg/kg) induction in mice
118	
3.4.1.3.b	Effects of intraperitoneal administration of <i>J. cursas</i> on clonic and tonic seizures following picrotoxin PTX (10 mg/kg) induction in mice
118	
3.4.1.4.a	Effects of oral administration of <i>J. cursas</i> on seizure latency and duration of seizure following pilocarpine (PILO) (300 mg/kg) induction in mice
125	



- 3.4.1.4.b Effects of intraperitoneal administration of *J. cursas* MJCL on seizure latency and duration of seizure following pilocarpine (PILO) (300 mg/kg) induction in mice  
125
- 3.4.1.5.a Effects of oral administration of *J. cursas* on onset and duration of hind limb tonic extension (HLTE) in maximal electroshock (MES) induction in mice  
125
- 3.4.1.5.b Effects of intraperitoneal administration of *J. cursas* on onset and duration of hind limb tonic extension (HLTE) in maximal electroshock (MES) induction in mice  
126
- 3.4.2.1.a Effects of oral administration of *A. sativum* on clonic and tonic seizures following strychnine (STR) (4 mg/kg) induction in mice.  
126
- 3.4.2.1.b Effects of intraperitoneal administration of *A. sativum* on on clonic and tonic seizures following strychnine (STR) (4 mg/kg) induction in mice  
126
- 3.4.2.2.a Effects of oral administration of *A. sativum* on clonic and tonic seizures following pentylenetetrazole (PTZ) (85 mg/kg) induction in mice.  
133
- 3.4.2.2.b Effects of intraperitoneal administration of *A. sativum* on clonic and tonic seizures following pentylenetetrazole (PTZ) (85 mg/kg) induction in mice.  
133
- 3.4.2.3.a Effects of oral administration of *A. sativum* on clonic and tonic seizures following



picrotoxin (PTX) (10 mg/kg) induction in mice.

133

3.4.2.3.b Effects of intraperitoneal administration of *A. sativum* on clonic and tonic seizures following picrotoxin PTX (10 mg/kg) induction in mice.

134

3.4.2.4.a Effects of oral administration of *A. sativum* on seizure latency and duration of seizure following pilocarpine (PILO) (300 mg/kg) induction in mice.

134

3.4.2.4.b Effects of intraperitoneal administration of *A. sativum* on seizure latency and duration of seizure following pilocarpine (PILO) (300 mg/kg) induction in mice.

134

3.4.2.5.a Effects of oral administration of *A. sativum* on onset and duration of hind limb tonic extension (HLTE) in maximal electroshock (MES) induction in mice.

141

3.4.2.5.b Effects of intraperitoneal administration of *A. sativum* on onset and duration of hind limb tonic extension (HLTE) in maximal electroshock (MES) induction in mice.

141

3.5 Anxiolytic Activity of the various fractions

144

3.5.1 Open Field Test

144

3.5.1a Effects of intraperitoneal administration of n-hexane fraction of methanolic leaf extract of

*J. curcas* (MJC<sub>L</sub>) on locomotor, rearing and grooming activities in OFT in mice

144

3.5.1b Effects of intraperitoneal administration of ethyl acetate fraction of methanolic leaf extract of

*J. curcas* (MJC<sub>L</sub>) on locomotor, rearing and grooming activities in OFT in mice

144

3.5.1c Effects of intraperitoneal administration of butanol fraction of methanolic leaf extract of

*J. curcas* (MJC<sub>L</sub>) on locomotor, rearing and grooming activities in OFT in mice

144

3.5.2 Elevated Plus Maze

148

3.5.2.a Effect of intraperitoneal administration of n-hexane fractions of *J. curcas* (MJC<sub>L</sub>) on Percentage Number of Open Arms Entries, duration in open arms and index of open arm

avoidance in EPM in mice

148

3.5.2.b Effect of intraperitoneal administration of ethyl acetate fraction of *J. curcas* (MJC<sub>L</sub>) on Percentage Number of Open Arms Entries, duration in open arms and index of open arm



avoidance in EPM in mice

148

3.5.2.c Effect of intraperitoneal administration of butanol fraction of *J. curcas* (MJC<sub>L</sub>)  
on Percentage Number of Open Arms Entries, duration in open arms and index of  
open arm

avoidance in EPM in mice

148

3.5.3. Holeboard (HB)

152

3.5.3.a Effect of intraperitoneal administration of n-hexane, ethyl acetate and butanol  
fractions

of methanolic leaf extract of *J. curcas* (MJC<sub>L</sub>) on number of head dips in HB in mice.

152

3.6. Hypnotic Activity

152

3.6.a Effect of intraperitoneal administration of n-hexane (HF) fraction of methanolic leaf  
extract

of *J. curcas* (MJC<sub>L</sub>) on sleep latency and duration of sleep in sodium pentobarbitone

- induced sleeping time in mice.

152

3.6.b Effect of intraperitoneal administration of ethyl acetate fraction of MJC<sub>L</sub> on sleep  
latency



- and duration of sleep in sodium pentobarbitone - induced sleeping time in mice
- 152
- 3.6.c Effect of intraperitoneal administration of butanol fraction of MJC<sub>L</sub> on sleep latency and duration of sleep in sodium pentobarbitone - induced sleeping time in mice
- 153
- 3.7 Anticonvulsant activity of the various fraction of MJC<sub>L</sub>
- 153
- 3.7.1 Effects of intraperitoneal administration of n-hexane (HF), ethyl acetate (EAF) and butanol (BF) of methanolic leaf extract of *J. curcas* MJC<sub>L</sub> on clonic and tonic seizures following pentylenetetrazole (PTZ) (85 mg/kg) induction in mice.
- 153
- 3.7.2 Effects of intraperitoneal administration of n-hexane (HF), ethyl acetate (EAF) and butanol (BF) of methanolic leaf extract of *J. curcas* (MJC<sub>L</sub>) on clonic and tonic seizures following picrotoxin (PTX) (10 mg/kg) induction in mice.
- 154
- 3.7.3 Effects of intraperitoneal administration of n-hexane (HF), ethyl acetate (EAF) and butanol (BF) of methanolic leaf extract of *J. curcas* (MJC<sub>L</sub>) on clonic and tonic seizures following pilocarpine (PILO) (300 mg/kg) induction in mice.
- 167
- 3.8 Effect of flumazenil on the anticonvulsant activity of ethyl acetate fraction of methanolic leaf extract of *J. curcas* MJC<sub>L</sub> following pentylenetetrazole (85 mg/kg) induction in mice
- 167

**CHAPTER FOUR****4.1. DISCUSSION AND CONCLUSION**

173

**4.1.1 Discussion**

173

**4.1.2 Conclusion**

188

**REFERENCES**

189

**APPENDICES**



## LIST OF TABLES

### Table

### Pages

Table 1:	Classification of antiepileptic drugs based on mechanism of action.	13
Table 2:	The various AEDS, their mechanisms of action and their therapeutic/clinical applications	17
Table 3:	The various AEDS, their dosage, adverse effects and contraindications.	18
Table 4:	Types of Anxiety Disorders that has to do with cognition, behavioural and physical manifestation	49
Table 5:	Effects of oral administration of MJC <sub>L</sub> on clonic and tonic seizures following strychnine (STR) (4 mg/kg) induction in mice.	119
Table 6:	Effects of intraperitoneal administration of MJC <sub>L</sub> on clonic and tonic seizures following strychnine (STR) (4 mg/kg) induction in mice.	120
Table 7:	Effects of oral administration of MJC <sub>L</sub> on clonic and tonic seizures following pentylenetetrazole (PTZ) (85 mg/kg) induction in mice .	121



Table 8: Effects of intraperitoneal administration of  $MJC_L$  on clonic and tonic seizures following pentylenetetrazole (PTZ) (85 mg/kg) induction in mice.

122

Table 9: Effects of oral administration of  $MJC_L$  on clonic and tonic seizures following picrotoxin (PTX) (10 mg/kg) induction in mice.

123

Table 10: Effects of intraperitoneal administration of  $MJC_L$  on clonic and tonic seizures following picrotoxin PTX (10 mg/kg) induction in mice.

124

Table 11: Effects of oral administration of  $MJC_L$  on seizure latency and duration of seizure following pilocarpine (PILO) (300 mg/kg) induction in mice

127

Table 12: Effects of intraperitoneal administration of  $MJC_L$  on seizure latency and duration of seizure following pilocarpine (PILO) (300 mg/kg) induction in mice.

128

Table 13: Effects of oral administration of  $MJC_L$  on onset and duration of hind limb tonic extension (HLTE) in maximal electroshock (MES) induction in mice.

129

Table 14: Effects of intraperitoneal administration of  $MJC_L$  on onset and duration of hind limb tonic extension (HLTE) in maximal electroshock (MES) induction in mice.

130

Table 15 Effects of oral administration of  $MAS_B$  on clonic and tonic seizures following



strychnine (STR) (4 mg/kg) induction in mice.

131

Table 16: Effects of intraperitoneal administration of MAS<sub>B</sub> on clonic and tonic seizures following strychnine (STR) (4 mg/kg) induction in mice.

132

Table 17: Effects of oral administration of MAS<sub>B</sub> on clonic and tonic seizures following pentylenetetrazole (PTZ) (85 mg/kg) induction in mice.

135

Table 18: Effects of intraperitoneal administration of MAS<sub>B</sub> on clonic and tonic seizures following pentylenetetrazole (PTZ) (85 mg/kg) induction in mice.

136

Table 19: Effects of oral administration of MAS<sub>B</sub> on clonic and tonic seizures following picrotoxin (PTX) (10 mg/kg) induction in mice.

137

Table 20: Effects of intraperitoneal administration of MAS<sub>B</sub> on clonic and tonic seizures following picrotoxin PTX (10 mg/kg) induction in mice.

138

Table 21: Effects of oral administration of MAS<sub>B</sub> on seizure latency and duration of seizure following pilocarpine (PILO) (300 mg/kg) induction in mice.

139

Table 22: Effects of intraperitoneal administration of MAS<sub>B</sub> on seizure latency and duration of seizure following pilocarpine (PILO) (300 mg/kg) induction in mice.

140

- Table 23: Effects of oral administration of MAS<sub>B</sub> on onset and duration of hind limb tonic extension (HLTE) in maximal electroshock (MES) induction in mice. 142
- Table 24: Effects of oral administration of MAS<sub>B</sub> on onset and duration of hind limb tonic extension (HLTE) in maximal electroshock (MES) induction in mice 143
- Table 25: Effects of intraperitoneal administration of n-hexane (HF) of MJC<sub>L</sub> on clonic and tonic seizures following pentylenetetrazole (PTZ) (85 mg/kg) induction in mice. 161
- Table 26: Effects of intraperitoneal administration of ethyl acetate (EAF) of MJC<sub>L</sub> on clonic and tonic seizures following pentylenetetrazole (PTZ) (85 mg/kg) induction in mice. 162
- Table 27: Effects of intraperitoneal administration of butanol (BF) of MJC<sub>L</sub> on clonic and tonic seizures following pentylenetetrazole (PTZ) (85 mg/kg) induction in mice. 163
- Table 28: Effects of intraperitoneal administration of n-hexane (HF) of MJC<sub>L</sub> on clonic and tonic seizures following picrotoxin (PTX) (10 mg/kg) induction in mice. 164
- Table 29: Effects of intraperitoneal administration of ethyl acetate (EAF) of MJC<sub>L</sub> on clonic and tonic seizures following picrotoxin (PTX) (10 mg/kg) induction in mice. 165
- Table 30: Effects of intraperitoneal administration of butanol (BF) of MJC<sub>L</sub> on clonic and



tonic seizures following picrotoxin (PTX) (10 mg/kg) induction in mice.

166

Table 31: Effects of intraperitoneal administration of n-hexane (HF) of MJC<sub>L</sub> on clonic and tonic seizures following pilocarpine (PILO) (300 mg/kg) induction in mice.

169

Table 32: Effects of intraperitoneal administration of ethyl acetate (EAF) of MJC<sub>L</sub> on clonic and tonic seizures following pilocarpine (PILO) (300 mg/kg) induction in mice.

170

Table 33: Effects of intraperitoneal administration of butanol (BF) of MJC<sub>L</sub> on clonic and tonic seizures following pilocarpine (PILO) (300 mg/kg) induction in mice.

171

Table 34: Effects of flumazenil on the anticonvulsant activity of ethyl acetate fraction (EAF) of MJC<sub>L</sub> on clonic and tonic seizures following pentylenetetrazole (PTZ) (85 mg/kg)

induction in mice.

172

## LIST OF FIGURES

### Figure

### Pages

Figure 1:	International league Against Epilepsy Nomenclature for describing seizures	5
Figure 2:	Systematic approach of drug treatment in a newly diagnosed patient	14
Figure 3:	Anti seizures heterocyclic ring structures	15
Figure 4:	<i>Jatropha curcas</i> Linn (Euphorbiaceae)	57
Figure 5:	<i>Allium sativum</i> Linn (Liiliaceae)	63
Figure 6A-C:	Anxiolytic effect of oral administration of <i>J. curcas</i> on Locomotor, Rearing and Grooming activities in OFT in mice	94
Figure 7A-C:	Anxiolytic effect of intraperitoneal administration of <i>J. curcas</i> on locomotor, Rearing and Grooming activities in OFT in mice.	95
Figure 8A-C:	Anxiolytic effect of oral administration of MAS <sub>B</sub> on Locomotor, Rearing and Grooming activities in OFT in mice.	96

Figure 9A-C: Anxiolytic effect of intraperitoneal administration of MAS<sub>B</sub> on locomotor,  
Rearing and Grooming activities in OFT in mice  
97

Figure 10A-C: Effects of oral administration of MJC<sub>L</sub> on Percentage Number of Open Arms  
Entries, Duration in open arms and Index of open arm avoidance in EPM in mice  
99

Figure 11A-C: Effects of intraperitoneal administration of MJC<sub>L</sub> on Percentage Number of Open  
Arms  
Entries, Duration in open arms and Index of open arm avoidance in EPM in mice  
101

Figure 12A-C: Effects of oral administration of MAS<sub>B</sub> on Percentage Number of Open Arms  
Entries, Duration in open arms and Index of open arm avoidance in EPM in mice  
103

Figure 13A-C: Effects of intraperitoneal administration of MAS<sub>B</sub> on Percentage Number of open  
Arms  
Entries, Duration in open arms and Index of open arm avoidance in EPM in mice  
105

Figure 14: Effect of oral administration of MJC<sub>L</sub> on number of head dips in HB in mice  
107

Figure 15: Effect of intraperitoneal administration of MJC<sub>L</sub> on number of head dips in HB in  
mice 108

Figure 16: Effect of oral administration of MAS<sub>B</sub> on number of head dips in HB in mice  
109

Figure 17: Effect of intraperitoneal administration of MAS<sub>B</sub> on number of head dips in HB  
in mice 110

Figure 18A-B: Effect of oral administration of MJC<sub>L</sub> on sleep latency and duration of sleep in  
sodium pentobarbitone induced sleeping time in mice  
112

Figure 19A-B: Effect of intraperitoneal administration of MJC<sub>L</sub> on sleep latency and duration of  
sleep in sodium pentobarbitone - induced sleeping time in mice  
113

Figure 20A-B: Effect of oral administration of MAS<sub>B</sub> on sleep latency and duration of sleep in  
sodium pentobarbitone - induced sleeping time in mice  
115

Figure 21A-B: Effect of intraperitoneal administration of MAS<sub>B</sub> on sleep latency and duration of  
sleep in sodium pentobarbitone - induced sleeping time in mice  
116

Figure 22A-C: Effects of i.p. administration of n-hexane (HF) fraction of methanolic leaf extract  
of *J. curcas* (MJC<sub>L</sub>) on locomotor, rearing and grooming activities in OFT in  
mice 145

Figure 23A-C: Effects of i.p. administration of ethylacetate (EAF) fraction of methanolic leaf  
extract  
of *J. curcas* (MJC<sub>L</sub>) on locomotor, rearing and grooming activities in OFT in  
mice 146



Figure 24A-C: Effects of i.p. administration of butanol (BF) fraction of methanolic leaf extract

of *J. curcas* (MJC<sub>L</sub>) on locomotor, rearing and grooming activities in OFT in mice 147

Figure 25A-C: Effect of i.p. administration of n-hexane (HF) fraction of MJC<sub>L</sub> on Percentage

Number of Open Arms Entries, duration in open arms and index of open arm avoidance

in EPM in mice 149

Figure 26A-C: Effect of i.p. administration of ethyl acetate (EAS) fraction of MJC<sub>L</sub> on Percentage

Number of Open Arms Entries, duration in open arms and index of open arm avoidance

in EPM in mice 150

Figure 27A-C: Effect of i.p. administration of butanol fraction (BF) of MJC<sub>L</sub> on Percentage

Number of Open Arms Entries, duration in open arms and index of open arm avoidance

in EPM in mice 151



Figure 28: Effect of intraperitoneal administration of n-hexane (HF) fraction of  $MJC_L$  on number

of head dips in HB in mice

155

Figure 29: Effect of intraperitoneal administration of ethyl acetate fraction of  $MJC_L$  on number of

head dips in HB in mice

156

Figure 30: Effect of intraperitoneal administration of butanol fraction of  $MJC_L$  on number of head

dips in HB in mice

157

Figure 31: Effect of intraperitoneal administration of n-hexane fraction (HF) of  $MJC_L$  on sleep

latency and duration of sleep in sodium pentobarbitone induced sleeping time in

mice 158

Figure 32: Effect of intraperitoneal administration of ethyl acetate fraction (EAF) of  $MJC_L$  on sleep

latency and duration of sleep in sodium pentobarbitone induced sleeping time in

mice 159

Figure 33: Effect of intraperitoneal administration of butanol fraction (BF) of  $MJC_L$  on sleep

latency and duration of sleep in sodium pentobarbitone induced sleeping time in

mice 160



## LIST OF ABBREVIATION

1. SE - Status epilepticus
2. PTZ – Pentylenetetrazole
3. PTX - Picrotoxin
4. STR – Strychnine
5. PILO –Pilocarpine
6. MES – Maximal electroshock
7. ILAE - International League Against Epilepsy
8. GABA – Gamma amino butyric acid
9. EEG – Electroencephalogram
10. JME- Juvenile myoclonic epilepsy
11. MRI – Magnetic resonance imaging
12. MRS – Magnetic resonance spectroscopy
13. fMRI – Functional magnet resonance imaging
14. PET – Position emission tomography
15. MEG – Magnetoencaphalography
16. EMU – Epilepsy monitoring unit
17. AEDS - Antiepileptic drugs
18. FDA - Federal Drug Administration
19. CNS – Central nervous system
20. REM - rapid-eye-movement
21. NREM - non- rapid-eye-movement



22. EMG - electromyogram
23. EOG - electro-oculogram
24. SCN - suprachiasmatic nuclei
25. ICSD - International Classification of Sleep Disorders
26. OSAS - Obstructive sleep apnoea syndrome
27. PLMS - periodic limb movements in sleep
28. RBD - REM behaviour disorder
29. RLS - Restless legs syndrome
30. DLBD - Diffuse Lewy- Body disease with dementia
31. FASPS - Familial advanced sleep-phase syndrome
32. PTSD - Post-traumatic stress disorder
33. GAD - Generalized anxiety disorder
34. OCD - Obsessive-compulsive disorder
35. CBT - Cognitive-behavioural Therapy
36. OFT – open field test
37. EPM – elevated plus maze
38. HB – Hole board
39. DMSO – Dimethylsulfoxide
40. L.D. – Lethal dose
41. ANOVA – Analysis of Variance
42. SEM – Standard error of mean
43. HLTE - hind limb tonic extension
44. I.P. - Intraperitoneal

45. P.O. - Per oral

OBAFEMI AWOLOWO UNIVERSITY

## ABSTRACT

This study investigated the anticonvulsant, anxiolytic and hypnotic effects of the methanolic extracts of *Jatropha curcas* Linn (Euphorbiaceae) leaf and *Allium sativum* Linn (Liliaceae) bulb in mice. It also determined the most active fraction of the two extracts. This was with a view to providing scientific information for their ethnomedicinal claims.

The cold methanolic extracts were obtained by maceration. The LD<sub>50</sub> of the extracts were determined using Lorke's method. Doses of (*J. curcas*: 500, 1000, 1500 mg/kg, per oral (p.o.) and 50, 100, 150 mg/kg, intraperitoneal (i.p.) and *A. sativum* 500, 1000, 2000 mg/kg, p.o., and 200, 400, 800 mg/kg, i.p.) were screened for anxiolytic, hypnotic and anticonvulsant activities. The anxiolytic effect was evaluated in Open Field Test (OFT), Elevated-Plus Maze (EPM) and Holeboard (HB), the hypnotic effect of the extracts was determined in pentobarbital - induced sleep model where the sleep latency and total sleeping time were assessed, while the anticonvulsant test was carried out using strychnine (STR), pentylenetetrazole (PTZ), picrotoxin (PTX), pilocarpine (PILO) and maximum electroshock (MES) - induced convulsion models. *J. curcas* extract with most potent anticonvulsant effect was separated into n-hexane, ethylacetate and butanol fractions. Each fraction (n-hexane 10, 15, 20 mg/kg, i.p., ethylacetate 10, 20 40 mg/kg, i.p. and butanol 100, 200 300 mg/kg, i.p.). Each fraction was evaluated for anxiolytic, hypnotic and anticonvulsant effects (PTZ, PTX and PILO - the models with highest anticonvulsant protection from the earlier models). The mechanism of action of the ethylacetate fraction that showed the highest anticonvulsant activity to pentylenetetrazole - induced convulsion was evaluated using appropriate antagonist, flumazenil. The results were analysed using one-way ANOVA followed by the Student- Newman keul-test.



The oral LD<sub>50</sub> of *J. curcas* and *A. sativum* were 3808 and  $\geq 5000$  mg/kg, respectively, and interperitoneally 346 and 2154 mg/kg, respectively in mice. The LD<sub>50</sub> of n-hexane, ethylacetate and butanol fractions of *J. curcas* also gave 49, 89 and 775 mg/kg, i.p. respectively in mice. The effect of the extracts in the OFT significantly ( $p < 0.05$ ) decreased locomotor, rearing and grooming activities and in HB there was a significant ( $p < 0.05$ ) decrease in number of head dips suggesting that the extracts possessed central nervous system depressant activity. The extracts also reduced sleep latency and prolonged sleeping time. The results showed that the extract of *J. curcas* gave a better anticonvulsant protection than *A. sativum* in PTX and PTZ – induced convulsion and significantly ( $p < 0.05$ ) prolonged onset of clonic tonic seizures and death latency. In the PILO-induced convulsion the duration of seizure was significantly ( $p < 0.05$ ) reduced and death latency was significantly prolonged. The three fractions of *J. curcas* that were evaluated showed varying degrees of anxiolytic, hypnotic and anticonvulsant effects. The ethylacetate fraction was found to be the most potent compared to the n-hexane and butanol fractions. The mechanism of action of the ethylacetate fraction is due to its interaction with GABA – benzodiazepine receptors.

The study concluded that *J. curcas* and *A. sativum* possessed anxiolytic, hypnotic and anticonvulsant activities. The anticonvulsant activity of *J. curcas* was higher than that of *A. sativum*, thus providing scientific evidence in support of the traditional use of the plant in the management of epilepsy.

## CHAPTER ONE

### 1.0 INTRODUCTION

In traditional medicine, various parts of plants such as the leaf, stem, bulb and root have been used for medicinal purposes over the centuries. These parts have been claimed to possess various clinical activities such as anti-ulcer (Devi *et al.*, 2008), anti-inflammatory, laxative, analgesic, anticancer, antibacterial, wound healing, anti-stress (Baliga *et al.*, 2013), sedative (Lopes *et al.*, 2011), antipyretic (Velazquez *et al.*, 2009), antimicrobial (Liolios *et al.*, 2010), anxiolytic (Ambawade *et al.*, 2001), anti-diarrhoeal (Aniagu *et al.*, 2005), antidiabetic, antioxidant (Nain *et al.*, 2012). In various parts of the world, many medicinal plants based on folk medicine or traditional uses have been investigated. Herbal products from medicinal plants have contributed immensely to the discovery of modern drugs and can also be used as an alternative source of therapy such as antiepileptic drugs because of better safety and efficacy compared to the problems associated with orthodox antiepileptic drugs as seen in the various side effects, dose-related problems, chronic toxicity, teratogenic effects (Mathur *et al.*, 2010; Aldenkamp, 2006), and the fact that some patients are never seizure free despite the use of these modern antiepileptic drugs (AEDS) (Raza *et al.*, 2003).

Convulsion or seizure is one of the most common neurological problems in our society today. The frequency is on the increase and this can no longer be overlooked. Studies have shown that the overall incidence of epilepsy in developed societies has been found to be around 50 cases per 100,000 persons per year and on a very high side in old age (Poole *et al.*, 2000; Ropper and Brown, 2005). Out of these number of patients, only about two-third, have their seizures well controlled with currently available antiepileptic drugs while in the remaining one-third patients



seizures are refractory to treatment suggesting that seizures are not well controlled by drugs (Bialer, 2006., Perucca *et al.*, 2007). For instance, neonatal seizures which were once treated effectively with phenobarbital and phenytoin suddenly became refractory in some patients, which brought about the use of other antiepileptic drugs such as levetiracetam and topiramate (Tulloch *et al.*, 2012). Also studies have shown that refractory status epilepticus occurs when status epilepticus (SE) fails to respond to appropriate typical antiepileptic drugs used in management of epilepsy (Synowiec *et al.*, 2013). Therefore, there is need for more research to help develop new strategies and medicinal agents for treating patients who become refractory to the medications and therapeutic targets currently used (Boyd *et al.*, 2012). The use of these available antiepileptic drugs produce many undesirable adverse effects in patients, which include dose related neurotoxic effects such as drowsiness, fatigue, dizziness, mental dullness, blurry vision, incoordination, idiosyncratic adverse effects like serious rash (Stevens-Johnson Syndrome, toxic epidermal necrolysis), hematologic changes, weight gain, severe hypertrophy of gums, hepatotoxicity, bone density loss and congenital malformations for instance teratogenesis in women of reproductive age (St. Louis, 2009). Looking at these numerous side effects, there is need for the discovery and developments of new antiepileptic drugs with improve seizure control and minimal side effects.

Therefore, considering the problems associated with modern antiepileptic drugs, herbal products may be of advantage over the orthodox drugs in the management of seizure. Hence, there is need for more research into medicinal plants with possible anticonvulsant activity based on traditional use. Thus, in line with this, the following plants *Jatropha curcas* and *Allium sativum* were screened for anticonvulsant effect using behavioural models for anticonvulsant such as

pentylentetrazole (PTZ), strychnine (STR), picrotoxin (PTX), pilocarpine (PILO) and maximal electroshock (MES).

## **1.1. LITERATURE REVIEW**

### **1.1.1. Seizures**

#### **1.1.1.1 Definition of seizures**

Seizures are central nervous system disorder characterized by sudden, excessive and abnormal electrical discharge from the brain cells resulting in a variety of events or symptoms such as feelings or loss of consciousness, alteration in awareness, abnormal movements, atypical or odd behavior, distorted perceptions that are of limited duration but recur if untreated (Harvey and Champe, 2009; Porter and Meldrum, 2009). The International League Against Epilepsy (ILAE) defines epilepsy as “a condition characterized by two or more recurrent epileptic seizures over a period longer than 24 hours, unprovoked by any immediate identified cause” (Sander, 1997). A modification in the definition was suggested later, the new definition is said to require the occurrence of at least one seizure plus a clear predisposing factor. This modification could have practical consequences because the prevalence of epilepsy can be overestimated (Fisher *et al.*, 2005).

#### **1.1.1.2 Epidemiology of Seizures**

Epilepsy is a very common disorder affecting as much as 50 million people worldwide and about eighty percent of them are living in developing countries, of which 90% do not have access to and hence do not receive appropriate treatment (Scott *et al.*, 2001). The prevalence of epilepsy in

the developed countries ranges from 4 to 10 cases per 1,000. Studies in the developing and tropical countries have reported a higher prevalence rate of epilepsy, ranging from 14 to 57 cases per 1,000 persons (Burneo *et al.*, 2005; Carpio and Hauser, 2009). The prevalence of epilepsy out of every 1000 population is 10-13 in Africa, 14-18 in Latin America, 5-8 in Nigeria and the prevalence is higher in males than females, 11 out of 16 in reviewing record data and 16 out of 29 in door to door evaluation studies (Banerjee *et al.*, 2009).