CHARACTERIZATION OF ANTIEPILEPTIC AND ANXIOLYTIC ACTIVITIES OF ETHYL ACETATE FRACTION FROM Swietenia macrophylla KING SEEDS

SAYYAD MUSTAK

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By

SAYYAD MUSTAK

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

May 2016

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DEDICATION

To my beloved parents, who have supported me in all of my life events, particularly in raising the decision to pursue higher studies

And

To my beloved wife Samiya Anees and our son Izaan, for giving soul to our life

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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May 2016

Chairman : Professor Rozita Binti Rosli, PhD
Faculty : Medicine and Health Sciences

Epilepsy is a serious brain disorder with approximately 2.4 million new cases each year globally, from which 80% of them are in the developing world. In addition, anxiety and depression are frequent co-morbid conditions associated with epilepsy patients, usually unrecognized and untreated in the majority of epilepsy sufferers. Thus, developing a new agent from a plant source which may be useful in the treatment of epilepsy, along with anxiety and depression is a worthwhile approach. A number of plants used in traditional medicine systems have been found to possess antiepileptic activity. *Swietenia macrophylla* is an important medicinal plant, has been reported for various activities, including antioxidant and antinociceptive activities. The main objective of this study was to characterize the potential neuropharmacological activity of ethyl acetate fraction of *Swietenia macrophylla* seeds (SMEAF) in experimental animal models. The *in vitro* assay was carried out to determine the neuroprotective properties using primary neuronal cells and cell viability was assessed using MTT assay. The results of assay suggested the ability of SMEAF in protecting primary neuronal cells against *tert*-Butyl hydroperoxide (TBHP) induced oxidative stress. An acute oral toxicity study was conducted in which the SMEAF was found to be safe up to the dose of 2000 mg/kg. Antiepileptic activity of SMEAF was evaluated in Pentylenetetrazole (PTZ) and Picrotoxin (PCT)-induced convulsion models in which important brain neurotransmitter, gamma-aminobutyric acid (GABA) levels was then estimated. SMEAF was found to have significant ($p \leq 0.05$, one-way ANOVA) anticonvulsant activity and exerted its property through multiple mechanisms, indicating its anticonvulsant property through GABA receptor and also by modulating the brain monoamine levels. Furthermore, the expression of selected epilepsy associated genes in the mouse brain was investigated, where it suppressed the mRNA expression levels of selected genes after PTZ treatment. Lastly, the effect of SMEAF on electroencephalogram (EEG) activity was determined, in which it corrected PTZ induced EEG disturbances. The antiepileptic activity may be due to limonoids and flavonoids which have also been reported to have various pharmacological activities in the central nervous system. The anxiolytic activity of SMEAF was evaluated in open field test and elevated plus

maze. SMEAF showed significant ($p \leq 0.05$, one-way ANOVA) anxiolytic activity in both tests. As SMEAF was shown to modulate the levels of GABA, this action might be contributing to the anxiolytic potential of SMEAF. The antidepressant activity was evaluated using tail suspension test, but showed no significant effect in animals treated with SMEAF as compared with the control group. Taken together, it is concluded that SMEAF may be developed as a potential therapeutic agent for the treatment of epilepsy along with anxiety.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENCIRIAN AKTIVITI ANTIEPILEPTIC DAN ANXIOLYTIC ETHYL ACETATE PECAHAN DARI BENIH SETAR *Swietenia macrophylla* KING BIJI

Oleh

SAYYAD MUSTAK

Mei 2016

Pengerusi : Profesor Rozita Binti Rosli, PhD
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Epilepsi adalah satu gangguan otak yang serius dengan kira-kira 2.4 juta kes baru setiap tahun di peringkat global, daripada 80% daripada mereka adalah di dunia membangun. Di samping itu, kebimbangan dan kemurungan adalah keadaan co-fobia yang kerap dikaitkan dengan pesakit epilepsi, biasanya tidak dikenali dan tidak dirawat dalam sebahagian besar penghidap epilepsi. Oleh itu, membangunkan ejen baru dari sumber tumbuhan yang berguna dalam rawatan epilepsi, bersama-sama dengan kebimbangan dan kemurungan adalah pendekatan yang berbaloi. Sejumlah tumbuh-tumbuhan yang digunakan dalam sistem perubatan tradisional telah didapati mempunyai aktiviti antiepileptic. Swietenia Setar adalah tumbuhan ubatan yang penting, telah dilaporkan untuk pelbagai aktiviti, termasuk aktiviti antinociceptive dan antioksidan. Objektif utama kajian ini adalah untuk mencirikan potensi aktiviti neuropharmacological ethyl acetate pecahan Swietenia Setar benih (SMEAF) dalam model haiwan eksperimen. Cerakin di dalam vitro telah dijalankan untuk menentukan sifat-sifat neuroprotective yang menggunakan sel memodulatkan utama dan daya maju sel telah dinilai menggunakan cerakin MTT. Keputusan cerakin dicadangkan keupayaan SMEAF dalam melindungi sel-sel memodulatkan utama terhadap tekanan oksidatif tert-Butyl hydroperoxide (TBHP) induced. Suatu kajian ketoksikan akut lisan dijalankan di mana SMEAF itu telah ditemui selamat sehingga dos yang 2000 mg/Kg. Antiepileptic aktiviti SMEAF dinilai dalam Pentylenetetrazole (PTZ) dan model konvulsi berpunca dari perbuatan Picrotoxin PCT di mana neurotransmitter otak penting, tahap gamma – aminobutyric asid (GABA) kemudian dianggarkan. SMEAF didapati mempunyai signifikan ($p \leq 0.05$, ANOVA satu hala) aktiviti anticonvulsant dan diberikan harta melalui pelbagai mekanisma, menunjukkan sifatnya anticonvulsant melalui penerima GABA dan juga modulating tahap monoamine otak. Selain itu, penyataan epilepsi terpilih berkaitan gen dalam otak tetikus disiasat, di mana ia ditindas peringkat ungkapan mRNA gen yang terpilih selepas rawatan PTZ. Akhir sekali, kesan SMEAF aktiviti electroencephalogram (EEG) telah ditentukan, iaitu ianya diperbetulkan PTZ induced gangguan EEG. Aktiviti antiepileptic mungkin disebabkan oleh limonoids dan flavonoid yang juga telah dilaporkan mempunyai aktiviti farmakologi dalam

sistem saraf pusat. Aktiviti anxiolytic SMEAF dinilai melalui ujian medan terbuka dan bertingkat plus maze. SMEAF menunjukkan penting ($p \leq 0.05$, ANOVA satu hala) anxiolytic aktiviti dalam kedua-dua ujian. Seperti yang SMEAF telah ditunjukkan kepada memodulatkan tahap GABA, tindakan ini mungkin menyumbang kepada potensi anxiolytic SMEAF. Aktiviti antidepressant dinilai menggunakan ujian penggantungan ekor, tetapi menunjukkan tiada kesan ketara pada haiwan yang dirawat dengan SMEAF berbanding dengan Kumpulan kawalan. Bersama, dapat disimpulkan bahawa SMEAF boleh dibangunkan sebagai potensi agen terapeutik untuk rawatan epilepsi bersama-sama dengan kebimbangan.

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Thesis was submitted to the senate of Universisti Putra Malaysia and has been accepted as fulfilment of the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

Arc	Activity-regulated cytoskeleton-associated protein
AEDs	Antiepileptic Drugs
Bdnf	Brain derived neurotrophic factor
Ca^{2+}	Calcium ion
EDTA	Ethylene diamine tetra acetic acid
EGR1	Early growth response 1
EEG	Electroencephalogram
EPA	Environmental Protection Agency
EPM	Elevated plus maze
DMSO	Dimethyl sulfoxide
DPX	Di-n-butylPhthalate in Xylene
FOS	FBJ osteosarcoma oncogene
GABA	Gamma amino butyric acid
H & E	Hematoxylene & eosin
5-HT	5-hydroxytryptamine
5-HT2A	5-hydroxytryptamine2A
LC-MS	Liquid chromatography-mass spectroscopy
MAO	Monoamine Oxidase
mRNA	Messenger RNA
MTT	3-(4, 5-Dimethylthiazol-2Y-1)-2, 5-Diphenyltetrazol Bromide
OECD	Organization for economic cooperation and development
OFT	Open field test
PCT	Picrotoxin

PTZ	Pentylenetetrazole
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SMEAF	*Swietenia macrophylla* ethyl acetate fraction
SNRI	Serotonin and norepinephrine reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
TST	Tail suspension test
TBHP	*tert*-Butyl hydroperoxide
T&CM	Traditional & Complementary Medicine

CHAPTER 1

INTRODUCTION

Neurological disorders represent a major global concern, affecting the daily life of many sufferers (Chin, *et al.*, 2014; Donald, *et al.*, 2010). According to global estimates, neurological disorders, including epilepsy constitute 6.3% of the global burden of disease and it has also been speculated that, there will be a 12 % increase in the global burden of neurological disorders by the year 2030 (WHO, 2005). Epilepsy is a serious brain disorder with approximately 2.4 million new cases are reported every year globally, as much as 80% of the individuals with epilepsy reside in developing world (Angalakuditi & Angalakuditi, 2011; Benerjee, *et al.*, 2010; Epilepsy.org, 2015; Malkki, 2014; Murray, 1996; WHO, 2005).

Epilepsy is a chronic neurological disorder that has complex interactions with social, vocational, and psychological functioning (Gilliam, *et al.*, 2004). In addition, psychiatric comorbidity associated with epilepsy sufferers adds extra burden of living with the disease (Johnson, *et al.*, 2004). Many epidemiological studies revealed that depression and anxiety are frequent co-morbid conditions among the persons with epilepsy (Manchanda, 2002; Rafnusson, *et al.*, 2001), and these psychiatric disorders are usually unrecognized and untreated in the majority of patients with epilepsy (Kimiskidis, *et al.*, 2007; Johnson, *et al.*, 2004). A number of controlled studies conducted at community setting, secondary care and specialist centers indicate that anxiety has prevalence rates ranging from 25%-50%, whereas depression ranging from 3-55% among epileptic subjects (Ettinger, *el al.,1999*; Jacoby, *et al.*, 1996; Jones, *et al.*, 2006; Lambert & Robertson, 1999). Thus, anxiety and depression exert a significantly negative effect on the health-related quality of life among epilepsy sufferers (Choi-kwan, *et al.*, 2003).

Many available therapies for epilepsy are developed to target origin and spreading of seizure, but not the actual processes causing epilepsy (Smith & Bleck, 2001). Hence, many available antiepileptic medications are incapable of affecting the natural history of the epileptic activity (Haernandez, 2007; Shinnar & Berg, 2006). Development of antiepileptic agents with multiple mechanisms of action, and also with low events of unwanted actions as compared to the currently available antiepileptic therapies are recommended (Morrell, 2011; Meinardi, 2005). In addition, the cost of new Antiepileptic drugs (AEDs) is a major concern and important factor which supports the need for developing new therapy, which can be affordable, especially in developing nations (Beghi, *et al.*, 2008; Britton & So, 2006; Cameron, *et al.*, 2012; Kochen, 2006; Krucik, 2014). Natural products used in traditional herbal medicine are important source of novel antiepileptic compounds (Chadwick, 1995). A number of plants used in traditional medicine systems have found to possess antiepileptic activity (Shinner & Berd, 2006 & 2004).

Medicines from natural origin have been used as a source of remedy for the prevention, cure and treatment of different ailments (Rates, 2001). Humans are extensively, taking advantage of plants as a basis for sophisticated traditional medicine among natural sources. There is enough documented evidence available to support their use in ancient time, and also continuous use in the modern era (Borris, 1996; Gurib-Fakim, 2006). Herbal medicines still play an important role in the current practice of medicine for some specific reasons such as economical, easily accessible and expected to have no to minimal unwanted effects (Katiyar, *et al.*, 2012). According to the World Health Organization report, about 80% of the global population primarily belonging to the developing countries relies on plant-based medicine to fulfill their health care needs (WHO, 2013).

The huge diversity of plant species undoubtedly contains a rich source of potentially therapeutic compounds with novel structures. Out of the estimated 250,000 to 300,000 species of plants, approximately only 5000 species have been thoroughly investigated for possible medicinal applications (Abelson, 1990). Thus, a natural wealth awaits to be explored scientifically for the benefit of mankind (Akerele, 1993; Balick, 1990; Plotkin, 1998).

Swietenia macrophylla mainly contains limonoids, polyphenols, and essential oils as major constituents (Chen, *et al.,* 2010). Traditionally, the seeds have been used to treat mild to moderate pain, and in the treatment of diabetes and hypertension (Moghadamtousi, *et al.,* 2013). Commercially, the fruits are used as a major ingredient in health care products for various skin conditions and improvement of blood circulation (Goh, *et al.,* 2012). The ethanolic fraction of *Swietenia macrophylla* seeds were shown to have antioxidant activity with efficient results in increased Vitamin C & E levels in the plasma and also, elevates the reduced glutathione level in major organs including kidney, liver and plasma (Kalpana, *et al.,* 2011). In a recent study, the ethanolic extract of *Swietenia macrophylla* fruits was revealed to possess antinociceptive activity (Das, *et al.,* 2009).

1.1 Problem Statement

Current literature shows that epilepsy is one the serious and commonly reported neurological disorder affecting many lives globally and needs attention, especially in developing countries (Cameron, *et al.,* 2012). Epilepsy is associated with psychiatric co-morbidity including anxiety and depression and these are usually unrecognized and untreated (Kimiskidis, *et al.,* 2007; Johnson, *et al.,* 2004). Also, the high cost of new antiepileptic drugs (AEDs) is demanding the need for agents from alternative sources (Beghi, *et al.,* 2008; Cameron, *et al.,* 2012; Krucik, 2014). Thus, developing a new agent from a plant source which possesses multiple mechanisms of action and can be useful for the treatment of epilepsy along with anxiety and depression is a worthwhile approach. In addition, this will also attend the problem associated with the cost of new AEDs.

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APPENDICES

Appendix A

APPROVAL LETTER FROM MONASH ANIMAL ETHICS COMMITTEE

MONASH University

APPLICATION FORM FOR THE USE OF ANIMALS FOR SCIENTIFIC PURPOSES IN RESEARCH AND TEACHING

MARP-1 **ANIMAL ETHICS COMMITTEE**

AEC NUMBER	**MARP/2015/040**
Project Type	☒ Research ☐ Undergraduate Teaching ☐ Training in Procedural Techniques
Project Title	Evaluation of pharmacological activity of ethyl acetate fraction of seeds from plant *Swietenia macrophylla*
Animal Use Categories (Refer to List of Categories attached)	1.1, 1.2, 1.4, 2.1, 4.2, 4.10
Standard Operating Procedures	☒ **No** ☐**Yes: Title/AEC Number:**

SOPs indicated will beto be read in conjunction with the application. Detail any variations from the SOP.

Proposed Start Date July 2015 actual start determined at time of AEC approval	**Proposed Finish Date Feb 2016** Actual finish determined at time of AEC approval

DECLARATION BY CHAIRPERSON OF AEC

I certify that the procedures/ personnel/ location in this project has been considered and approved by the Animal Ethics Committee **for the period ...19/08/15.. to ..31/12/17.**

Digitally signed by Rick Lang
DN: cn=Rick Lang, o=MARP, ou=MARP, email=animal.ethics@monash.edu, c=AU
Date: 2015.08.19 13:40:08 +10'00'

....MARP-1... .....19/08/2015.....
Chairperson's signature AEC Date

Conditions of Approval:

1. Monash University Investigators must not deviate from approved application without a written AEC approval and must adhere to all requirements of the AEC;
2. Any variation proposed to the project, and the reasons for that change, must be submitted to the AEC for approval and must not be implemented until written approval is granted;
3. All matters pertaining to the conduct of the approved project will beto be reported to the AEC, which maintains oversight in accordance with Monash University licence conditions;
4. A record of details of any animals used in the project must be retained;
5. Project must only be conducted in approved premises;
6. The AEC must be notified any incidents and adverse events that may impact on the welfwill beof the animals and any changes to approved investigators;
7. Annual Progress Report & Victorian government Animal Use Return must be provided to the AEC at the end of each calendar year and a Completed Report within 6 months of end of approval.

Appendix B

HAEMATOXYLIN AND EOSIN STAINING

Xylene I (5 minutes)
↓
Xylene II (5 minutes)
↓
Alcohol 100%
Alcohol 95%
Alcohol 80%
Alcohol 70%
↓
Wash with running water until clean
↓
Haematoxylin (5-18 minutes)
↓
Wash and soak in running water
↓
Immerse in alakaline alcohol (3 wash)
↓
Wash and soak in running water for 6 minutes
↓
Eosin (5 minutes)
↓
Alcohol 70%
Alcohol 80%
Alcohol 95%
Alcohol 100%
Alcohol 100%
↓
Xylene I
↓
Xylene II
↓
Xylene III
↓
Mount with DPX and cover with glass cover slips

Appendix C

SET UP FOR RECORDING THE EXPERIMENTS

a. The camcorder was fixed onto the camcorder stand.

b. The open cage box was placed below the camcorder.

c. The camcorder stand was extended so that the cage is within view through the camera including the ends of the cage (to ensure the subject is clearly visible to record its behavior throughout the experiment).

d. The cage was disinfected with 70% ethanol and debris was removed from the cage.

e. Finally, the subjects to be tested were acclimatized by transferring them into the test room at least one hour prior to the experiment.

Appendix D

ESTIMETION OF GABA USING LC-MS ANALYTICAL TECHNIQUE

Optimized Standard Protocol for LCMS system

Method creator: Thermo TSQ
Instrument: Accela Pump
Injection volume (uL) 10.000
Flush volume (uL): 400
Common settings:
Tray temp control is on. Temp(C): 4.000
Column oven control is on. Temp(C): 30.000

Mobile phase:
Solvent A: 0.1%FA + H2O
Solvent B: 0.1%FA + ACN
Start settings: Surveyor AS injection logic
Method finalizing: First line conditions
Operating mode: Low pressure (0.~7000 PSI)
Min pressure: 0.00
Max pressure: 400.00

Appendix E

GC–MS ANALYSIS PERFORMED USING AGILENT TECHNOLOGIES 6980N EQUIPPED WITH 5979 MASS SELECTIVE DETECTOR

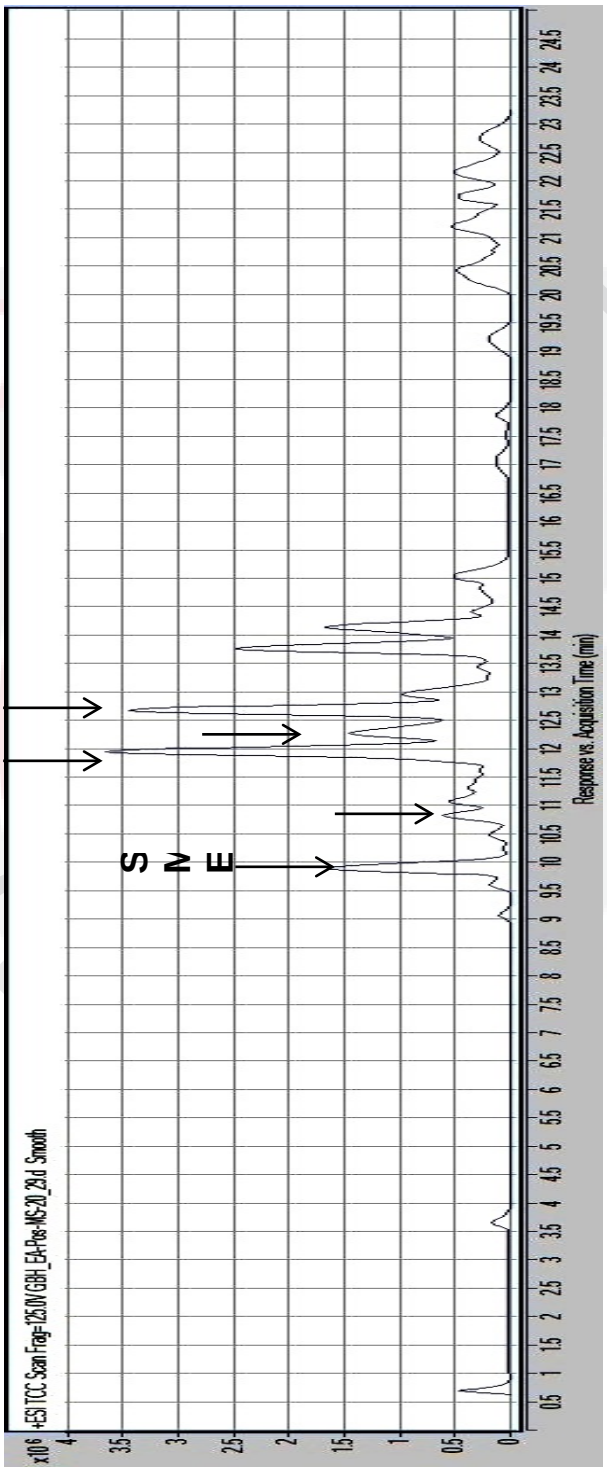
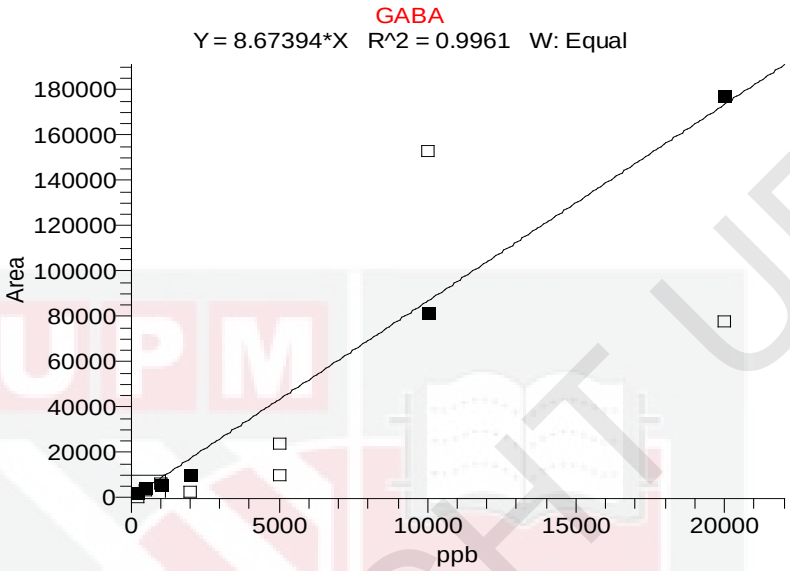


Figure 4.1 - Compounds from SMEAF. Swietenolide (1), 3-O-acetylswietenolide (2), Swietenine (3), Methyl angolensate (4), and Diacetyl swietenolide (5).

Appendix F

ESTIMETION OF GABA USING LC-MS ANALYTICAL TECHNIQUE

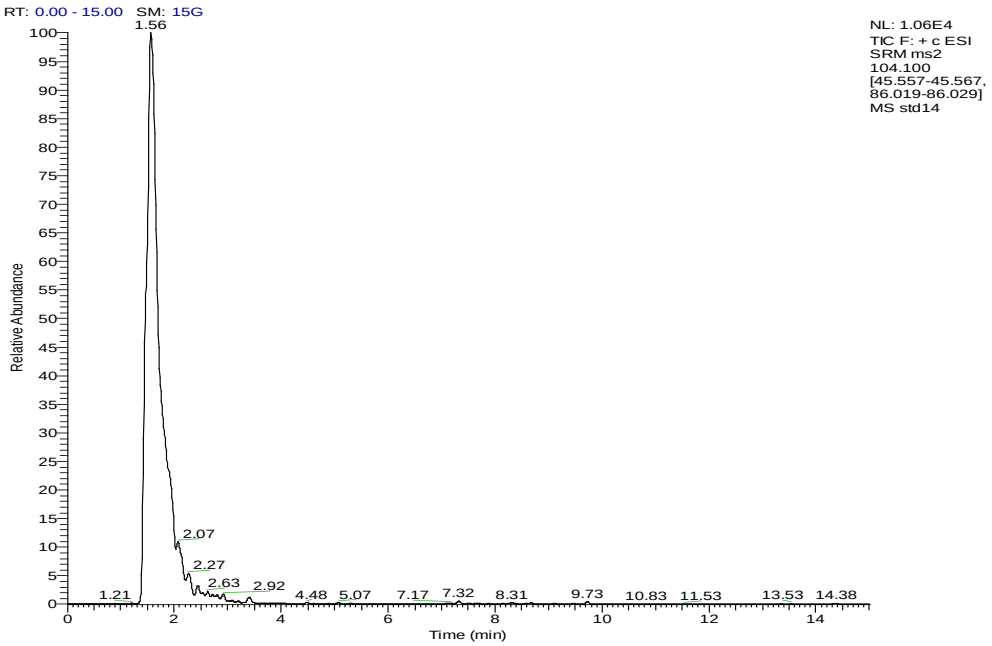


Standard GABA calibration curve

Component Name	**Equation**
GABA	Y = 8.67394*X R^2 = 0.9961

Sample Name	**Exp Amt**	**Calc Amt**	**Units**	**%Diff**	**Level**	**Area**	**Height**	**RT**	**S/N**
std 0.25ppm-2	250.000	249.130	ppb	0%	cal1	2160.94	412.53	1.46	434.32
std 0.5ppm-1	500.000	493.092	ppb	-1%	cal2	4277.05	342.99	1.57	406.99
std 1ppm-1	1000.000	656.487	ppb	-34%	cal3	5694.33	356.20	1.52	106.47
std 2ppm-1	2000.000	1171.573	ppb	-41%	cal4	10162.16	1104.56	1.47	50.02
std 10ppm-1	10000.000	9410.829	ppb	-6%	cal6	81628.99	7087.33	1.55	185.38
std 20ppm-2	20000.000	20394.788	ppb	2%	cal7	176903.22	10954.88	1.56	360.29

Standard GABA 20ppm



Concentration of GABA

Component Name
GABA

Equation
Y = 8.67394*X R^2 = 0.9961

Filename	Sample Name	Calc Amt	Units	Area	Height	RT
sample1	control1-1	26.768	ppb	238.12	113.24	1.61
sample2	control1-2	23.768	ppb	211.43	96.74	1.58
sample3	control2-1	23.547	ppb	209.46	106.07	1.58
sample4	control2-2	20.891	ppb	185.84	63.66	1.58
sample5	NEG control1-1	15.483	ppb	137.73	42.45	1.62
sample6	NEG control1-2	15.648	ppb	139.20	57.32	1.47
sample7	NEG control2-1	15.857	ppb	141.06	40.22	1.48
sample8	NEG control2-2	13.286	ppb	118.19	16.39	1.45
sample9	POS control1-1	19.055	ppb	165.28	36.42	1.54
sample10	POS control1-2	20.064	ppb	174.04	42.90	1.52
sample11	POS control2-1	20.003	ppb	173.50	69.89	1.61
sample12	POS control2-2	20.415	ppb	177.08	38.36	1.62
sample13	treatment GRP1-1	25.913	ppb	224.77	29.67	1.58
sample14	treatment GRP1-2	27.888	ppb	241.90	120.96	1.59
sample15	treatment GRP2-1	20.483	ppb	177.67	32.94	1.66
sample16	treatment GRP2-2	20.706	ppb	179.60	30.24	1.61

LIST OF PUBLICATIONS

Sayyad, M., Ning, T., Kumari, Y., Hing, G.B., Jaiswal, Y., Rosli, R., Williams, L., Farooq Shaikh, Mohd., 2016. Acute toxicity profiling of the ethyl acetate fraction of Swietenia macrophylla seeds and in-vitro neuroprotection studies, *SaudiPharmaceutical Journal*. http://dx.doi.org/10.1016/j.jsps.2016.05.002

Bakhtiar, A.,# Sayyad, M.,# Rosli, Maruyama, A., Chowdhury, E.H., 2014. Intracellular Delivery of Potential Therapeutic Genes: Prospects in Cancer Gene Therapy. *Current Gene Therapy*, 14:247-257. # Equal contribution.

Stanislaus, A., Kunnath, A.P., Tiash, S., Fatemian, T., Kamaruzman, N.I., Bakhtiar, A., Sayyad, M., Hossain, S., Akaike, T., Chowdhury, E.H., 2013. Intracellular delivery of NF-κB small interfering RNA for modulating therapeutic activities of classical anti-cancer drugs in human cervical cancer cells. *Drugs and Therapy Studies*, volume 3:e7.

Sayyad, M., 2012. Comparative study of Tephrosia purpurea (Linn) leaves and Lovastatin on cholesterol level of hyperlipidemic wistar rats. *IOSR Journal of Pharmacy and Biological Sciences (IOSRJPBS)*, 1(2):25-30.

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