IN VITRO ANTIOXIDANT, ANTIANGIOGENIC AND VASORELAXANT STUDIES OF *CLERODENDRON SERRATUM* (SPRENG.) LEAVES EXTRACTS

by

ALI JIMALE MOHAMED

Thesis submitted in fulfillment of the requirements for the degree of Master of Science

UNIVERSITI SAINS MALAYSIA

JUNE 2011

This thesis is dedicated to...

Anab Nur Hilowle

ACKNOWLEDGEMENTS

All praise and thanks are due to Allah, the almighty, for giving me the strength and health to accomplish this work. Many people are responsible for my completion of this project, far too many to name, but few of you are deserve singling out. First and foremost, I respectfully express my sincere and deepest gratitude to my supervisor Prof. Mohd Zaini Asmawi for providing me invaluable advice, all research materials, generous side of help, and genuine scholarly guidance during my research work.

Ass. Prof. Dr. Norhayati Binti Ismail, my co-supervisor is gratefully acknowledged for her endless effort in ensuring this work has achieved the desired standard. Moreover, I also wish to extend my heartfelt appreciation to my co-supervisor, Ass. Prof. Dr. Amin Malik Shah for his guidance, magnanimous invitations and wonderful trips that created a pleasant working atmosphere for me.

It gives me immense pleasure to express my deep appreciation to the Islamic development Bank (IDB), for organization of Islamic conference (OIC), for providing me with two and 1/2-years sponsorship (IDB N0: SO/2004/016), to study Universiti Sains Malaysia. Honestly, I can say that without IDB support my ambition/dream to get higher education would not have been possible to be achieved.

I express my sincere thanks to all my colleagues who assisted me to complete my research work, especially, Daud Moalin Ishaq, Yam Mun Fei, Omar Ziad Ameer, Lim Chunping, Hayder B. Sahib, Abdalrahim F. A. Aisha, Norshirin Idris, Dr. Khalid Hussein and Dr. Saddiqui for who in one way or another have contributed in

success of this study. I also express my appreciation to the ever-ready assistance, Rosli Hassan, laboratory Technician for his excellent technical assistance and other laboratory staffs such as Ms. Yong, Selva, and Abdirahim Abdullah are appreciated.

I am highly thankful and my sincere gratitude goes to my best friend Mohamed-rashid Ahmed Mohamed Haras, for his ideas and advice that have been most valuable to me during my study in USM. In addition to that, all the cheerful group of Somalian fellow in a USM such as Dr. Ahmed Osman Awil, Ass. Prof. Abdullahi Ali Mohamed, Dr. Ali Warsame, Sayid Mohamed Abdulle, Sharif Yusof Gedi, Bashir Ali Warsame, Abdulaziz Ahmed Digale, Omar Salad, Yahye Abbas Amin, Shukri Abbas Amin, Amina Ahmed Haras, Abdilahi Nadif Muse, Mohamed Ahmed Ibrahim, and Abdinasir Tahir Abdi for their help during difficult times.

Last but not least, my warmest thanks are due to my parents (*Hawa Muhammed*) for her continuous prayers and all my family members, patience and support over the last three years, in particularly, Mohamed Hassan Jimale, Maryam Mahmoud Gure, and Mohamed Ali Abdi. In this Occasion, I would like to express my special thanks to Shukri Jimale Mohamed who bolstered me morally, understanding and never failing support during all these years, while she was in war town of Mogadishu. Last in line but first in my mind to my beloved one, Anab Nur Hilowle for her love, morally support, and caring she provided me in various forms during my study.

Special thanks, tribute and appreciation to all those their names do not appear here who have contributed to the successful completion of this study.

TABLE OF CONTENTS

CHAPTER THREE: ANTIOXIDANT ACTIVITY STUDY

CHAPTER FOUR: ANTIANGIOGENIC ACTIVITY STUDY

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

°C	Degree celsius
λ	Lambda
%	Percent
±	Plus minus
5-HT	5-hydroxy tryptamine
AA	Arachidonic acid
ACE	Angiotensin-converting enzyme
ACEH	Angiotensin-converting enzyme homolog
ACEI	Angiotensin-converting enzyme inhibitor
ADP	Adenosine diphosphate
AIDS	Acquired immunodeficiency syndrome
Ang I	Angiotensin I
Ang II	Angiotensin II
ANOVA	Analysis of variance
AT-1	Angiotensin type I receptor
ATP	Adenosine triphosphate
BP	Blood pressure
BHT	Butylated hydroxytoluene
bFGF	Basic fibroblast growth factor
BRNs	Baroreceptor neurons
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
cAMP-PKA	Cyclic adenosine monophosphate- dependent protein kinase A
cGMP-PKG	Cyclic adenosine monophosphate- dependent protein kinase G
CAT	Catalase
CC	Column chromatography
CE-CS	Chloroform extract of *C. serratum*
CFME-CS	Chloroform fraction of methanol extract of *C. serratum*
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
CHF	Congestive heart failure
ChTX	Charybdotoxin
Cl^{-}	Chloride ion
Cl_{Ca}	Calcium-activated chloride channel
CNS	Central nervous system
cm	Centimeter
COX	Cyclooxgenase
COX-2	Cyclooxgenase *2*
CRP	C-reactive protein
CVD	Cardiovascular disease
CYP	Cytochrome
DG	Diacylglycerol
$DPPH^{\bullet}$	2,2'-diphenyl-1-picrylhydrazyl
DHET	Dihydroxyeicosatrienoic acids
DNA	Deoxyribonucleic acid
DMPP	1,1-dimethyl-4-phenylpiperazinium iodide
EAFME-CS	Ethyl acetate fraction of methanolic extract of *C. serratum*
EC	Endothelial cells

ECM	Extracellular matrix
EC_{50}	Half maximal effective concentration
ecSOD	Extracellular superoxide dismutase
EDCF	Endothelium-derived contracting factor
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxation factor
EET	Epoxyeicosatrienoic acid
eNOS	Endothelial nitric oxide synthase
ET	Endothelin
et al.	And others
ET_A	Endothelin receptor A
ET_B	Endothelin receptor B
ETCE	Endothelin converting enzyme
F	Flow
FABP	Fatty acid binding proteins
FRSA	Free radical scavenging activity
g	Gram
GPL	Glycerophospholipids
GPX	Glutathione peroxidase
h	Hour
HETE	Hydroxyeicosatetraenoic acids
Hg	Mercury
HGF	Hepatocyte Growth Factor
HIF	Hypoxia-inducible factor
HREC	Human retinal endothelial cells
H_2O_2	Hydrogen peroxide
$HO^{\bullet}$	Hydroxyl racial
HOCl	Hypochlorous acid
HREC	Human retinal endothelial cells
HVA	High voltage-activated
IC_{50}	Half maximal inhibitory concentrations
IL	Interleukin
IK_{Ca}	Intermediate-conductance calcium-sensitive potassium channel
IP_3	Inositoltriphosphate
JG	Juxtaglomerular
K^+	Potassium ion
K_{ATP}	ATP-sensitive potassium channel
K_{Ca}	Calcium-activated potassium channel
kg	Kilogram
KI	Potasium iodide
L	Liter
LOXs	Lipoxygenases
m	Meter
M	Molar
MAPK	Mitogen-activated protein kinase
ME-CS	Methanol extract of *C. serratum*
mg	Milligram
Mg^{+2}	Magnesium ion
min	Minute
mL	Milliliter

MLC	Myosin light chain
MLCP	Myosin light chain phosphatase
mm	Millimeter
mM	Millimolar
MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
mV	Millivolt
n	Number of determination
n-BFME-CS	*n*-Butanol fraction of methanolic extract of *C. serratum*
n-HFME-CS	*n*-Hexane fraction of methanolic extract of *C. serratum*
Na^+	Sodium ion
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NA	Noradrenaline
NO	Nitric oxide
$NO^{\bullet}$	Nitric oxide radical
NOS	Nitric oxide synthase
NP/PEG	Natural products-polyethylene glycol
NSAIDs	Non-steroidal anti-inflammatory drugs
$O_2^{-\bullet}$	Superoxide anion
$OH^{\bullet}$	Hydroxyl radicals
$ONOO^-$	Peroxynitrite
$ONOOCO_2$	Peroxynitrite carbonate adduct
OXST	Oxidative stress
P	Pressure
P	Probability
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PEG-400	Polyethylene glycol-400
PE-CS	Petroleum ether extract of *C. serratum*
PGs	Prostaglandins
PGH_2	Prostaglandin H_2
PGG_2	prostaglandin G_2
PGHS	Prostaglandin endoperoxide H synthase
PGI_2	Prostacyclin I_2
PIP_2	Phosphatidyl inositol-(4,5)-biphosphate
PK	Protein kinase
PKA	Protein kinase A
PKC	Protein kinase C
PL	Phospholipase
PLC	Phospholipase C
PPAR	Peroxisome proliferator-activated receptors
RAAS	Renin-angiotensin-aldosterone system
RAS	Renin-angiotensin system
RhoGEF	Rho guanine nucleotide exchange factor
R_{max}	Maximum response
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
$ROO^{\bullet}$	Peroxyl radical

ROS	Reactive oxygen species
RSA	Radical scavenging activity
s	Second
S.E.M.	Standard error of mean
SMC	Smooth muscle cells
SD	Sprague Dawley
sEH	Soluble epoxide hydrolase
SERCA	Sarcoplasmic reticulum ATPase
SNS	Sympathetic nervous system
Na_2SO_4	Sodium sulfate
SOD	Superoxide dismutase
SPSS	Statistical package for the social sciences
SVR	Systemic vascular resistance
TAA	Total antioxidant activity
TEAC	Trolox equivalent antioxidant capacity
THF-diols	Tetrahydrofuran-diols
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor alpha
TX	Thromboxane
TXA_2	Thromboxane A_2
µg	Microgram
µL	Microliter
µM	Micromolar
µm	Micrometer
UV	Ultraviolet
v/v	Volume over volume
VEGF	Vascular endothelial growth factors
VEGF-R2	Vascular endothelial growth factors-receptor-2
VCAM-1	Vascular cell adhesion molecule
vs.	Versus
VSM	Vascular smooth muscle
VSMCs	Vascular smooth muscle cells
WE-CS	Water extract of *C. serratum*
WFME-CS	Water fraction of methanolic extract of *C. serratum*
WHO	World Health Organisation
w/v	Weight over volume
w/w	Weight over weight

LIST OF PUBLICATIONS

1- **Ali Jimale Mohamed**, Elsnoussi Ali Hussin Mohamed, Abdalrahim F. A. Aisha, Omar Ziad Ameer, Zhari Ismail, Norhayati Ismail, Amin Malik Shah Abdulmajid, Mohd Zaini Asmawi and Mun Fei Yam (2011). Antioxidant, Antiangiogenic, and vasorelaxant activities of methanolic extract of *Clerodendron serratum* (Spreng.) leaves. *Journal of Medicinal Plants Research* (Accepted).

2- **Ali Jimale Mohamed**, Norhayati binti Ismail, and Mohd Zaini Asmawi. *In vitro* vasorelaxant effect of *n*-hexane, chloroform and *n*-butanol fractions of the *Clerodendrum serratum* (Spreng.) leaves in rat aortic ring preparation. 4th Life Sciences Postgraduate Conference, 18-20th June 2008. Universiti sains Malaysia, Penang, Malaysia.

3- **Ali Jimale Mohamed**, Norhayati binti Ismail, and Mohd Zaini Asmawi. Vasorelaxant effect of *Clerodendrum serratum* (Spreng.) leaves. 22nd MSPP Scientific Meeting, 5-6th April 2008. University of Malaya, Kuala lumpur, Malaysia.

KAJIAN *IN VITRO* ANTIOKSIDAN, ANTIANGIOGENIK DAN VASORELAKSAN EKSTRAK DAUN *CLERODENDRON SERRATUM* (SPRENG.)

ABSTRAK

Clerodendron serratum (Spreng.) daun dari keluarga Verbenaceae, dikenali sebagai "Timba Tasek" dalam bahasa Malaysia adalah sejenis tumbuhan ubatan teradisional Melayu yang digunakan untuk merawat berbagai jenis penyakit. Walau pun begitu, penyelidikan ini terfokus kepada mengevaluasi aktiviti antiangiogenik, vasorelaksan dan antioksidan serta kandungan kimia ektrak daun *C. serratum*. Serbuk daun kering *C. serratum* di maserasi secara bersiri dengan eter petroleum (langkah menyah lemak), kloroform, methanol dan air. Aktiviti antiangiogenik (kesan perencatan ekstrak ini keatas pertumbuhan/pembentukan salur darah) telah diselidiki menggunakan assai cincin aorta tikus. Aktiviti vasorelaksan telah diselidiki dengan memeriksa kesan perencatan ekstrak daun *C. serratum* keatas gerakbalas penguncupan dos kumulatif noradrenalin sediaan terasing cincin aorta tikus. Kesan antioksidan pula telah ditentukan menggunakan assai "DPPH* radical scavenging activity" dan "Trolox equivalent antioxidant capacity (TEAC)". Didapati diantara ekstrak-ekstrak, ekstrak metanol (ME-CS) menunjukan kesan antiangiogenik, vasorelaksan dan antioksidan yang paling poten. Oleh itu, ME-CS telah difraksinasi menggunakan *n*-heksan, kloroform, etil asetat, *n*-butanol dan air. Diantara fraksi-fraksi, didapati fraksi *n*-butanol dari ME-CS menunjukan kesan antiangiogenik, vasorelaksan dan antioksidan yang paling poten. Kajian fitokimia menunjukan fraksi *n*-butanol mengandungi sebatian polifenol yang relatif tinggi dan sedikit terpenoid dan saponin. Oleh kerana aktiviti paling poten untuk ketiga-tiga aktiviti (antioksidan, antiangiogenik dan vasorelaksan) terdapat dalam fraksi yang sama (fraksi *n*-butanol

dari ME-CS), besar kemungkinan yang kesemua aktiviti di hasilkan oleh kumpulan bahan kimia yang sama. Aktiviti antioksidan mungkin terlibat dalam menghasilkan aktiviti antiangiogenik dan vasorelaksan daun *C. serratum*.

IN VITRO ANTIOXIDANT, ANTIANGIOGENIC AND VASORELAXANT STUDIES OF *CLERODENDRON SERRATUM* (SPRENG.) LEAVES EXTRACTS

ABSTRACT

Clerodendron serratum (Spreng.) leaves from Verbenacea family, locally known as "Timba Tasek" is a traditional Malay medicinal plant used for treating various diseases. However, the present works were focused on evaluating its antiangiogenic, vasorelaxant and antioxidant activities as well as chemical profiles of the leaves extracts. The dried powdered leaves of *C. serratum* were serially macerated with petroleum ether (a defatting step), chloroform, methanol and water. The antiangiogenic activity (the inhibitory effects of these extracts on blood vessel growth/formation) was studied using rat aortic ring assay. The vasorelaxant activity was studied by examining the inhibitory effect of *C. serratum* extract on contractile responses of cumulative doses of noradrenaline in isolated rat aortic ring preparation. The antioxidant activity was determine using $DPPH^{\bullet}$ radical scavenging activity and Trolox equivalent antioxidant capacity (TEAC) assays. It was found that amongst the extracts, the methanolic extract (ME-CS) showed the most potent antiangiogenic, vasorelaxant and antioxidant activities. Therefore, the methanolic extract was fractionated using *n*-hexane, chloroform, ethyl acetate, *n*-butanol and water. Amongst the fractions, the *n*-butanol fraction of ME-CS showed the most potent antiangiogenic, vasorelaxant and antioxidant activities. Phythochemical study revealed that *n*-butanol fraction contains relatively large amount of polyphenolics compounds and some terpenoids and saponins. Since, all three activities (antioxidant, antiangiogenic and vasorelaxant) end up in the same fraction, it is very likely that all

the activities were contributed by the same group of chemical compounds. The antioxidant activity may be involved in its antiangiogenic and vasorelaxant activities of *C. serratum* leaves.

CHAPTER ONE

GENERAL INTRODUCTION

1.1. The Usage of Medicinal Plants

Traditional medicines, particularly herbal medicines have been used for thousands of years in maintaining health as an alternative to or in conjunction with modern medicines. The World Health Organization (WHO) has defined herbs to include crude plant materials such as leaves, flowers, fruits, seeds, stems, wood, barks, roots, rhizomes or other parts of plant which may be entirely fragmented or powdered. On the other hand, herbal products consist of herbal preparations made from one or more than one herb. Herbal medicine is defined as the use of crude materials of plant origin to treat illness or to promote health. It may contain excipients in addition to the active ingredients. In their unprocessed state, these herbal drugs are usually in the dried form but are sometimes stored fresh. Certain exudates may also be considered as herbal drugs (WHO, 2007a).

As we know, the use of plants in herbal therapy has been based on humankind's attempt to free itself from diseases descending from empirical findings of hundreds and thousands of years to provide new therapy. The traditional cultures of herbal medicine may not understand the scientific rationale behind their medicines, but they developed through trial and error from personal experience or collective tradition and the memories of their parents and grandparents. All scientific disciplines now recognize the importance of herbal plants as sources of medicine and have initiated active research programmes either to isolate new compounds or to produce standardized extracts as a source of medicines. It is a fact that 25% of all medical prescriptions are based on substances derived from herbal drugs of plant

origin or plant-derived synthetic analogues (Gurib-Fakim, 2006; Vitalini et al., 2009).

However, during the later part of 20th century, as science advanced, drugs were synthesized and herbalism declined. Newly developed principles of organic chemistry made it possible to replicate plant-produced chemicals leading to the synthesis of new compounds that preserved the beneficial properties of the natural chemical, but minimized its toxic effects (Swarbrick, 2007). Many drugs available in the market today are from herbal origin. In the world, 80% of people depend on medicinal plants for medicine, lack of access to medicines prescribed by modern medicine but also because these plants often have a real impact (Zabri et al., 2009), among modern medicines.

Today, natural products compounds discovered from medicinal plants (and their analogues thereof) have provided numerous clinically useful medicines (Jachak & Saklani, 2007). In 2001 and 2002, approximately one quarter of the best-selling drugs worldwide were natural products or derived from natural products (Balunas & Kinghorn, 2005). Herbal medicine is one of the most important areas in which compounds isolated from plants source have contributed successfully in cardiovascular research. Aspirin, atropine, ephedrine, digoxin, morphine, quinine, reserpine and tubocurarine are a few examples of plant-based drugs, which were originally discovered through the study of traditional medicine (Gilani & Atta ur, 2005).

Herbal plants have a long history of use in the treatment of cancer. Accordingly, there is a considerable scientific and commercial interest in the continuing discovery of new anticancer agents from all natural product sources, including plant secondary metabolites (Gurib-Fakim, 2006). Now, it is well established that herbal plants have been a useful source for clinically used anticancer drugs (Gordon et al., 2007). A number of promising new agents are under clinical development based on selective activity of these drugs against cancer-related molecular targets. Examples of plant active constituents used as anticancer agents are vinblastine, vincristine, camptothecin derivatives, topotecan and irinotecan, etoposide (derived from *epipodophyllotoxin*), and paclitaxel (Taxol®) (derived from the bark of the Pacific Yew, *Taxus brevifolia* Nutt) (Cragg & Newman, 2005). The natural-derived products, paclitaxel and camptothecin were estimated to account for nearly one-third of the global market for anticancer or about three billion USD of nine billion USD in total annually in 2002 (Oberlies & Kroll, 2004).

The use of plants, plant extracts, or plant-derived pure chemicals to treat disease is a therapeutic approach, which has stood the test of time. Indeed today many pharmacological classes of drugs include a natural product prototype. The search for new molecules could be undertaken within the plant and animal biodiversity using ethnopharmacology data. This approach allows us to select plants potentially active and increase significantly the number of discovery of new products assets (Zabri et al., 2009). A number of drugs of plant origin have recently been introduced to the market, including artemether, galantamine, nitisinone, and tiotropium, or are currently involved in late-phase clinical trials (Balunas & Kinghorn, 2005).

1.2. The Plant Literature Review: *Clerodendron serratum* (Spreng.)

1.2.1. Botanical Description

The *C. serratum* is a herbaceous shrub or treelet which is distributed from Pakistan, eastward to central of India, southern China, Myanmar, Laos, Vietnam, Philippines, Cambodia, Thailand, Malaysia, Singapore, Indonesia, and to Southern part of Africa (such as Mauritius, Madagascar and South Africa). The *C. serratum* is found in grasslands, thickets, and secondary forest from sea-level up to 1700 m altitude. This plant can attain a height up to 4 m tall or sometimes climping. Its stems are relatively stout, mostly unbranched, nodes not annulate; leaves are elliptical to obovate, 7-22 cm x 3-8 cm, base acute to subcuneate, apex acute or short acuminate, margin serrate, glabrous on both surfaces, petiole 0.3-1.2 cm long; axillary cymes 3-5 cm long (van Valkenburg & Bunypraphatsara, 2002).

The classification of the *Clerodendron serratum* (Spreng.) according to (Burkill, 1966) is as follows:

Division	*Spermatophyta*
Sub division	*Magnoliophyta*
Class	*Magnoliopsida*
Sub class	*Asteridae*
Order	*Lamiales*
Family	*Verbenaceae*
Genus	*Clerodendron*
Species	*Clerodendron serratum* (Spreng.).

The Malay name for *C. serratum* is *timba tasek* (the bucket of the lake), *tẽnjal tasek* (the plant with its foot on the lake), *tambun tasek*, *taman tasek*, *sunga tasek*, *sẽnggugor* (plant for miscarriage), *lambin budak*, and *mata kẽsang* (Burkill, 1966). Plate 1.1 shows the picture of *Clerodendron serratum* (Spreng.) leaves.

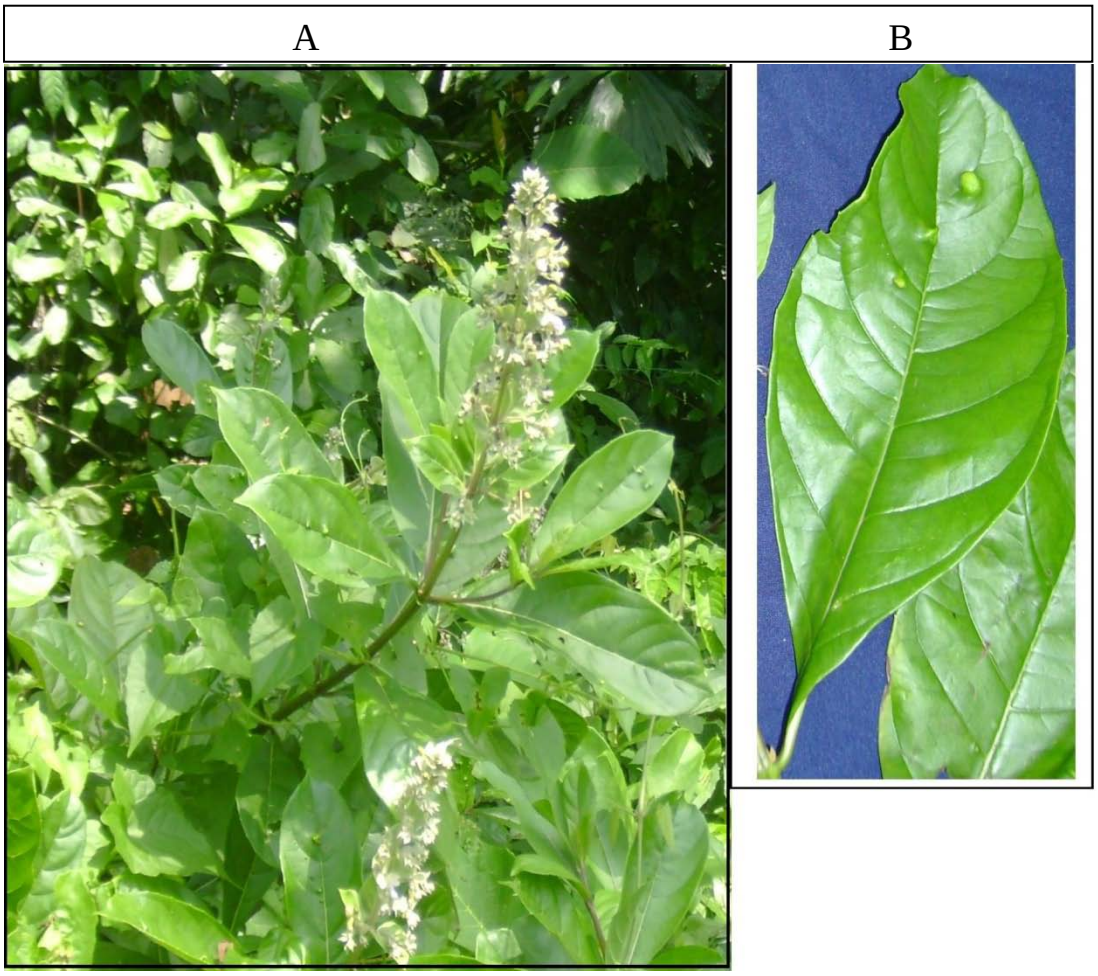


Plate 1.1. Picture of *Clerodendron serratum* (Spreng.): (A) plant and (B) leaves.

1.2.2. Biological Activity

In Malaysia, the Malays use this plant more externally than internally. Poultice of *C. serratum* is used to treat ringworm, leprosy, headache, and persistent fever, foment for puru and embrocate for stiffness in the joints. The Malays give a decoction internally for colic. In India, decoction of the roots with ginger and coriander is taken for nausea and the tender leaves are eaten as a salad. Dutch authorities said that in the Dutch Indies, the roots were eaten to clear voice, the leaves were eaten during labour, an extract was given for an extended stomach and ripe and unripe fruits were chewed for coughs (Burkill, 1966). In addition to that, this plant is useful in treating pain (fever), inflammation, rheumatism, respiratory disorders and malarial infections (Narayanan et al., 1999). Moreover, this plant is consider as a central nervous system depressant, antihistaminic, hypotensive, bronchoconstrictor, antiallergic, antibiotic, antifertility and stomachic medicine as mentioned by Sharma et al., (2000).

Narayanan et al., (1999) also reported that the ethanolic extract of *C. serratum* showed antinociceptive, anti-inflammatory and antipyretic effects in experimental animals. Vidya et al., (2007) reported that ethanolic extract of the *C. serratum* roots showed hepatoprotective activity. Asmawi et al., (1989) also reported that extract obtained from this plant, administered intravenously into anaesthetised rats lowered the blood pressure. In addition to that Nyman et al., (1998) also reported that root extracts of this plant inhibited angiotensin converting enzyme. Ethanolic extract of root of this plant were tested on two gram-positive and six gram-negative bacterial strains and found significantly inhibited the growth of *Streptococcus pyogenes-A* and *Proteus mirabilis* (Narayanan et al., 2004).

1.2.3. Phytochemistry

A number of compounds have been isolated from *C. serratum*. The root bark contains triterpenoids, mainly sapogenins, oleanolic acid, queretaroic acid, and serratagenic acid (Rangaswami & Sarangan, 1969), whereas the leaves contain flavonoids, and phenolic acid (Sharma et al., 2009). Several other substances have been isolated and identified in *C. serratum*, including *D*-mannitol (Garg & Verma, 1967; Banerjee et al., 1969), γ-sitosterol (Banerjee et al., 1969), plantarenaloside, euphroside, β-amyrin, apigenin, luteolin, and nepetin (van Valkenburg & Bunypraphatsara, 2002).

In addition to that, triterpenes (bauer-9-en-3-one), steroids (spinasterol), steroid glucoside (spinasteryl-β-D-glucopyranoside), and disaccharide (sucrose) were isolated from twigs and stems of methanolic extract of *C. serratum* (Boonsri, 2004). Vidya et al., (2007) reported that another triterpene, ursolic acid, was isolated from the ethanolic extract of *C. serratum* roots. Ravikumar et al., (2008) isolated triterpines, serratin and lupeol from the essential oil of *C. serratum*. Flavonoids such as ferulic acid, scutellarein and baicalein were found in this plant (Sharma et al., 2000). Hui et al., (2000) isolated monoterpene acid with monosaccharide derivatives named surratumin A, *cis*-cinnamic acid, *trans*-cinnamic acid, *p*-coumaric acid, 5, 7, 8, 4-tetrahydroxy-6-methoxyflavone and 5, 6, 7-trihydroxy-4-methoxyflavone-7-0-D-glucopyranoside. Wei et al., (2000) reported to find 7-β-coumaroyloxyugandoside, and 7-β-cinnamoyloxyugandoside. Banerjee, et al (1969) reported that the root bark of *C. serratum* does not contain any alkaloidal contents. Furthermore, Cannon et al., (1980) again reported that this plant does not contain any alkaloids. Some chemical structures of *C. serratum* constituents are presented in Figure 1.1.

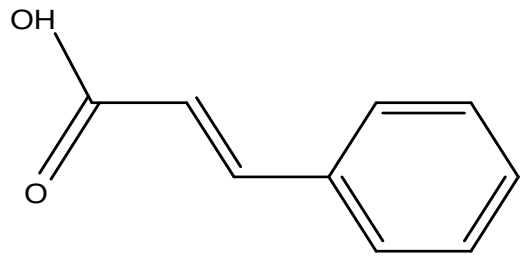
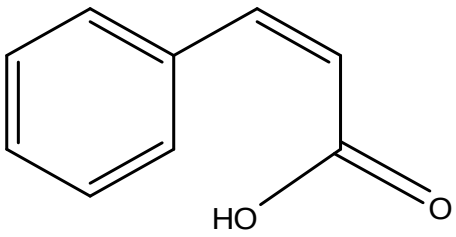
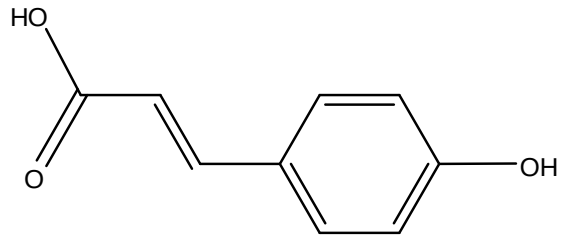
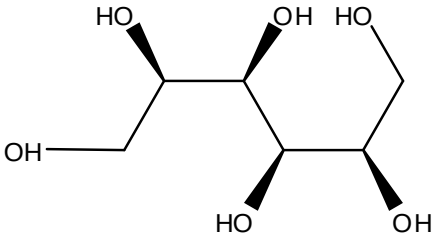
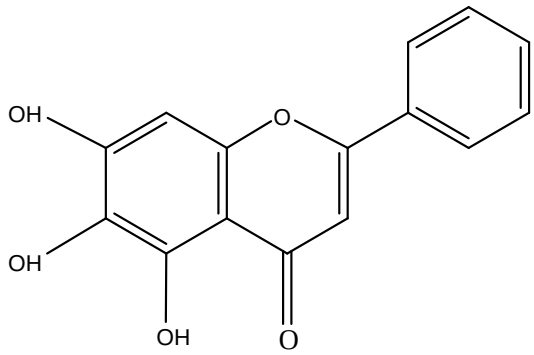
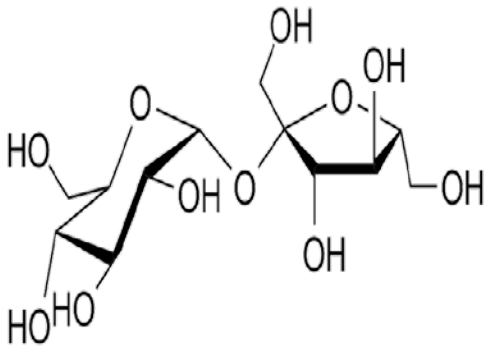
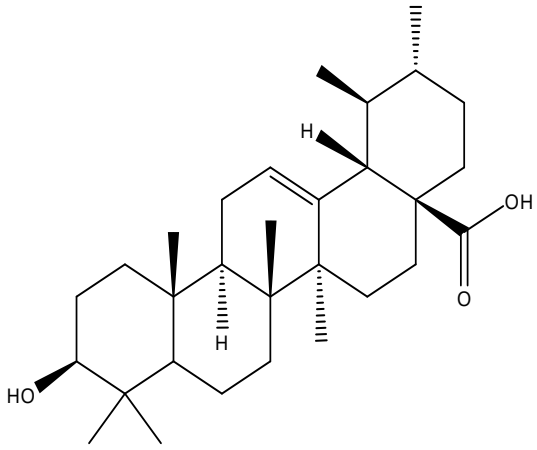
 Trans-cinnamic acid (Hui et al., 2000).	 Cis-cinnamic acid (Hui et al., 2000).
 P-coumaric acid (Hui et al., 2000).	 D-mannitol (Banerjee et al., 1969; Garg & Verma, 1967).
 Baicalein (Sharma et al., 2000).	 Sucrose (Boonsri, 2004).

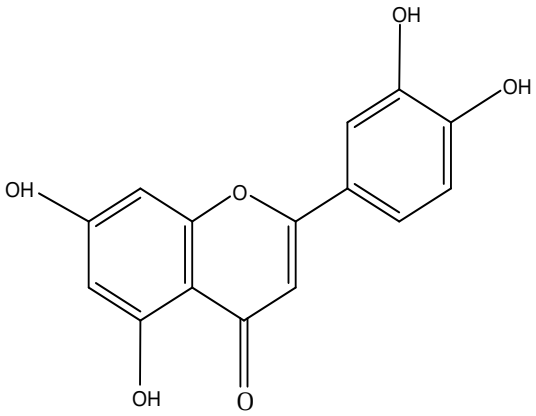
Figure 1.1. Chemical structures of some of the *C. serratum* constituents

Figure 1.1. (Continue.....)



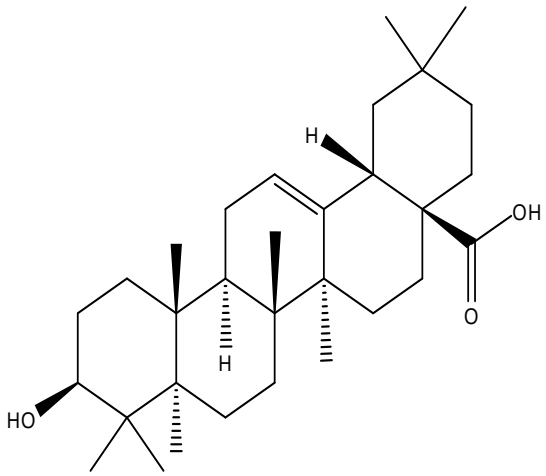
Ursolic acid

(Vidya et al., 2007).



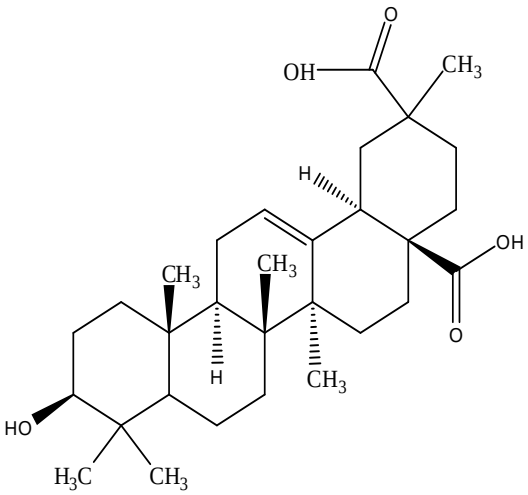
Luteolin

(van Valkenburg & Bunypraphatsara, 2002).



Oleanolic acid

(Rangaswami & Sarangan, 1969).



Serratagenic acid

(Rangaswami & Sarangan, 1969).

Figure 1.1. Chemical structures of some of the *C. serratum* constituents

1.1.4. Overall Objectives:

1. To serially extract the leaves of *C. serratum* with different solvents using petroleum ether, chloroform, methanol, and water respectively by maceration method for further pharmacological evaluation.
2. To fractionate the most potent pharmacologically active extract by solvent-solvent partition method using separatory funnel.
3. To perform phytochemical screening study of chemical groups present in most active extract/fraction by thin layer chromatography (TLC).
4. To quantify the total phenolic and flavonoid contents of *C. serratum* leaves extracts and its fractions.
5. To evaluate antioxidant activity of *C. serratum* leaves extracts and its fractions.
6. To screen the antiangiogenic activity of *C. serratum* leaves extracts and its fractions.
7. To determine the half maximal inhibitory concentration (IC_{50}) value on blood vessels growth inhibition of most active extract/fraction of *C. serratum* leaves.
8. To evaluate a possible vasorelaxant activity of *C. serratum* leaves extracts against noradrenaline (NA)-induced contraction of isolated rat aortic ring preparations.
9. To perform the vasorelaxant activity study of different fractions of methanolic extract of *C. serratum* leaves against NA-induced contraction of isolated rat aortic ring preparations.

CHAPTER TWO

PHYTOCHEMICAL SCREENING STUDY

2.1. Introduction

The first step of the extraction method is to release and solubilize the smaller secondary metabolites by a thorough solvent or aqueous extraction. This can be performed by a series of stepwise extractions, using different solvents of increasing polarity which should dissolve most natural products (Cannell, 1998). Hence, the extract is initially separated into various fractions containing compounds of relatively similar polarities. These fractions may be obvious, physically discrete divisions, such as the two phases of a liquid‑liquid partition method. However, it is more difficult to design an isolation protocol for extract or fraction where the types of compounds present are totally unknown. In this situation, it is advisable to carry out qualitative tests for the presence of various types of compounds, for example, terpenoids, tannins, saponins, alkaloids, flavonoids, and so forth, as well as analytical thin-layer chromatography (TLC), followed by gravity column chromatography for isolation purpose of target compound (Sarker et al., 2005).

2.2. Objectives

1. To serially extract the leaves of *C. serratum* with different solvents (using petroleum ether, chloroform, methanol, and water respectively) by maceration method to test pharmacological evaluation.
2. To fractionate the most potent pharmacologically active extract by solvent-solvent partition method using separatory funnel.
3. To perform phytochemical screening study of chemical groups present in most active extract/fraction by TLC.

2.3. Materials and Methods

2.3.1. Instruments

Fluorescence Analysis Cabinet, Model CX-20 (Long wave UV 365nm, Short wave UV 254nm)	Westbury, USA.
Rotary evaporator	Büchi, Switzerland
Water bath shaker model 903	Brotech, Malaysia.

2.3.2. Chemicals

Anisaldehyde reagent [$C_8H_8O_2$]	Sigma-Aldrich, Germany
Chloroform	Fisher Scientific, UK
Dragendroff reagent	Sigma-Aldrich, USA
Glacial acetic acid	R & M Chem, UK
Lead acetate [$Pb(CH_3COO)_2.3H_2O$]	Bensoden, Laboratory Chemicals
Methanol	Fisher Scientific, UK
Polyethylene glycol 4000 [PEG 4000]	Merck, Germany
Potassium hydroxide [KOH]	Merck, Germany
Petroleum ether	Fisher Scientific, UK
Silica gel 60 (70–230 mesh ASTM)	Merck, Germany
Sulphuric acid	R & M Chem, UK

2.3.3. Preparation of the Plant Materials

The fresh green leaves of *C. serratum* leaves, locally known as '*Timba tasek*', were collected between lowland of Maxwell Hill and Agriculture Department of District Larut, Taiping, Perak, Malaysia (from sea-level up to ±3.5 m altitude). The plant was collected in September 2006. The fresh green leaves of *C. serratum*, were washed under running tap water, separated from twigs, cleaned off adulterants, and

then dried in hot air oven at 40 ^{0}C for two days. The dried leaves were then grounded to fine powder using an electrically driven grinder. The plant was taxonomically identified as *Clerodendron serratum*, with the help of the late Abdulmajid Ahmed (May Allah have mercy on him) a staff of School of Pharmaceutical Sciences, Universiti Sains Malaysia. A voucher speciment of the plant (Reference number: 10935) has been deposited at the Herbarium School of Biological Sciences, Universiti Sains Malaysia.

2.3.4. Preparation of Extracts and Fractions of *C. serratum* Leaves

2.3.4.1. Maceration Method

The dried powdered leaves of *C. serratum* leaves (100g) were macerated sequentially with solvents of increasing polarity from petroleum ether (a defatting step), chloroform, methanol and water respectively in flasks (250 mL) in water bath shaker model 903 (Brotech, Malaysia) at 40 ^{0}C for 8 hours. The extraction procedure with each solvent was repeated three times. The combined filtrate obtained was filtered using Whatman filter paper and then the solvent was removed using a rotary evaporator (Büchi, Switzerland) under reduced pressure (Figure 2.1). The concentrated extracts were kept in a freezer at -70 ^{0}C for 48 hours and freezed dried for 48 hours. The dried extracts obtained were kept in tightly covered, labelled glass bottles and stored in desiccators.

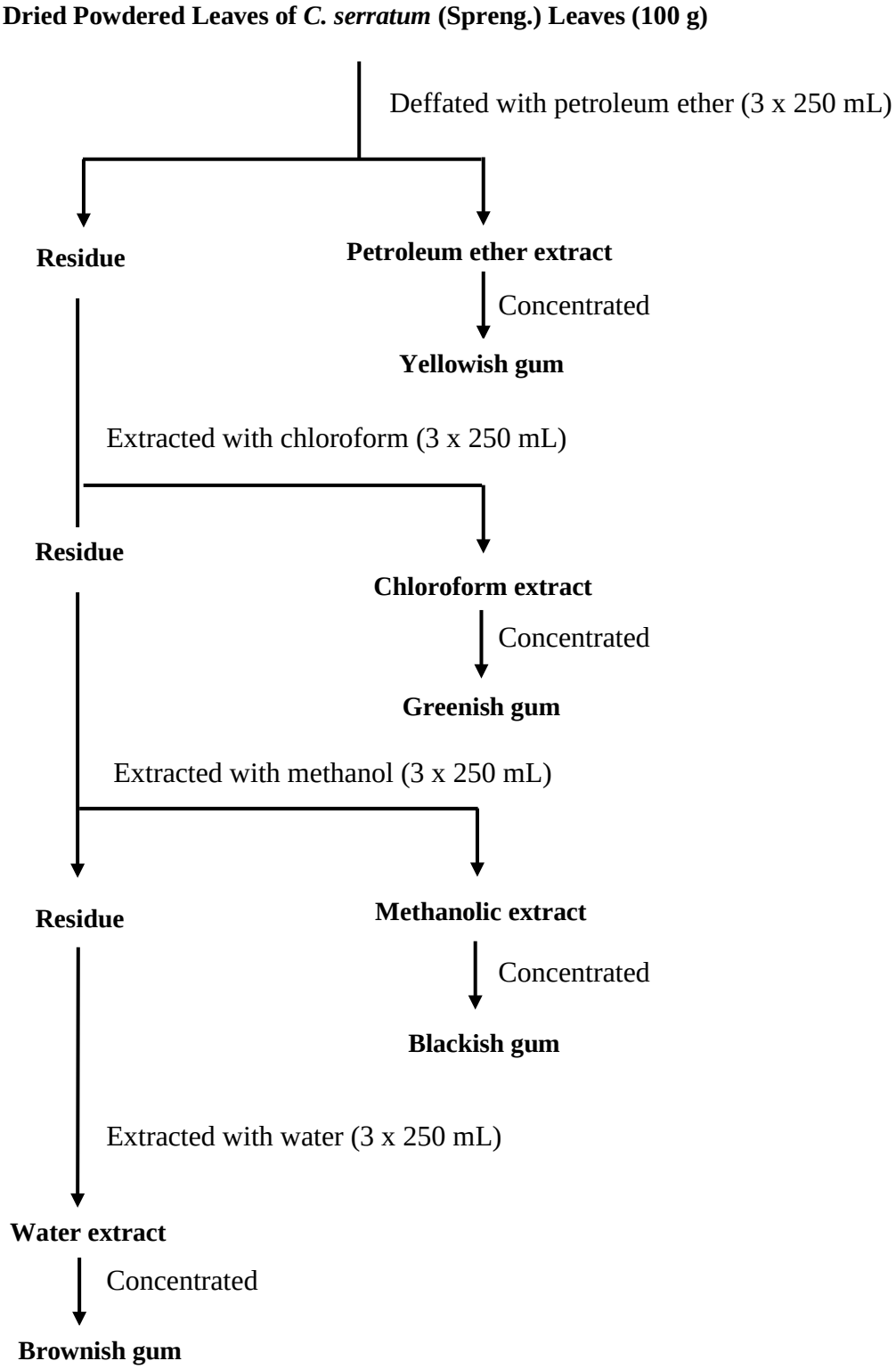


Figure 2.1. Schematic diagram showing the extraction procedure of *Clerodendron serratum* (Spreng.) leaves.

2.3.4.2. Partition Method

Since, methanolic extract of *C. serratum* leaves shown to be the most potent antioxidant, antiangiogenic and vasorelaxant effect amongst the four extracts. Then, the methanolic extract was subjected to a solvent-solvent partition process using solvents of increasing polarity from *n*-hexane, chloroform, ethyl acetate, *n*-butanol and water fraction respectively (Figure 2.2).

The methanolic extract (200 g) was initially suspended in approximately 500 mL of distilled water (37 ^{0}C) in a beaker. A glass rod was used to stir the mixture. The solvent obtained was then transferred into a separatory funnel. The solution was partitioned (equal volumes), with 3 x 250 mL of *n*-hexane using in a 1 litter volume separatory funnel. The resultant *n*-hexane layer was dried over using anhydrous sodium sulfate [Na_2SO_4]. The upper (*n*-hexane fraction) layer was poured out from the top of the separatory funnel to avoid contamination from the aqueous layer. The combined *n*-hexane filtrate fractions obtained was evaporated under reduced pressure using a rotary evaporator (Büchi, Switzerland) to yield a yellowish gum.

The aqueous layer (water miscible organic portion) was subjected to partition process with 3 x 250 mL of chloroform. The bottom (chloroform fraction) layer was separated into a flask. The combined chloroform filtrate fractions obtained was evaporated under reduced pressure using a rotatory evaporator (Büchi, Switzerland) to yield a greenish gum.

The aqueous layer was then partitioned with 3 x 250 mL of ethyl acetate. The upper (ethyl acetate fraction) layer was poured out from the top of the separatory

funnel to avoid contamination from the aqueous layer. The resultant ethyl acetate layer was dried over using anhydrous sodium sulfate [Na_2SO_4]. The combined ethyl acetate fraction obtained was concentrated using a rotatory evaporator (Büchi, Switzerland) under reduced pressure and a yellowish gum was obtained.

The aqueous layer was then extracted with 3 x 250 mL of *n*-butanol. The resultant *n*-butanol layer was dried over using anhydrous sodium sulfate [Na_2SO_4]. The upper (*n*-butanol fraction) layer was poured out from the top of the separatory funnel to minimize contamination from the aqueous layer. The combined *n*-butanol fraction obtained was concentrated on a rotatory evaporator (Büchi, Switzerland) under reduced pressure to yield a blackish gum.

The remaining aqueous solution (aqueous phase) obtained was concentrated using a rotatory evaporator (Büchi, Switzerland) under reduced pressure to yield a brownish gum of water fraction. The concentrated extracts obtained were kept in a freezer at -70 ^{0}C for 48 hours and freezed dried under reduced pressure at -40 ^{0}C for 48 hours to ensure complete dryness. The weights of the dried extracts obtained are shown in Table 2.2. These extracts were kept in tightly covered labelled glass bottles and stored in a desiccator.

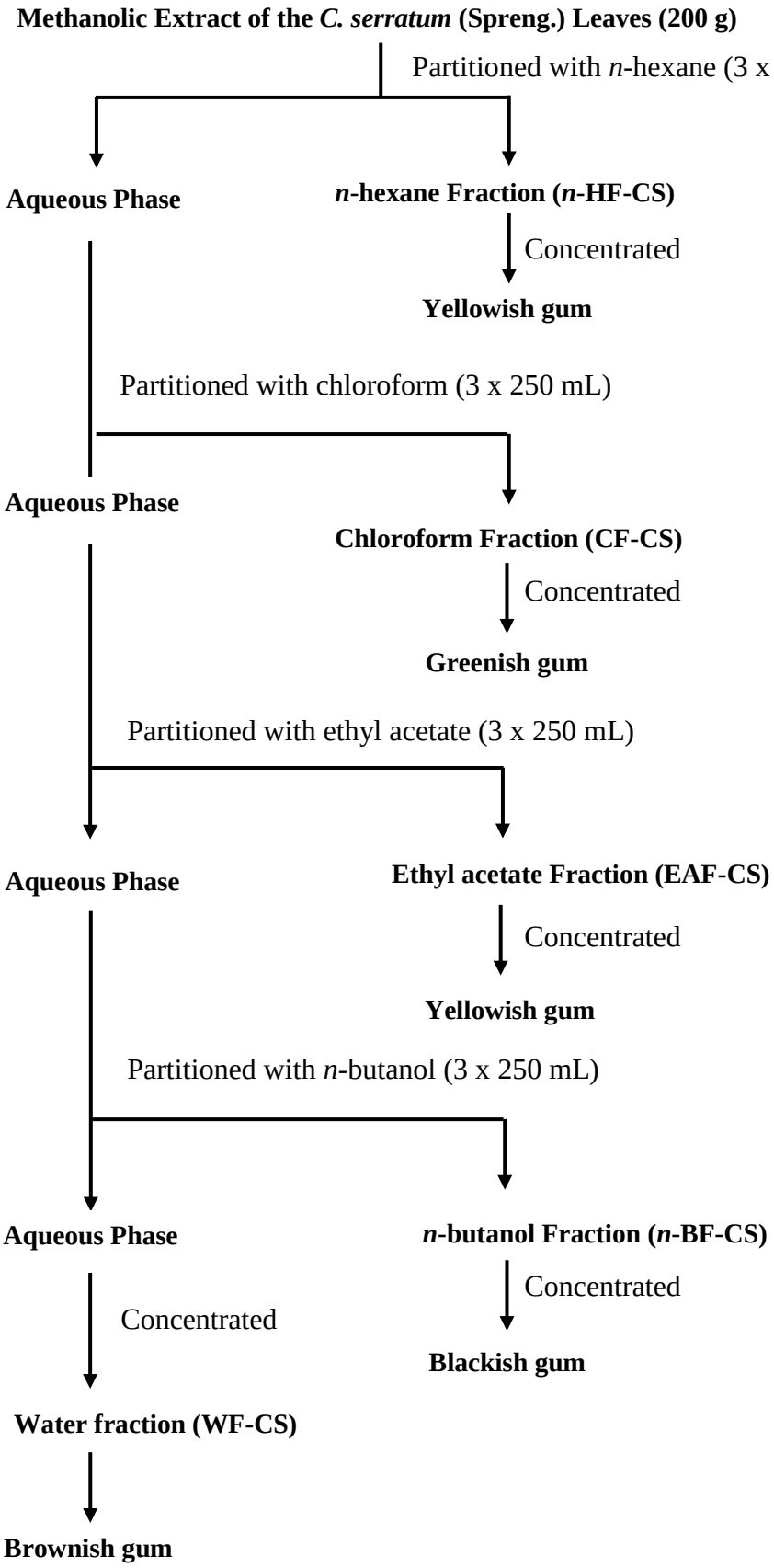


Figure 2.2. Schematic diagram showing fractionation method of the methanolic extract of *Clerodendron serratum* (Spreng.) leaves.

2.3.5. Sub-fractionations of *n*-Butanol Fraction of Methanolic Extract of *C. serratum* Leaves by Gravity Column Chromatography

2.3.5.1. Preparation of Gravity Column Chromatography

A sintered glass column chromatography measuring 105 cm long and 7 cm in diameter was attached to a ring stand and fastened in vertical position in a fumehood. A suspension of silica gel-60 (Cat No: 1.07734.1000, Merck, Germany) was prepared. The column was packed by filling about half-way with chloroform as eluting solvent system. Using a powder funnel, the translucent slurry was carefully transferred over a sintered glass column chromatography while swirling. A pasteur pipet was used to rinse, any silica that stucked on the sides of the column, with small amount of chloroform solvent. A recycled solvent was poured through the column several times until the column was firmly packed and silica gel becomes translucent. Then, the surface of silica gel was permanently kept covered with an eluting solvent system (Pavia et al., 1998; Fessenden et al., 2001).

2.3.5.2. Pre-adsorption and Sub-fractionation of the Sample

The *n*-butanol fraction (50g) was pre-adsorbed onto the adsorbent (1:2), silica gel-60 (70-230 mesh ASTM), by dissolving the extract in methanol. Using a round bottom flask, the solvent was removed from the slurry on a rotary evaporator under reduced pressure. The sample mixture was scraped down the sides of the flask before the last solvent was removed from the flask. When the sample was dried in oven at 40 ^{0}C, it was transferred to a mortar and grounded into a fine powder. The resultant dried fine powder of extract was placed onto the top of the column bed using a powder funnel. After loading the sample, a piece of small amount of non-absorbent cotton was added to the top of the column bed to prevent sample from being disturbed when fresh solvent is added. The successive eluates (100 mL) of sample

were collected into different pre-labeled conical flasks (Pavia et al., 1998; Fessenden et al., 2001). The eluting solvent system used as mobile phase in gravity column chromatography is shown in Table 2.1.

Table 2.1. Ratio of Different Solvents Used as Mobile Phase in Gravity Column Chromatography.

Number	Ratio of eluting solvent system			Total volume (L)
	Methanol	Chloroform	Water	
1	5.0	95.0	0.0	2.0
2	10.0	90.0	0.0	2.0
3	15.0	85.0	0.0	2.0
4	20.0	80.0	0.0	2.0
5	25.0	75.0	0.0	2.0
6	30.0	70.0	0.0	2.0
8	35.0	65.0	0.0	2.0
9	40.0	60.0	0.0	2.0
10	45.0	55.0	0.0	2.0
11	50.0	50.0	0.0	2.0
12	65.0	35	10	2.0

2.3.6. Phytochemical Screening of Methanolic Extract and *n*-Butanol Fraction of *C. serratum* Leaves

Preliminary phytochemical screening was performed to detect the presence of alkoloids, flavonoids, triterpenoids, saponins, and tannins. A few milligrams of each sample were dissolved in methanol and 5-10 µL was spotted on thin layer chromatography (TLC) plates (5 x 10 cm). The TLC plates were developed using upper phase of *n*-butanol, acetic acid and water (5:1:4 v/v) in TLC chamber at room temperature. The developed chromatogram of test sample was sprayed with various specific spray reagents for detection of the respective classes of compounds.

2.3.6.1. Detection of Alkaloids

The developed TLC plate was sprayed with Dragendorff's reagent [(Solution A: 0.85 g of basic bismuth nitrate was dissolved in 10 mL of glacial acetic acid and 40 mL distilled water), (Solution B: 8g of potasium iodide (KI) was dissolved in 30 mL distilled water)]. The solution A was mixed with solution B (1:1) and used as stock solution. The Dragendorff's reagent was prepared by mixing 5 mL of stock solution with 10 mL of glacial acetic acid and diluted with it to 50 mL of distilled water. Unstable orange-brown spots appear immediately under visible light indicates the presence of alkoloids (Wagner et al., 1984).

2.3.6.2. Detection of Terpenoids

The developed TLC plate was sprayed with anisaldehyde-sulphuric acid reagent and then heated for 5 min at 100 ^{0}C. The anisaldehyde-sulphuric acid reagent was prepared by mixing 0.5 mL of anisaldehyde with 10 mL glacial acetic acid, followed by 85 mL methanol and 5 mL concentrated sulphuric acid. The appearance

of blue-violet fourescence to red violet spots under visible light and UV 365 nm indicates the presence of triterpenes (Wagner et al., 1984).

2.3.6.3. Detection of Saponins

Saponins, due to their surface-active properties, they may be recognized by regular shaking an aqueous solution of the sample and observing the production of foam in a test tube, which is stable approximately for 15 minutes (Wagner et al., 1984; Silva et al., 1998; Tona et al., 1998). Forthing which persisted on warming indicates the presence of saponins (Owoyele et al., 2008).

2.3.6.4. Detection of Flavonoids

The developed TLC plate was sprayed with 10 mL of natural products-polyethylene glycol reagent (NP/PEG) and then examined under UV 365 nm. The NP/PEG was prepared by mixing 10 mL of solution A (1% diphenylboric acid β-ethylamino ester in methanol) followed by 8 mL of solution B (5% polyethylene glycol-4000 in ethanol). The flourensence intensity is produced either immediately or 15 minutes after spraying. The presence of dark yellow, blue and green flourescence under UV 365 nm indicates the presence of flavonoids (Wagner et al., 1984).

2.3.6.5. Detection of Tannins

Almost an aqueous solution of extracts or fractions that contains hydrolysable tannins are precipitated by 1 mL (10% w/v) of lead acetate in addition of 1 mL (10% v/v) of acetic acid, while non-hydrolysable tannins (Condensed) are soluble in

addition of 1 mL (10% v/v) of acetic acid (El Sissi & El Sherbeiny, 1967; Wagner et al., 1984).

2.4. Results

2.4.1. Extraction Yield of *C. serratum* Leaves

The extraction yield obtained from maceration of *C. serratum* leaves using various solvents is summarized in Table 2.2. Amongst the four solvents, methanol produced the highest extraction yield.

Table 2.2. The Extraction Yield Obtained from 100 g of Dried Powder Leaves of *C. serratum*.

Number	Extract	Weight (g)	% (w/w)
1	Petroleum ether	4.50	4.5
2	Chloroform	7.20	7.2
3	Methanol	28.50	28.5
4	Water	13.60	13.6

2.4.2. Partition Yield of Methanolic Extract Fractions of *C. serratum* Leaves

The methanolic extract of *C. serratum* leaves was fractionated using solvents of increasing polarity from *n*-hexane, chloroform, ethyl acetate, *n*-butanol and water respectively. The yield obtained from fractionation of methanolic extract of *C. serratum* leaves is shown in Table 2.3.

Table 2.3. The Fractionation Yield Obtained From the Methanolic Extract (200 g) of *C. serratum* Leaves Using Solvent-solvent Partition Method.

Number	Fraction	Weight (g)	% (w/w)
1	*n*-hexane	1.10	0.5
2	Chloroform	21.1	10.55
3	Ethyl acetate	6.90	3.45
4	*n*-butanol	78.9	39.45
5	Water	83.7	41.85

2.4.3. Column Chromatography

The *n*-butanol fraction of methanolic extract of *C. serratum* was chromatographed and eluted with eluting solvent system mentioned in Table 2.1. The eluate in each flask was concentrated to about 50 mL using rotary evaporator. Then, an aliquot of each fraction were spotted onto a thin layer chromatography (TLC) plate. TLC plates were developed using the upper phase of *n*-butanol, acetic acid and water (5:1:4 v/v) as mobile phase. Those eluates giving the same profiles were pooled together. The combination of eluates yielded ten sub-fractions which were not fully dried and labelled as (A) 1.20, (B) 1.70, (C) 1.14, (D) 3.43, (E) 0.70, (F) 8.00, (G) 15.50, (H) 1.50, (I) 11.23 and (J) 1.30 g respectively. The sub-fraction I (12.23 g) that showed as targeted compound by thin layer chromatography have been successfully isolated from *n*-butanol fraction of *C. serratum* leaves but need further purification steps.

2.4.4. Phytochemical Screening of Methanolic Extract, *n*-Butanol Fraction and Sub-fraction of *C. serratum* Leaves

2.4.4.1. Detection of the Presence of Alkaloids

The developed chromatograms of the methanolic extract, *n*-butanol fraction and *n*-butanol sub-fraction of *C. serratum* leaves were sprayed with Dragendorff's reagent and examined under visible light. The result showed that there was no orange-brown zone observed in methanol extract, *n*-butanol fraction and *n*-butanol sub-fraction (i) of *C. serratum* leaves which indicated the absence of any alkaloids (Plate 2.1).

2.4.4.2. Detection of the Presence of Flavonoids

The developed chromatograms of the methanolic extract, *n*-butanol fraction and *n*-butanol sub-fraction (i), of *C. serratum* leaves was first examined under UV 365nm and then was sprayed with natural product reagent (NP/PEG) in order to intensify the fluorescence quenching. The presence of blue coloured spots under UV 365 nm before and after spraying indicated the presence of flavonoids (Plate 2.2).

2.4.4.3. Detection of the Presence of Terpenoids

The developed chromatograms of the methanolic extract, *n*-butanol fraction and *n*-butanol sub-fraction (i) of *C. serratum* leaves were sprayed with anisaldehyde-sulphuric acid reagent. Then they were examined under visible light and UV 365 nm. After being sprayed with anisaldehyde-sulphuric acid reagent the appearance of blue and green coloured spots under UV 365 nm indicated the presence of terpenoids (Plate 2.3).