PHYTOCHEMICAL AND BIOLOGICAL ACTIVITY STUDIES ON *GARCINIA ATROVIRIDIS* GRIFF. EX T. ANDERS.

by

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LIST OF ABBREVIATIONS AND SYMBOLS

AChE	Acetylcholinesterase
BChE	Butyrylcholinesterase
CC	Column Chromatography
CD	Circular Dichroism
COSY	Correlation Spectroscopy
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
d	Doublet
dd	Doublet Of Doublets
DEPT	Distortionless Enhancement By Polarization Transfer
DP-MS	Direct Probe Mass Spectrometry
DPPH	Diphenylpicryl-hydrazyl
EI-MS	Electron Ionization Mass Spectrometry
eV	Electron Volt
FID	Flame Ionization Detector
FT-IR	Fourier-Transform Infrared
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HMBC	Hetronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
INT	*p*-Iodonitrotetrazolium Violet
IR	Infrared
J	Coupling Constant

MIC	Minimum Inhibitory Concentrations
m/z	Mass/Charge
NMR	Nuclear Magnetic Resonance
MS	Mass Spectrometry
NOESY	Nuclear Overhauser Enhancement Spectroscopy
ppm	Part Per Million
q	Quartet
RF	Response Factor
RI	Retention Index
RT	Retention Time (Minutes)
s	Singlet
t	Triplet
TLC	Thin Layer Chromatography
UV	Ultra-Violet

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KAJIAN FITOKIMIA DAN AKTIVITI BIOLOGI KE ATAS *GARCINIA ATROVIRIDIS* GRIFF. EX T. ANDERS.

ABSTRAK

Minyak mudah meruap daripada buah *Garcinia atroviridis* Griff. ex T. Anders. telah dipencilkan melalui kaedah penghidrosulingan dan dianalisa dengan kaedah kapilari GC dan GC-MS menggunakan tiga turus yang mempunyai kekutuban yang berbeza. Minyak mudah meruap mengandungi terutamanya seskuiterpena dengan komponen utama (-)-*β*-kariofilena (23.8 %), alkohol *β*-kariofilena (15.6 %) dan *α*-humulena (10.7 %). Tiga komponen daripada minyak mudah meruap, iaitu alkohol *β*-kariofilena (**68**), ginsenol (**70**) dan (*1S,2S,5S,8S*)-4,4,8-trimetiltrisiklo[6.3.1.$0^{1,5}$]dodekan-2-ol (**71**) telah disintesis dan dipencilkan melalui kaedah GC preparatif bagi tujuan pengesahan struktur.

Kulit batang *G. atroviridis* yang dikeringudara telah diekstrak secara berturutan menggunakan radas Sokslet, dengan heksana, diklorometana dan metanol. Ekstrak metanol kemudiannya diperingkatkan dengan klorofom. Setiap ekstrak ditulenkan melalui kaedah kromatografi turus dengan menggunakan gel silika atau Sefadeks LH-20 dan memberikan 11 sebatian tulen. Struktur sebatian tersebut telah ditentukan secara kaedah spektroskopi seperti FT-IR, 1D NMR, 2D NMR, UV, DP-MS dan TOF LC-MS. Stigmasta-5,22-dien-3-ol (**90**) dan stigmasta-5,22-dien-3-*O*-*β*-glukopiranosida (**91**) telah dipencilkan daripada ekstrak heksana manakala ekstrak diklorometana memberikan 3*β*-asetoksi-11*α*,12*α*-epoksiolenan-28,13*β*-olida (**92**), 2,6-dimetoksi-*p*-benzokuinon (**93**), 1,3,5-trihidroksi-2-metoksizanton (**94**), 1,3,7-

trihidroksizanton (**95**), 3,5,7,4’-tetrahidroksiflavon (**96**) dan 3,5,7,3’,4’-pentahidroksiflavon (**97**). Tiga hasil semula jadi yang baru, iaitu, 5,10-dihidroksi-12-metoksi-2,2-dimetil-2*H*,6*H*-pirano[3,2-b]zanten-6-on (**98**), 3,3’’,4’’’,5,5’’-pentahidroksi-(4’-7’’)-oksi-7-*O*-(11,15-dihidroksi-19-okso-13-zantil)biflavon (**100**) dan (*2S,2’’S,2’’’’S*)-4’’’’’,5,5’’,5’’’’-tetrahidroksi-(4’-7’’, 4’’’-7’’’’)-dioksi-7-*O*-(11,17-dihidroksi-19-okso-13-zantil)triflavon (**101**) telah dipencilkan daripada ekstrak klorofom.

Minyak mudah meruap dan tiga komponen daripadanya, ekstrak mentah dan semua sebatian yang dipencilkan telah dianalisa untuk aktiviti biologi (antibakteria, perencat enzim kolinsterase, antioksidan dan antiradang). Minyak mudah meruap dan tiga komponen daripadanya menunjukkan aktiviti antibakteria dan antiradang yang baik. Sebatian **97** dan **98** yang dipencilkan daripada kulit batang adalah aktif terhadap aktiviti perencat enzim asetilkolinsteras dan butirilkolinsteras, masing-masing. Mengenai aktiviti perangkap radikal bebas DPPH, sebatian **97** dan **95** telah menunjukkan potensi yang baik bagi aktiviti antioksida.

PHYTOCHEMICAL AND BIOLOGICAL ACTIVITY STUDIES ON *GARCINIA ATROVIRIDIS* GRIFF. EX T. ANDERS.

ABSTRACT

The volatile oil of the fruit of *Garcinia atroviridis* Griff. ex T. Anders. was isolated by hydrodistillation and analysed by capillary GC and GC-MS, using three columns of different polarity. The oil was dominated by sesquiterpenoids, with the most abundant components being (-)-*β*-caryophyllene (23.8 %), *β*-caryophyllene alcohol (15.6 %) and *α*-humulene (10.7 %). Three of the volatile constituents, *β*-caryophyllene alcohol (**68**), ginsenol (**70**) and (*1S,2S,5S,8S*)-4,4,8-trimethyltricyclo[6.3.1.$0^{1,5}$]dodecan-2-ol (**71**) were synthesized and isolated by preparative GC for authentication purposes.

The air-dried stem bark of *G. atroviridis* was sequentially extracted in a Soxhlet apparatus using hexane, dichloromethane and methanol. The methanol extract was then partitioned with chloroform. Each extract was purified by repeated column chromatography over either silica gel or Sephadex LH-20, giving 11 pure compounds. Their structures were elucidated by spectroscopic methods such as FT-IR, 1D NMR, 2D NMR, UV, DP-MS and TOF LC-MS. Stigmasta-5,22-dien-3-ol (**90**) and stigmasta-5,22-dien-3-*O*-*β*-glucopyranoside (**91**) were isolated from the hexane extract, while the dichloromethane extract gave 3*β*-acetoxy-11*α*,12*α*-epoxyoleanan-28,13*β*-olide (**92**), 2,6-dimethoxy-*p*-benzoquinone (**93**), 1,3,5-trihydroxy-2-methoxyxanthone (**94**), 1,3,7-trihydroxyxanthone (**95**), 3,5,7,4’-tetrahydroxyflavone (**96**) and 3,5,7,3’,4’-pentahydroxyflavone (**97**). Three new natural products, namely,

5,10-dihydroxy-12-methoxy-2,2-dimethyl-*2H*,*6H*-pyrano[3,2-b]xanthen-6-one (**98**), 3,3’’,4’’’,5,5’’-pentahydroxy-(4’-7’’)-oxy-7-*O*-(11,15-dihydroxy-19-oxo-13-xanthyl)biflavone (**100**) and (*2S*,*2’’S*,*2’’’’S*)-4’’’’’,5,5’’,5’’’’-tetrahydroxy-(4’-7’’, 4’’’-7’’’’)-dioxy-7-*O*-(11,17-dihydroxy-19-oxo-13-xanthyl)triflavone (**101**) were isolated from the chloroform extract.

The volatile oil and three of its constituents, crude extracts and all the isolated compounds were assayed for biological activities (antibacterial, cholinesterase enzyme inhibitory, antioxidant and anti-inflammatory). The volatile oil and three of the constituents showed remarkable antibacterial and anti-inflammatory activities. Compounds **97** and **98** isolated from the stem bark are active against acetylcholinesterase and butyrylcholinesterase enzymes inhibitory activity, respectively. Regarding the DPPH free radical scavenging activity, compounds **97** and **95** were shown to possess remarkable potential for antioxidant acitivity.

CHAPTER ONE

INTRODUCTION

1.1 Natural Products

The term ‘natural products’ is applied to materials derived from plants, microorganisms, invertebrates and vertebrates, which are fine biochemical factories for the biosynthesis of both primary and secondary metabolites. Natural products include steroids, terpenoids, fatty lipids and prostaglandins, alkaloids, amino acids, proteins, nucleic acids, carbohydrates, insect and plant growth regulators, phenolic compounds, natural dyes and pigments, vitamins, hormones, etc. Due to multidirectional promising aspects, the interest in natural products continues to this very day. The chemical diversity and unique biological activities of a wide variety of natural products have propelled many discoveries in chemical and biological sciences, and provided therapeutic agents to treat various diseases as well as offered leads for the development of valuable medicines. With the advent of improved chromatographic separation techniques, the separation of various natural products is achieved routinely. Newer spectroscopic techniques such as two-dimensional nuclear magnetic resonance spectrocopy, infrared and Raman spectroscopy, X-ray crystallography, and mass spectroscopy have simplified the structure elucidation of new natural products.

1.2 The Clusiaceae Family

The Clusiaceae (or Guttiferae) family of trees is naturally distributed in tropical zones between the latitudes of 10º north and 10º south and comprises about 40 genera and 1000 species (Corner, 1988; Pangsuban, Bamroongrugsa, Kanchanapoom,

& Nualsri, 2009). Among them, four genera (*Garcinia*, *Calophyllum*, *Mesua* and *Mammea*) and about 120 species are found in Peninsular Malaysia (Corner, 1988). Trees or shrubs of this family contain yellow or white resinous latex, and the leaves are opposite, simple and mostly entire, with stipules absent (Corner, 1988). The flowers are often bright and showy, variously arranged but generally clustered and axillary (Corner, 1988). Clusiaceae are all woody plants and the trunks may be buttressed, or there may be knee or prop roots (Stevens, 2007). Several species of the Clusiaceae family are well-known for their hulls manufacture due to the quality of their wood, and for the healing properties of their latex which is used traditionally for its effectiveness against dermatoses (Poumale et al., 2011). One unique feature of plants in Clusiaceae is the presence of natural, secondary metabolites called xanthones (Lim, 2012).

1.3 The Genus *Garcinia*

Garcinia is the biggest genus in the family of Clusiaceae, with over 400 species in the tropics of Africa and Asia, and about 50 species found in the lowland and mountains of Peninsular Malaysia (Corner, 1988; Pangsuban et al., 2009). *Garcinia* are evergreen, lactiferous, and dioecious trees and shrubs, generally of medium height and seldom exceeding 18 m (Corner, 1988; Lim, 2012). The flowers are singly or in small clusters in the leaf-axils and occasionally in short racemes (Corner, 1988). The fruit of *Garcinia* is large, fleshy and often with a rind (Corner, 1988). Of the edible *Garcinia* species, most notable ones from an economic standpoint are *Garcinia mangostana*, dubbed the Queen of fruits, and *Garcinia gummi-gutta*, source of hydroxycitric acid for weight loss (Lim, 2012).

The genus *Garcinia* comprises of a group of medicinal plants with potential therapeutic agents (Hemshekhar et al., 2011). The different plant parts such as fruit, fruit rind, flowers, leaves, bark and stem have been used globally as an ethnomedicine to treat several disorders such as inflammation, oxidative stress, microbial infection, cancer and obesity (Hemshekhar et al., 2011).

1.4 *Garcinia atroviridis* Griff. ex T. Anders. And Its Medicinal Uses

Garcinia atroviridis Griff. ex T. Anders. (Fig. 1.1) is a medium-sized fruit tree found wild in the forest of Peninsular Malaysia, Thailand, Myanmar, India and sometimes in a half-cultivated state in villages (Burkill, 1966; Lim, 2012). It is commonly known as 'Asam Gelugor' in Malaysia or 'Som-Khaek' in Thailand (Lim, 2012). The tree can grow to a height of 27 m with 70 cm girth and a long trunk. The crown of the tree is narrowly conical with numerous slender, terete drooping branches (Lim, 2012).

The young fruits are green and turn to bright yellow when ripe. They are depressed globose, 6 − 10 cm in diameter with broadly sunken concave apex, containing 12 – 16 ribs and shallow grooves giving it a fluted form with persistent petals and sepals and a thick rind. The fruits often separate off into pegs or segments (Lim, 2012). A mature fruit can weigh up to 2 kg (Lim, 2012). The fruit contains several flattened seeds 1.5 cm long, which are surrounded by acidic, bright orange pulp (Lim, 2012) (Fig. 1.2 & 1.3).

G. atroviridis is gynodioecious and is commonly propagated by seeds (Pangsuban et al., 2009). The trees begin to flower 5 − 6 years after planting, and maximum fruit

yield is usually attained after 6 − 7 years (Pangsuban et al., 2009). The female trees produce fruits, while the hermaphrodite trees sometimes bear only a few fruits or have no fruit-set (Pangsuban et al., 2009).

Sun-dried slices of the fruits, locally known as ‘asam keping’, are commercially available and are popularly used as a seasoning in curries, sour relish, dressing fish or stewed in plenty of sugar and eaten (Lim, 2012; Mackeen et al., 2000). The dried fruit slices are used to give acidity to cooked dishes in place of tamarind. The dried rind is also used in herbal health teas (Lim, 2012). Occasionally, the young leaves are used for culinary purposes and as traditional vegetable (Mackeen et al., 2000). In the East Coast states of Peninsular Malaysia, fresh fish are steamed with the leaves of *G. atroviridis* to delay spoilage (Mackeen et al., 2000).

Fig. 1.1 *G. atroviridis* Tree Found In Botanical Garden, Penang

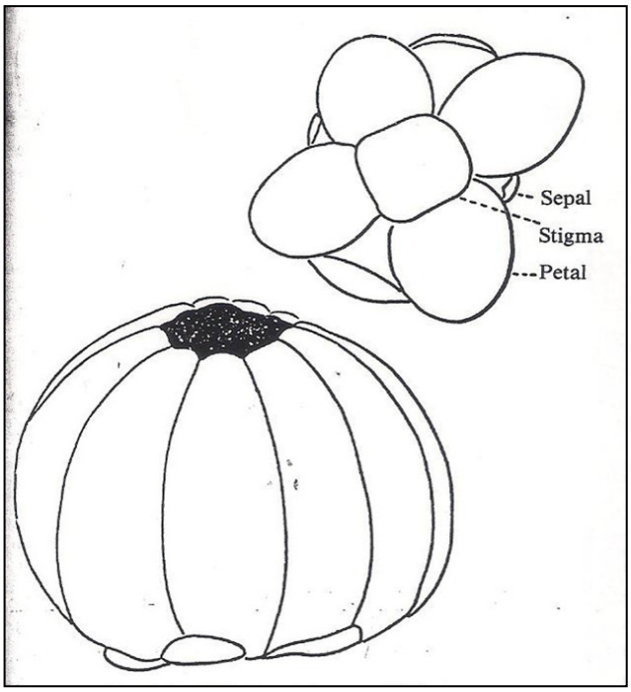


Fig. 1.2 The Fruit Of *Garcinia atroviridis* (Corner, 1988)

Fig. 1.3 *Garcinia atroviridis* Fruit

In folkloric medicine, *G. atroviridis* has been used as a postpartum medication agent as well as an agent to treat earache, throat irritation, cough, dandruff and certain stomach aches associated with pregnancy (Amran, Zaiton, Faizah, & Morat, 2009). In Peninsular Malaysia, the fruit is used in a lotion made with vinegar, which is rubbed upon the abdomen of a woman after confinement (Burkill, 1966). Juice from the leaves is given to a woman after childbirth in one of the numerous protective preparations which the Malays employ (Burkill, 1966). It is recorded that decoction of the leaves and roots is dropped into the ear for earache (Burkill, 1966). In Thailand, the dried fruit of *G. atroviridis* is used for improving blood circulation, as an expectorant, treatment of coughs and as laxative (Lim, 2012).

CHAPTER TWO

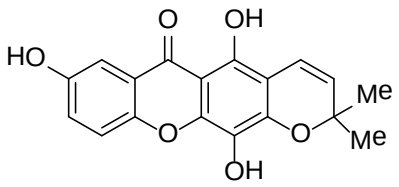
LITERATURE REVIEW

2.1 Previous Studies On *Garcinia atroviridis*

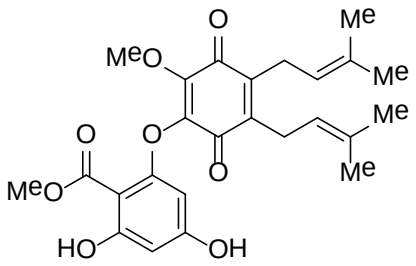
A xanthone, atroviridin (**1**) was isolated by Kosin et al. (1998) from the chloroform extract of the stem bark of *G. atroviridis*. From the methanol extract of the dried roots, a prenylated benzoquinone, atrovirinone (**2**), and a prenylated depsidone, atrovirisidone (**3**), have been isolated (Permana et al., 2001). In bioassay for antimicrobial activity, atrovirinone (**2**) and atrovirisidone (**3**) were found to exhibit significant inhibitory activity against *Staphylococcus aureus* and *Bacillus cereus* (both Gram positive) but not against *Escherichia coli* (Gram negative), *Aspergillus ochraceus* (fungus), and *Candida albicans* (yeast) at the dose of 10 µg per disk (Permana et al., 2001). Whereas in cytotoxic activity assay, only atrovirinone (**2**) showed cytotoxicity toward HeLa cells with an IC_{50} of 15 µg/mL, which was comparable to the standards, Doxorubicin (IC_{50} 11 µg/mL) and Colchicine (IC_{50} 21 µg/mL) (Permana et al., 2001). In a continuing study on the roots, Permana et al. (2003) had identified a prenylated hydroquinone: 4-methylhydroatrovirinone (**4**) from the methanol extract of the plant. This compound was believed to be the intermediate involved in the biosynthesis of atrovirinone (**2**) and atrovirisidone (**3**) (Permana et al., 2003). In addition, *cis*-14-docosenoic acid (**5**), morelloflavone (**6**) and its glucosidic derivative, fukugiside (**7**), were also isolated (Permana et al., 2003). Further investigation on the roots, a prenylated depsidone: atrovirisidone B (**8**) was isolated (Permana et al., 2005). In the *in vitro* cytotoxicity assay, atrovirisidone B (**8**) has shown to be active against human breast cancer (MCF-7), human prostate cancer

(DU-145) and human lung cancer (H-460) cell lines. Besides, naringenin (**9**) and 3,8''-binaringenin (**10**) were also isolated in the same study (Permana et al., 2005).

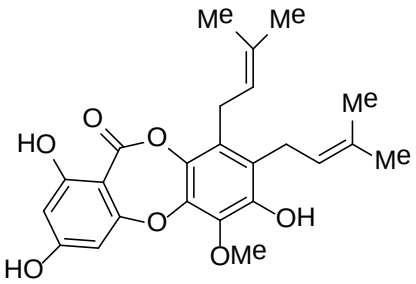
Previous studies on the fruits have reported the isolation of garcinia acid (identical to (-)-hyroxycitric acid) (**11**), and its dibutyl methyl esters, and *β*- and *γ*-lactone derivatives, as well as the identification of several saturated fatty acids such as pentadecanoic, octadecanoic, nonadecanoic and dodecanoic acids by gas chromatography-mass spectrometry (Permana et al., 2001; Jena, Jayaprakasha, Singh & Sakariah, 2002; Amran et al., 2009; Lim, 2012). The fruit also contains fruit acids such as citric acid (**12**), tartaric acid (**13**) and ascorbic acid (**14**) that have antioxidant properties (Amran et al., 2009). The hydroxycitric acid and flavonoids present in the fruit extract have been reported to exhibit hypolipidemic activity that can promote weight loss, by lowering lipogenesis and increasing glycogen development, thus lowering appetite (Amran et al., 2009).



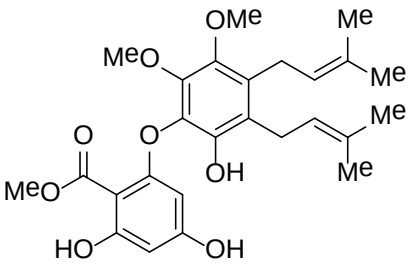
(**1**)



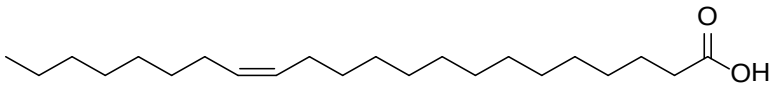
(**2**)



(**3**)



(**4**)



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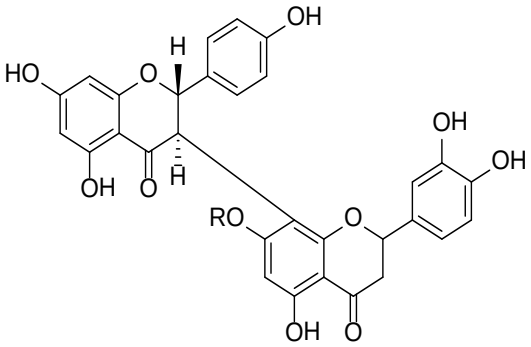
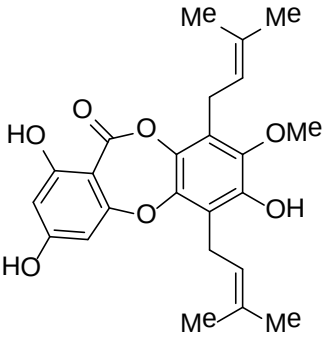
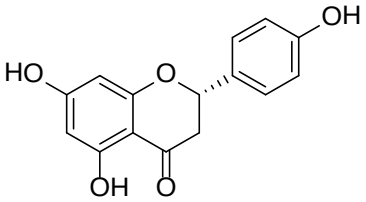


(**6**) R = H

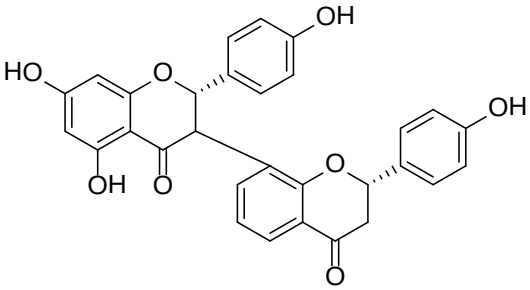
(**7**) R = Glucosyl



(**8**)



(**9**)



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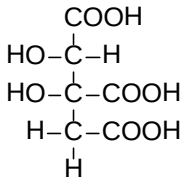
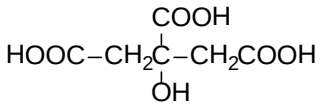


(**11**)



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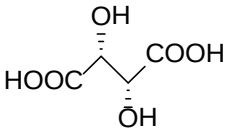
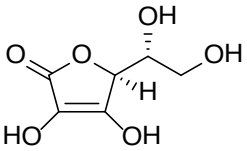


(**13**)

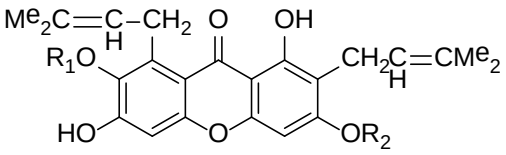


(**14**)

2.2 Studies On Other *Garcinia* Species

A well-known species from the genus *Garcinia* is *Garcinia mangostana* L., commonly known as mangosteen. This tropical fruit tree is extensively distributed in Southeast Asia (Obolskiy, Pischel, N.Siriwatanametanon, & Heinrich, 2009; Zhang, Song, Hao, Qiu, & Xu, 2010). Phytochemical investigations on *Garcinia mangostana* have led to the isolation of xanthone-type compounds as the major secondary metabolites (Chin & Kinghorn, 2008). Xanthones, a class of polyphenolic compounds with the skeleton of a xanthen-9-one, have been isolated from the pericarp, whole fruit, bark, and leaves of *G. mangostana* (Pedraza-Chaverri, Cardenas-Rodriguez, Orozco-Ibarra, & Perez-Rojas, 2008; Obolskiy et al., 2009). Studies on the pericarp of the mangosteen fruit have afforded *α*-mangostin (**15**) (Schmid, 1855; Dragendorff, 1930; Murakami, 1932; Yates & Stout, 1958), *β*-mangostin (**16**) (Dragendorff, 1930; Yates & Bhat, 1968), *γ*-mangosteen (**17**) (Jefferson, Quillinan, Scheinmann, & Sim, 1970), gartanin (**18**) (Govindachari, Kalyanaraman, Muthukumaraswamy, & Pai, 1971) and 8-deoxygartanin (**19**) (Govindachari et al., 1971) as the most abundant representatives. Recently,

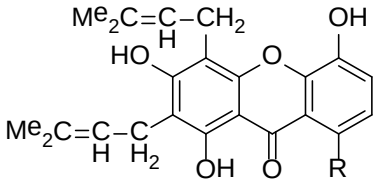
garcimangosxanthone A (**20**), B (**21**) and C (**22**) were isolated from the pericarp of *Garcinia mangostana* (Zhang et al., 2010). Among them, garcimangosxanthone A (**20**) and B (**21**) were reported to exhibit *in vitro* cytotoxicity against human lung cancer (A549), human pulmonary carcinoma (LAC) and human hepatoma (A375) cell lines with IC_{50} values of 5.7 − 24.9 µM, which were comparable to those of Doxorubicin (Zhang et al., 2010). From the ethanol extract of the stem bark of *Garcinia mangostana*, a xanthone, mangosharin (**23**) has been isolated (Ee, Daud, Taufiq-Yap, Ismail, & Rahmani, 2006).



(**15**) R_1 = Me, R_2 = H

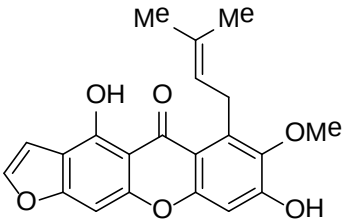
(**16**) R_1 = Me, R_2 = Me

(**17**) R_1 = H, R_2 = H

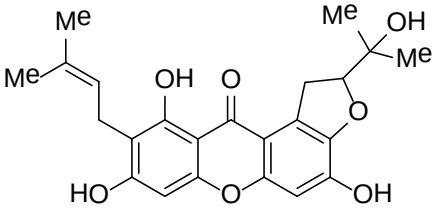


(**18**) R = OH

(**19**) R = H



(**20**)



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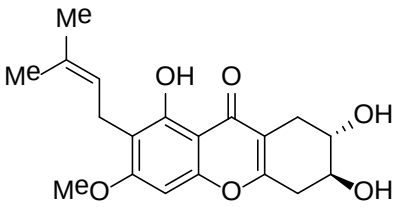
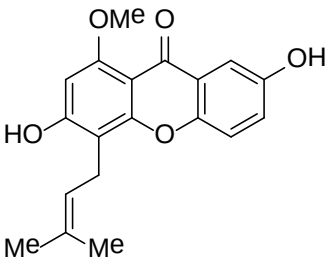
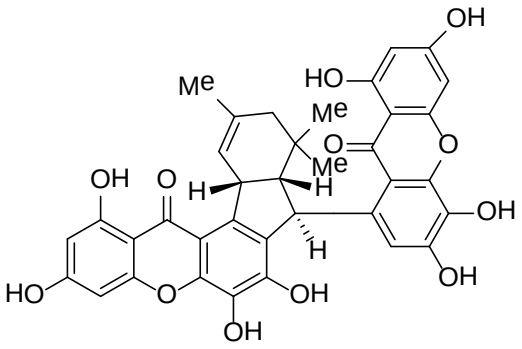


(**22**)

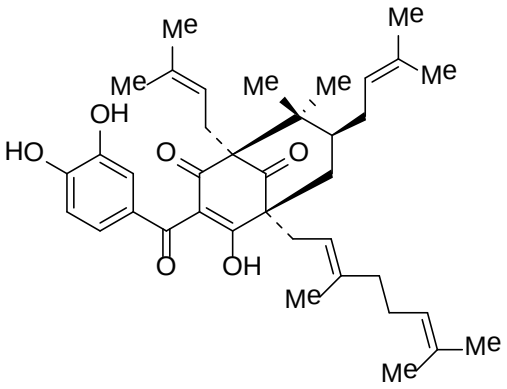


(**23**)

Garcinia griffithii T. Anders. is a moderate-size tree found in Peninsular Malaysia (Burkill, 1966). Griffipavixanthone (**24**), a novel bixanthone, has been isolated from its bark (Xu et al., 1998). This compound showed high *in vitro* cytotoxicity against P388, LL/2 and Wehi 164 cell lines. In a continuing study on the stem bark of *Garcinia griffithii*, Nilar et al. (2005) had isolated a polyisoprenylated benzophenone, guttiferone I (**25**).



(**24**)



(**25**)

2.3 Problem Statement

Reported investigations on *Garcinia* species have shown that this genus is rich with many pharmacologically and medicinally important chemical entities. The isolated compounds from different plant parts of genus *Garcinia* have been widely studied for their various therapeutic and pharmacological activities such as antioxidant, anti-inflammatory, anti-bacterial, anti-viral, anti-protozoan, anti-ulcer, anti-cancer and hypolipidemic properties (Hemshekhar et al., 2011).

However, there is lack of information from *G. atroviridis* on its volatile and non-volatile constituents though this plant is widely found in Peninsular Malaysia. There

is a need to examine the constituents present in *G. atroviridis* in an attempt to discover new natural products that may possess important biochemical and pharmacological effects.

2.4 Objectives Of The Study

The objectives of this study are divided into three parts. The first part is to identify the volatile constituents from the fruits of *Garcinia atroviridis.* The second part is to isolate and characterize the non-volatile constituents from the stem barks of *G. atroviridis*. The final part is the biological activity studies including antibacterial, cholinesterase enzyme inhibitory, antioxidant and anti-inflammatory.

1. To identify and quantify the volatile flavour components of the fruit of *Garcinia atroviridis* using capillary GC and GC-MS techniques.
2. To synthesize the authentic flavour components, namely, *β*-caryophyllene alcohol (**68**), ginsenol (**70**) and (*1S,2S,5S,8S*)-4,4,8-trimethyltricyclo[6.3.1.$0^{1,5}$]dodecan-2-ol (**71**).
3. To isolate and elucidate the structures of the non-volatile constituents in the stem bark of *Garcinia atroviridis*, using various spectroscopic techniques (FT-IR, 1D and 2D NMR, UV, DP-MS and TOF LC-MS).
4. To assay the fruit volatile constituents, all plant extracts and isolated pure components for biological activities (antibacterial, cholinesterase enzyme inhibitory, antioxidant and anti-inflammatory).

2.4 Scopes Of The Study

The samples for this study are the fruits and stem barks of *G. atroviridis* which were collected from Kedah, where a voucher specimen USM 11201 was deposited. Identitification of volatile constituents was carried out by hydrodistillation using Clevenger apparatus. Sequentially solvent extraction using Soxhlet was used to obtain a series of plant extracts. Fractionation and purification of the extracts using thin layer chromatography (TLC) and column choromatography (CC) gave pure compounds. The structural elucidation of pure compounds is confirmed using analytical and spectroscopic methods including FT-IR, 1D and 2D NMR, UV, DP-MS and TOF LC-MS. The biological activity assays such as antibacterial, cholinesterase enzyme inhibitory, antioxidant and anti-inflammatory were performed on the volatile oils, crude extracts and pure compounds.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection Of Plant Material

The fruits and stem barks of *G. atroviridis* were collected in August 2009 from Kedah, Malaysia and identified by Mr. Baharuddin Sulaiman from the School of Biological Sciences, Universiti Sains Malaysia. A voucher specimen (USM 11201) has been deposited at the herbarium of Universiti Sains Malaysia, Penang, Malaysia.

3.2 Chemicals And Reagents

Chloroform-D1, with 0.03 vol. % TMS, deuteration degree min. 99.8 % for NMR spectroscopy (stabilized with silver) (Merck, Germany), Methanol-D4, deuteration degree min. 99.8 % for NMR spectroscopy (Merck, Germany), Acetone-D6, deuteration degree min. 99.9 % for NMR spectroscopy (Merck, Germany), Hexane, AR grade (QReC, Malaysia), Chloroform, AR and HPLC grade (QReC, Malaysia), Dichloromethane, AR grade (QReC, Malaysia), Ethyl acetate, AR grade (QReC, Malaysia), Acetone, AR grade (QReC, Malaysia), Methanol, AR and HPLC grade (QReC, Malaysia), DMSO, AR Grade (QReC, Malaysia), Ethanol 99.7 %, AR grade (QReC, Malaysia), *Pentane, AR grade (QReC, Malaysia), Sephadex LH-20 (Sigma-Aldrich, USA), Silica Gel 60 for column chromatography, (0.040 - 0.063 mm), (230 - 400 mesh ASTM) (Merck, Germany), TLC aluminium sheets, Silica Gel 60 F_{254}, 20cm × 20cm (Merck, Germany), Indomethacin (Sigma-Aldrich, USA), *p*-Iodonitrotetrazolium violet (Sigma-Aldrich, USA), Gentamicin sulphate (Sigma-Aldrich, USA), Nutrient Broth (Merck, Germany), Cyclooxygenase (COX) inhibitor screening assay kit (No. 560131; Cayman Chemical, USA), Acetylthiocholine

iodide (Sigma-Aldrich, USA), *S*-butyrylthiocholine chloride (Sigma-Aldrich, USA), Dithiobisnitrobenzoate (Sigma-Aldrich, USA), Physostigmine (Sigma-Aldrich, USA), Sodium hydrogen phosphate (R&M Chemical, UK), Disodium hydrogen phosphate (R&M Chemical, UK), DPPH free radical (Sigma-Aldrich, USA), Ascorbic Acid (Sigma-Aldrich, USA), Arachidonic Acid (Sigma-Aldrich, USA)

* Solvent was distilled prior to use

3.3 Isolation And Analysis Of Volatile Oil

3.3.1 Isolation Of Volatile Oil

Freshly-picked fruits were subjected to hydrodistillation to isolate the volatile oil, which was carried out using an all-glass apparatus similar to that described in the British Pharmacopoaeia (British Pharmacopoeia, 1993).

The fruits were washed with distilled water and their seeds removed. The rind and pulp (850 g) were cut into small pieces and pulverized for 30 s in a blender, after the addition of 450 mL of distilled water. The resultant puree was placed in a 2 L round bottom flask. Ice-cold water was circulated through the condenser during the hydrodistillation process. Hydrodistillation was carried out for 5 hours. A small volume of distilled pentane was added from time to time to ensure that the volume of pentane was maintained at about 5 mL.

At the end of the hydrodistillation process, the glass apparatus was left to cool and the volatile oil solution in pentane was drained through the stop cock into a separatory funnel where water was removed. The resulting solution was collected into a glass vial (5 mL), concentrated to an appropriate concentration by a gentle

stream of nitrogen gas at room temperature, and stored in the refrigerator until required for GC and GC-MS analyses.

3.3.2 Gas Chromatographic Analysis Of The Volatile Oil

The volatile oil isolated from the fruits was analysed by capillary gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS).

3.3.2 (a) Gas Chromatography (GC)

GC analysis was carried out using a Thermo-Finnigan instrument equipped with a flame ionization detector (FID). Three fused silica capillary columns of different polarity were employed: SPB-1 (bonded poly(dimethylsiloxane), 30 m × 0.25 mm i.d., film thickness, 0.25 μm), HP-5 (bonded poly(5% diphenyl/95% dimethylsiloxane), 30 m × 0.32 mm i.d., film thickness, 0.25 μm) and Supelcowax 10 (bonded poly(ethylene glycol), 30 m × 0.25 mm i.d., film thickness, 0.25 μm). The operating conditions of the three columns were as follows: initial oven temperature, 40°C for 1 min, then to 220°C at 5°C/min and held for 10 min; injector port and detector temperatures, 250°C; carrier gas, 2.0 mL/min He; injection volume, 0.4 μL; split ratio, 30:1. Peak areas were obtained with a Hitachi D2500 Chromato-Integrator.

The peak area percentages of individual components were the average values of two separate injections of the isolates, one from each of the two samples obtained from two independent extractions, while the absolute amounts of the components were calculated from the peak area percentages, taking into consideration the response factors of the FID for these compounds (Rome & McIntyre, 2012). For the

determination of the response factors needed for the calculation of the absolute amounts of the selected components, a solution in pentane containing known weights of (-)-*β*-caryophyllene, *α*-humulene, *β*-selinene, *α*-selinene, *β*-caryophyllene alcohol, humulol, ginsenol, (*1S,2S,5S,8S*)-4,4,8-trimethyltricyclo[6.3.1.$0^{1,5}$]dodecan-2-ol, tetradecanoic acid, hexadecanoic acid and internal standards (pentadecane, (-)-globulol, octadecanoic acid) was injected under the same analytical conditions in a separate experiment. For some of the other components which were sesquiterpenes, semi-quantitative determination of their absolute amounts was carried out, using pentadecane as the internal standard, without considering the calibration factors. The experimental retention indices (RIs) were calculated relative to those of *n*-alkanes, a mixture containing a homologous series $C_5 - C_{32}$, which was injected immediately after GC analysis of volatile oil under identical operating conditions.

For a temperature-programmed GC, the retention index (RI) of a component in the volatile oil was calculated using the following equation (Dool & Kratz, 1963):

$$RI = 100\, i \left[\frac{t - t_{(n)}}{t_{(n+i)} - t_{(n)}} \right] + 100\, n$$

under the condition that $t_{(n)} < t < t_{(n+i)}$.

t = retention time of a volatile oil component.

$t_{(n)}$ = retention time of the alkane with *n* carbon atoms which was eluted just before the component.

$t_{(n+i)}$ = retention time of the alkane with $(n+i)$ carbon atoms which was eluted just after the component.

i = difference in the number of carbon atoms between the two alkanes.

n = the number of carbon atoms in the alkane.

and this was used to calculate the *RI* values in the present work.

3.3.2 (b) Gas Chromatography - Mass Spectrometry (GC-MS)

GC-MS analysis was performed using a Perkin-Elmer Clarus 600T equipped with the Wiley 8th and NIST 08 libraries. The same capillary columns and GC operating conditions were employed as described in Section 3.3.2.1. The injection port was set at 250°C. Significant quadrupole MS operating parameters were as follows: interface temperature, 250°C; electron impact ionization at 70 eV with scan mass range of 28 − 400 *m/z* at a sampling rate of 1.0 scan/s.

3.3.3 Identification Of The Volatile Oil Components

The components of volatile oil were identified by comparison of their mass spectra and retention indices with those of commercially available standards or compounds synthesized by established methods. For some constituents, standards were unavailable for positive identification. These compounds were identified by matching their mass spectra with those recorded in the Wiley 8th and NIST 08 libraries, and also by comparing the retention indices of these components with published data (Adams, 2001).

3.3.4 Chiral Separation Of *β*-Caryophyllene

The correct *β*-caryophyllene isomer in the volatile oil was determined by GC and GC-MS using a Gamma-Dex™ capillary column (non-bonded; 25% 2,3-di-*O*-methyl-6-*O*-TBDMS-*γ*-cyclodextrin in SPB-20 poly(20% diphenyl/80% dimethylsiloxane) phase, 30 m × 0.25 mm i.d., film thickness, 0.25 μm; Supelco Inc.). GC-FID analysis was carried out using a Thermo Finnigan instrument. The operating conditions of the column in GC were as follows: initial oven temperature, 40°C for 1 min, then to 220°C at 5°C/min and held for 10 min; injector port and detector temperatures, 250°C; carrier gas, 2.0 mL/min He; injection volume, 0.4 μL; split ratio, 30:1. Peak areas were obtained with a Hitachi D2500 Chromato-Integrator. GC-MS analysis was performed using a Perkin-Elmer Clarus 600 T with the same capillary GC conditions as described above. The injection port was set at 250°C. Significant quadrupole MS operating parameters were as follows: interface temperature, 250°C; electron impact ionization at 70 eV with scan mass range of 28 − 400 *m/z* at a sampling rate of 1.0 scan/s.

3.3.5 Synthesis Of Authentic Compounds

The authentic compounds (**68**, **70** and **71**) were prepared by the acid-catalysed rearrangement of (-)-*β*-caryophyllene according to the procedure of Fitjer et al. (1995). A solution of 96 % sulfuric acid (0.78 mL) in ether (5 mL) was added to a solution of (-)-*β*-caryophyllene (1.11 mL, purity ≥ 98.5 %) in ether (5 mL) at 0 °C with stirring. The mixture was allowed to warm to room temperature. A saturated solution of sodium bicarbonate (30 mL) was added after 30 min. The mixture was then extracted with ether (3 × 30 mL), the combined ether layers were dried with $MgSO_4$ anhydrous, and the solvent was evaporated (bath temperature 20 °C) to yield

a colourless oil (1.2 g). Constituents **68**, **70** and **71** were isolated from the mixture by preparative GC using a Shimadzu gas chromatograph equipped with a glass column (2.1 m × 3.2 mm i.d.) packed with 10 % Carbowax 20M on Chromosorb WAW DMCS 80/100 (Supelco Inc.) and operated isothermally at 220°C with N_2 at 50 mL/min as carrier gas.

(**68**). ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.21 − 2.27 (m, H2), 1.80 − 1.85 (m, H10), 1.74 − 1.79 (m, H5), 1.64 − 1.79 (m, H5, H15), 1.58 − 1.63 (m, H4), 1.53 − 1.56 (m, H1), 1.50 − 1.52 (m, H8, H9), 1.46 − 1.49 (m, H1), 1.34 − 1.38 (m, H6), 1.30 − 1.33 (m, H9), 1.28 − 1.29 (m, H4), 1.10 − 1.14 (m, H8), 1.07 (d, *J* = 5.0 Hz, H6), 1.02 − 1.05 (m, H15), 1.01 (s, H12), 1.00 (s, H13), 0.89 (s, H14). ^{13}C NMR (125 MHz, $CDCl_3$): δ 71.14 (C, C3), 48.72 (CH_2, C15), 44.79 (CH, C10), 39.54 (CH, C2), 38.58 (CH_2, C4), 37.45 (CH_2, C6), 36.65 (CH_2, C8), 34.97 (C, C7), 34.80 (C, C11), 34.45 (CH_2, C1), 33.25 (CH_3, C14), 30.53 (CH_3, C12), 21.93 (CH_2, C9), 20.86 (CH_2, C5), 20.82 (CH_3, C13). Results for the ^{1}H and ^{13}C NMR are in good agreement with data reported for this compound (Rocha, Rodrigues, Kozhevnikov, & Gusevskaya, 2010). Mass spectrometry, *m/z* %: 222 (M^+, 1), 166 (100), 123 (49), 85 (30), 95 (29), 81 (29), 41 (27), 55 (23), 135 (23), 93 (22), 137 (22).

(**70**). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.99 (d, *J* = 14.0 Hz, H2), 1.93 − 1.96 (m, H5), 1.82 − 1.87 (m, H9), 1.79 − 1.82 (m, H10), 1.76 − 1.79 (m, H10, H6), 1.70 (d, *J* = 14.0 Hz, H2), 1.55 − 1.61 (m, H6), 1.48 − 1.53 (m, H5), 1.38 − 1.45 (m, H4, H8, H9), 1.27 (s, H12), 1.20 (s, H15), 1.16 (dd, *J* = 5.0, 12.0 Hz, H8), 1.04 (s, H13), 0.87 (s, H14). ^{13}C NMR (125 MHz, $CDCl_3$): δ 83.27 (C, C1), 57.15 (CH, C4), 48.06 (CH_2, C2), 45.39 (C, C11), 38.34 (C, C7), 35.48 (C, C3), 34.56 (CH_2, C6), 34.48 (CH_2,

C8), 34.34 (CH_3, C12), 33.62 (CH_2, C10), 29.15 (CH_3, C15), 28.59 (CH_3, C13), 26.29 (CH_2, C5), 24.65 (CH_3, C14), 21.86 (CH_2, C9). Results for the ^1H and ^{13}C NMR are in good agreement with data reported for this compound (Iwabuchi, Yoshikura, & Kamisako, 1988). Mass spectrometry, *m/z* %: 222 (M^+, 1), 207 (100), 123 (26), 41 (20), 43 (17), 95 (16), 55 (16), 69 (14), 109 (14), 125 (14), 81 (13).

(**71**). ^1H NMR (500 MHz, $CDCl_3$): δ 3.76 (dd, J = 6.0, 10.0 Hz, H2), 1.73 (dd, J = 6.0, 12.0Hz, H3), 1.59 − 1.63 (m, H6), 1.50 (dd, J = 10.0, 12.0 Hz, H3), 1.41 − 1.43 (m, H10), 1.37 − 1.39 (m, H5, H6, H9), 1.21 − 1.29 (m) (m, H7, H9, H10, H11), 1.18 (d, J = 12.0 Hz, H12), 1.04 (s, H13), 1.01 (d, J = 5.0 Hz, H11), 0.98 (d, J = 12.0 Hz, H12), 0.89 (s, H14), 0.86 (s, H15). ^{13}C NMR (125 MHz, $CDCl_3$): δ 81.37 (CH, C2), 50.88 (CH, C5), 47.81 (CH_2, C3), 44.64 (C, C1), 43.17 (CH_2, C12), 40.61 (CH_2, C11), 37.36 (C, C4), 33.45 (CH_2, C9), 32.99 (CH_3, C14), 32.02 (CH_2, C7), 31.68 (CH_3, C13), 30.15 (C, C8), 25.67 (CH_3, C15), 20.82 (CH_2, C10), 19.11 (CH_2, C6). Results for the ^1H and ^{13}C NMR are in good agreement with data reported for this compound (Fitjer et al., 1995). Mass spectrometry, *m/z* %: 222 (M^+, 1), 166 (100), 123 (49), 85 (30), 95 (29), 81 (29), 41 (27), 55 (23), 135 (23), 93 (22), 137 (22).

3.4 Isolation And Characterization Of The Non-Volatile Constituents In The Stem Bark of *Garcinia atroviridis*.

3.4.1 Extraction Procedure

Air-dried stem bark of *G. atroviridis* (6.00 kg) was ground and sequentially extracted in a Soxhlet apparatus with hexane, dichloromethane and methanol. The resulting extracts were evaporated to dryness at 40°C under reduced pressure, using a rotary evaporator, to afford greenish-yellow hexane extract (18.00 g), a brown

dichloromethane extract (17.00 g) and a reddish-brown methanol extract (100.00 g). The methanol extract was then partitioned with chloroform to give a dark brown chloroform extract (17.00 g).

3.4.2 Separation Techniques

Preliminary investigation of the chromatographic separation of the crude extracts was carried out using TLC silica gel 60 F_{254} (5 cm × 2 cm). Different solvent system was tested to find the one with the best separation for each of the mixtures. The selected solvent systems were utilized later in the column chromatographic separation of the crude extracts to isolate the components. Developed TLC plates were visualized using a UV lamp (365 nm), or dipped in reagents such as 1 % $FeCl_3$ (heating not required), or 5 % methanolic H_2SO_4, followed by heating at 100 − 105 °C until full development of colour has occurred to aid visualization.

Column chromatography using either silica gel or Sephadex LH-20 was employed for component isolation or purification. When silica gel was used as the adsorbent, elution was carried out using either isocratic or gradient solvent systems. In the case of Sephadex LH-20, methanol was used. Eluates were collected in fractions and the composition of each fraction was monitored by TLC. Fractions showing similar TLC profiles were pooled, and the solvents were evaporated off. Repeated purification using the same technique was carried out until a pure compound was isolated.

3.4.3 Isolation And Purification

3.4.3 (a) Hexane Extract

The hexane extract (15.00 g) was chromatographed on a silica gel column. Elution was carried out using solvents of hexane:choloroform / chloroform:ethyl acetate / ethyl acetate:methanol gradient system to afford 50 fractions (H1 − H50). The solid from fraction H15 (0.35 g) was recrystallised from methanol:chloroform (1:1) to yield **90 (20 mg)**. Fraction H20 (0.25 g) was rechromatographed on a silica gel column, eluted successively using solvents of hexane:chloroform / chloroform:methanol gradient system to afford 8 major fractions H20A − H20F. Fraction H20D (0.03 g) was further purified by silica gel column using an isocratic solvent system of chloroform:methanol (9:1) (v/v) to yield **91 (15 mg)**.