PHYTOCHEMICAL AND BIOLOGICAL ACTIVITY STUDIES OF *GARCINIA HOMBRONIANA*

by

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LIST OF ABBREVIATIONS/SYMBOLS

Abbreviations

MeOD-d_4	Deuterated Methanol
$CDCl_3$	Deuterated Chloroform
DMSO-d_6	Deuterated Dimethyl Sulfoxide
Acetone-d_6	Deuterated Acetone
C_5D_5N	Deuterated Pyridine
AR	Analytical Reagent
HPLC	High Performance Liquid Chromatography
EI-MS	Electron Ionization Mass Spectrometry
ESI-MS	Electrospray Ionization Mass Spectrometry
HR-ESI-MS	High Resolution Electrospray Ionization Mass Spectrometry
LR-ESI-MS	Low Resolution Electrospray Ionization Mass Spectrometry
FT-IR	Fourier Transformer Infrared
1D-NMR	One Dimensional Nuclear Magnetic Resonance
2D-NMR	Two Dimensional Nuclear Magnetic Resonance
COSY	Correlation Spectroscopy
DQF COSY	Double Quantum Filtered Correlation Spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
HSQC	Heteronuclear Single Quantum Correlation
NOESY	Nuclear Overhauser Enhancement Spectroscopy

NOE	Nuclear Overhauser Effect
ROESY	Rotating Frame Overhauser Enhancement Spectroscopy
LDL	Low Density Lipoproteins
MRSA	Methicillin Resistant S*taphylococcus aureus*
MIC	Minimum Inhibitory Concentration
AChE	Acetylcholinesterase Enzyme
BChE	Butyrylcholinesterase Enzyme

Symbols

m/*z*	mass/charge
w/v	weight/volume
v/v	volume/volume
R_f	retention factor
nm	nano meter
MHz	mega Hertz
s	singlet
br. *s*	broad singlet
d	doublet
br. *d*	broad doublet
dd	doublet of doublets
ddd	doublet of doublets of doublets
t	triplet
dt	doublet of triplet
q	quartet
dq	doublet of quartet

m	multiplet
α	alpha
β	beta
µg	microgram
ppm	parts per million
SD	standard deviation
cm^{-1}	per centimeter
°C	degree Celsius
δ	chemical shift
g	gram
µg/mL	microgram per millilitre
µL	microlitre
µM	micromole
IC_{50}	concentration required inhibiting 50% of effect
EC_{50}	effective concentration required to induce a 50% effect
λ_{max}	Lambda (Maximum Wavelength)

LIST OF APPENDICES

KAJIAN FITOKIMIA DAN AKTIVITI BIOLOGI BAGI *GARCINIA HOMBRONIANA*

ABSTRAK

Kajian ini ialah laporan pertama yang menggambarkan kajian fitokimia dan aktiviti biologi bagi kulit batang *G. hombroniana*. Ekstrak diklorometana dan etil asetat bagi kulit pokok *Garcinia hombroniana* yang dipisahkan secara kromatografi turus telah memberikan 4 sebatian baru (**DN8**, **DN9**, **EN2** dan **EN5**) dan 14 sebatian yang telah diketahui (**DN1**-**DN7**, **DN10**, **EN1**, **EN3**, **EN4**, **EN6**-**EN8**). Struktur bagi semua sebatian baru telah dicirikan secara kaedah spektroskopi UV, IR dan 1D dan 2D NMR, EI-MS dan ESI-MS. Kesemua sebatian yang diketahui telah dikenalpasti dan ditentukan secara perbandingan data spektroskopi sebatian ini dengan data yang telah dilaporkan.

Ekstrak mentah telah dikaji bagi menentukan jumlah kandungan fenolik dan jumlah kandungan flavonoid. Antioksidan, antibakteria dan aktiviti perencat kolinesterase bagi ekstrak tersebut dan beberapa sebatian tulen turut dikaji. Sebatian **DN6** dan **EN2** juga telah dikaji bagi aktiviti sitotoksik. Ekstrak etil asetat dan metanol telah menunjukkan kandungan fenolik yang sama manakala kandungan flavonoid didapati lebih tinggi dalam ekstrak etil asetat. Kesemua ekstrak polar telah menunjukkan aktiviti antioksidan yang ketara dalam ujian DPPH, ABTS dan FRAP. Bagi sebatian tulen yang dikaji **EN2**, **EN3**, **EN4**, **EN8** dan **DN9** telah menunjukkan aktiviti memerangkap radikal yang paling tinggi dalam cerakin DPPH. Aktiviti tersebut adalah lebih tinggi berbanding aktiviti memerangkap radical bagi rujukan piawai, Trolox. Sebatian **EN2**, **EN8** dan **EN3** juga menunjukkan aktiviti

pemerangkapan yang paling tinggi dalam ujian ABTS, iaitu tiga kali lebih tinggi berbanding aktiviti Trolox tetapi hampir sama dengan aktiviti pememerangkapan sebatian rujukan, asid gallik.

Dalam asai aktiviti antibakteria terhadap dua bakteria Gram positif, *Staphylococcus aureus*, *Bacillus subtilis* dan dua bakteria Gram negatif, *Pseudomonas aeruginosa* dan *Escherichia coli*, ekstrak diklorometana telah merencatkan pertumbuhan *B. subtilis* dan *P. aeruginosa*, dengan nilai MIC 62.5 µg/mL. Akan tetapi, ekstrak metanol dan air didapati tidak aktif terhadap kesemua strain bakteria yang dikaji. Aktiviti antibakteria bagi sebatian tulen **DN1**-**DN10**, **EN1** dan **EN2** juga telah dikaji terhadap strain bakteria yang disebutkan di atas tetapi tiada yang menunjukkan aktiviti antibakteria.

Dalam asai aktiviti perencatan kolinesterase, ekstrak etil asetat telah menunjukkan perencatan yang paling tinggi terhadap AChE dan BChE tetapi ekstrak metanol dan air telah menunjukkan perencatan yang sangat lemah. Antara sebatian tulen yang diuji (**DN1**-**DN10** dan **EN1**-**EN7**), tulen **DN4**-**DN7**, **DN9**, **DN10** dan **EN2** yang menunjukkan aktiviti perencatan terhadap AChE dan BChE.

Sebatian tulen **DN6** dan **EN2** telah menunjukkan aktiviti sitotoksik yang lebih tinggi terhadap sel DBTRG berbanding sel PC-3, U2OS dan MCF-7. Aktiviti sitotoksik bagi sebatian tulen ini didapati bergantung kepada faktor leluhur sel, masa dan kepekatan.

Keputusan kajian ini telah menonjolkan potensi kegunaan *G. hombroniana* dalam bidang perubatan tradisional dan penemuan ubat-ubatan.

PHYTOCHEMICAL AND BIOLOGICAL ACTIVITY STUDIES OF *GARCINIA HOMBRONIANA*

ABSTRACT

This study is the first report describing the phytochemical investigation and biological activities of the bark of *G. hombroniana.* The dichloromethane and ethyl acetate extracts of the bark of *Garcinia hombroniana* were separated by column chromatography to yield 4 new (**DN8**, **DN9**, **EN2** and **EN5**) and 14 known (**DN1-DN7**, **DN10**, **EN1**, **EN3**, **EN4**, **EN6-EN8**) compounds. The structures of the new compounds were characterized by spectroscopic techniques such as UV, IR, and 1D and 2D NMR, together with EI and ESI-MS spectrometric methods. The known compounds were identified and confirmed by comparison of their spectroscopic data with the reported literature.

Crude extracts were examined for total phenolic and total flavonoids contents. Antioxidant, antibacterial and cholinesterase inhibitory activities of these extracts together with some selected pure compounds were also examined. Compounds **DN6** and **EN2** were further tested for cytotoxic activities. The ethyl acetate and methanol extracts showed equal phenolic contents, while flavonoids content was found to be higher in the ethyl acetate extract. All the polar extracts showed significant antioxidant activities by DPPH, ABTS and FRAP assays. For pure compounds **EN2**, **EN3**, **EN4**, **EN8** and **DN9**, they showed the strongest scavenging activities in DPPH assay, all of which were higher than that of the referenced standard, Trolox. Compounds **EN2**, **EN3** and **EN8** also showed the

strongest scavenging activities in ABTS assay which is three times higher than that of Trolox and almost equal to that of the referenced compound, gallic acid.

In the antibacterial activity assays which were carried out against two Gram positive bacteria, *Staphylococcus aureus*, *Bacillus subtilis*, and two Gram negative bacteria, *Pseudomonas aeruginosa* and *Escherichia coli*, the dichloromethane extract inhibited the growth of *B. subtilis* and *P. aeruginosa*, with a MIC value of 62.5 μg/mL. However, the methanol and water extracts were found to be inactive towards all the tested bacterial strains. The pure compounds **DN1**-**DN10**, **EN1** and **EN2** were also tested for antibacterial activities against the aforementioned bacteria strain but none of them showed antibacterial activity.

In cholinesterase inhibitory activity assays, ethyl acetate extract showed the strongest inhibition against AChE and BChE but methanol and water extracts showed very weak inhibition. Among the tested pure compounds (**DN1**-**DN10** and **EN1**-**EN7**), compounds **DN4**-**DN7**, **DN9**, **DN10** and **EN2** showed inhibition against AChE and BChE.

Pure compounds **DN6** and **EN2** possessed significant cytotoxic activities against DBTRG cells as compared with PC-3, U2OS and MCF-7 cells. The cytotoxic activities of these compounds were found to be cell-line, time and concentration dependent.

Results of this study concluded the potential uses of *G. hombroniana* in traditional medicine and drug discovery.

CHAPTER 1

INTRODUCTION

1.1 Natural Products

The name “natural products” is given to compounds derived from natural sources such as plants, animals and microorganisms, which are used for self-defense and to enhance survival period (Williams et al., 1989). More than 80,000 of 250,000 species of plants are used as traditional medicine for healing purposes (Newman, 2007). They can be either in crude forms or as pure compounds, derived from a whole plant or certain parts of a plant, or from exudates of plants (Joy et al., 2001). Today, about three-quarter of the world’s population used herbal medicine in their health care needs (Moerman, 1996). However, only a small percentage of plants have been evaluated for their phytochemical and pharmacological potential (Tringali et al., 2001) while a large portion of which remains unknown. Interests in the use of traditional medicine are increasing rapidly due to their easy accessibility, a relatively low cost and low side effects to be used against various diseases including cancer, HIV/AIDS, and etcetera (Liang & Fang, 2006; WHO, 2002). A rapid development of natural products chemistry has resulted in a remarkable effect on the discoveries of bioactive components in natural sources. The present research work focuses on a plant named *Garcinia hombroniana* (from the Clusiaceae family). The bark of *G. hombroniana* was used in the extraction and isolation of some pure compounds. Characterization of their chemical structures was carried out using various spectroscopic techniques. These compounds were also assayed for their antioxidant, antibacterial, anticholinesterase and cytotoxic activities.

1.2 The Clusiaceae (Guttiferae) Family

The Clusiaceae family in Peninsular Malaysia is composed of approximately 37 genera and 1610 species. The plants are trees, shrubs or herbs which can grow from small to medium sized woody plants producing resinous white to yellow viscous exudates. Several plants of the Clusiaceae family produce important commercial products, edible fruits and more than 15% of oil from the seeds, and the timbers of many of its species are used for building houses and making furniture (Xiwen & Yan-hui, 1990).

1.3 The Genus *Garcinia*

Garcinia is the largest genus of the Clusiaceae family with about 450 species, indigenous to tropical Asia, tropical and southern Africa, Madagascar, North East Australia, West Polynesia and tropical America (Xiwen & Yan-hui 1990). It is a genus of small to medium sized trees which can be found from seashore to the lowland and up to the mountain forests. It is traditionally used in the treatment of abdominal pain, dysentery, diarrhea, infected wound, leucorrhoea, chronic ulcer and gonorrhea (Balemba et al., 2010; Braide, 1993; Moongkarndi et al., 2004a).

1.4 Botanical Characterization of *Garcinia*

Trees in this genus are either dioecious or polygamous. The flowers may be solitary, fascicled and umbelled or panicled, usually with four to five decussate or imbricate and free sepals, and four to five imbricate petals. The male flowers have two to four lobed free or joined stamens. Anthers are straight with a horse shoe shape. Female flowers have 2-12 celled ovaries. The stigma is visible and sessile.

The berry is covered by tough peel and contains numerous large seeds suspended in a pulpy inner part (Xiwen & Yan-hui, 1990).

1.5 *Garcinia hombroniana* Pierre

G. hombroniana is widely distributed and is native to Peninsular Malaysia where it can be found in the coastal regions, from the lowland forests near the sea to the lower mountain forests and the highlands, upper parts of Borneo, Cambodia, Thailand, Andaman, Nicobar Islands and Vietnam. The name *Garcinia hombroniana* was given by Pierre (1882-1885), after a French physician J. B. Hombron, who had collected this plant while travelling from Singapore to Malacca in the Peninsular Malaysia (Nazre, 2010).

1.6 Botanical Characterization of *G. hombroniana*

G. hombroniana, a seashore mangosteen (English) or manggis hutan (Malay) is an evergreen fast growing medium sized dioecious tree, reaching to a height of 4.6-6.0 m and 1.8 m girth (Figure 1.1). It is the closest species to mangosteen (*G. mangostana*), and is thought to be one of its ancestors but interestingly the flowering time is much more different from that of mangosteen (Hammer, 2001). Due to the physical similarities of these two species, sometimes the nurseries mistakenly sell the seedlings as those of mangosteen. *G. hombroniana* has straight stem with horizontal and opposite dense branches. Young twigs are smooth and green (Figure 1.2a) but the older bark is dark brown and rough with white latex (Figure 1.2b). Leaves are bright yellowish green which become glossy green on maturity (Figure 1.2c).

Figure 1.1. *Garcinia hombroniana* Pierre

The fruit (Figure 1.2d) is smooth and spherical with a pinkish red peel. The internal part is segmented, with yellowish pulp and has a good flavour. The flowers (Figure 1.2e) are whitish, with four sepals and petals, and 2.5 cm in diameter. The berry (Figure 1.2f) is ash-green in the beginning but turns crimson red on ripening. The pericarp (Figure 1.2d) is relatively thin. The seeds are about 6-8 in number and the ripened berry is full with soft, sour, juicy and pale creamish arillus of a peach flavour (John et al., 2008).

1.7 Uses of *G. hombroniana*

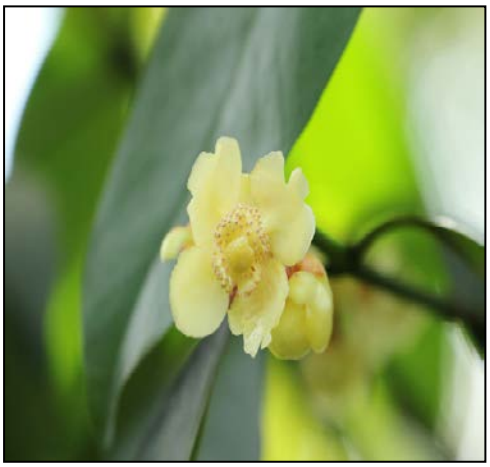
The fruit of *G. hombroniana* is aromatic with thin and sour flesh. It is used in making juices and jellies. The timber is used to make houses and oars. The roots and leaves are used medicinally to relieve itching and as a protective medicine after childbirth (Pierre, 1882-1885).

(**a**) Young twig

(**b**) Bark

(**c**) Leaves

(**d**) Fruit and pericarp



(**e**) Flower

(**f**) Unripened berry

Figure 1.2. Parts of *G. hombroniana*

CHAPTER 2

LITERATURE REVIEW

2.1 Phytochemical Studies of *Garcinia*

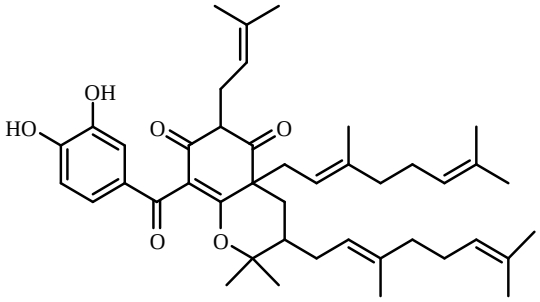
Garcinia is a rich source of bioactive secondary metabolites including oxygenated prenylated xanthones (Peres et al., 2000), benzophenones (Magadulla et al., 2008), flavonoids and biflavonoids (Harati et al., 2007), and triterpenes (Rukachaisirikul et al., 2005a). These phenolic compounds possess various pharmacological properties such as antioxidative, anti-inflammatory, antiallergic, antimicrobial, antimalarial, antihepatotoxic and HIV inhibitory activities (Gustafson et al., 1992; Hay et al., 2004).

2.2 Phytochemical Investigation of *G. hombroniana*

Previous phytochemical investigation of the different parts of *G. hombroniana* such as stem wood, pericarp, leaves and twigs had resulted in the isolation of different classes of compounds including xanthones, flavonoids, biflavonoids and triterpenes. The chemical constituents isolated from *G. hombroniana* up to the year 2013 are reported in this thesis using Scifinder database.

2.2.1 Chemical Constituents Isolated from the Stem Wood of *G. hombroniana*

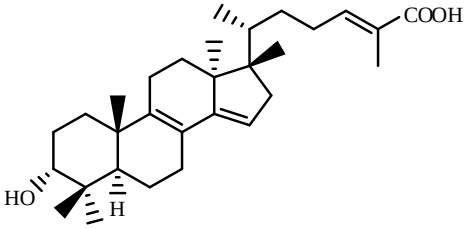
Bronianone (**1**) is the only compound isolated by Ollis et al. (1969) from the stem wood of *G. hombroniana*.



(**1**)

2.2.2 Chemical Constituents Isolated from the Pericarp of *G. hombroniana*

Rukachaisirikul et al. (2000) reported the isolation of triterpenes as the major compounds from dichloromethane extract of the pericarp of *G. hombroniana*. The compounds include (24*E*)-3α-hydroxy-17,14-friedolanostan-8,14,24-dien-26-oic acid (**2**), garcihombronane B (**3**), garcihombronane C (**4**), garcihombronane D (**5**) and garcihombronane E (**6**).



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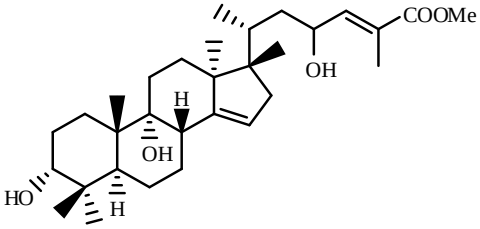

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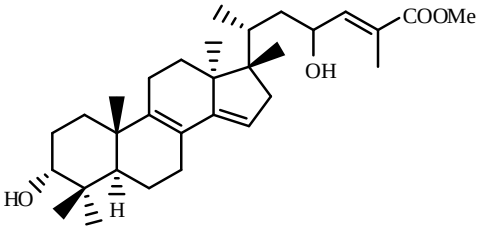

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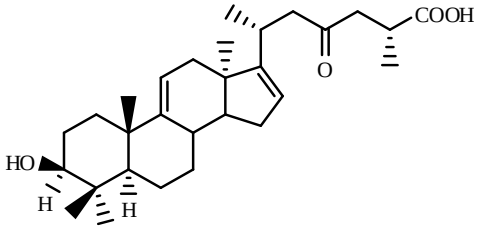

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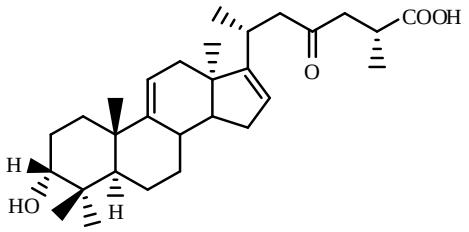
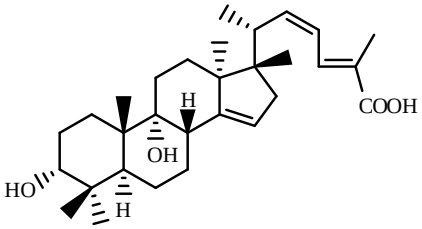


(**6**)

2.2.3 Chemical Constituents Isolated from the Leaves of *G. hombroniana*

Rukachaisirikul et al. (2005a) further reported the isolation of triterpenoids along with some other classes of compounds from the leaves of *G. hombroniana*. The compounds include garcihombronane F (**7**), garcihombronane G (**8**), garcihombronane H (**9**), monoacetate of garcihombronane H (**10**), garcihombronane I (**11**), monoacetate of garcihombronane I (**12**), garcihombronane J (**13**), methyl (25*R*)-3*β*-hydroxy-23-ox-9,15-lanostadien-26-oate (**14**), vitexin (**15**), isovitexin (**16**), blum-inol-C-9-*O*-*β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranoside (**17**), vomifoliol-9-*O*-*β*-D -apiofuranosyl (1→6)-*β*-D-glucopyranoside (**18**) and garcihombronanes B-E.



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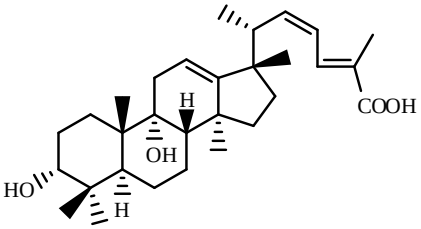

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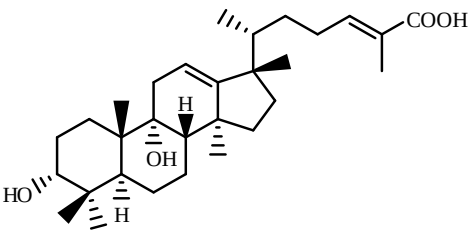

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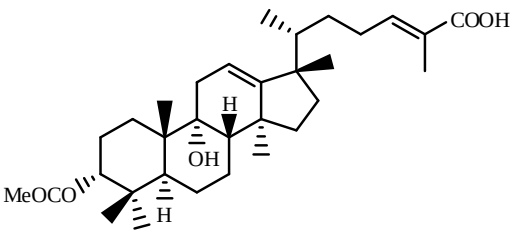

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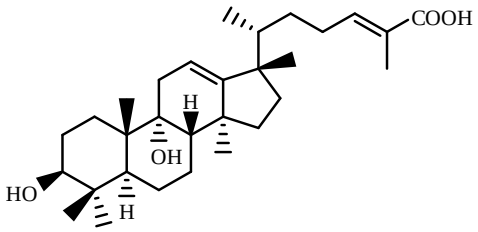

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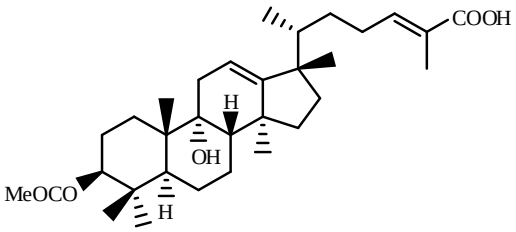

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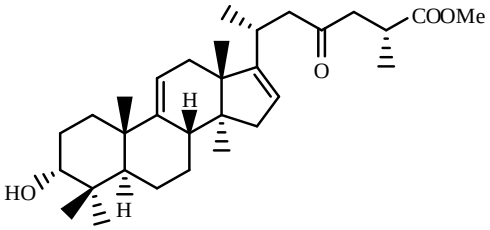

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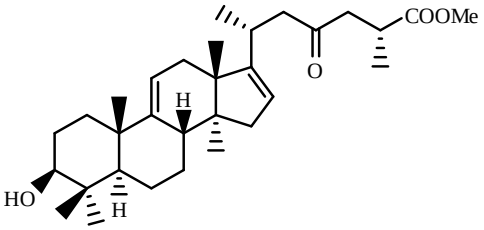

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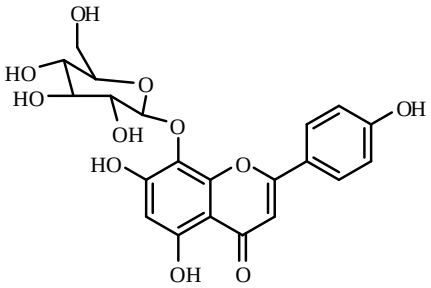

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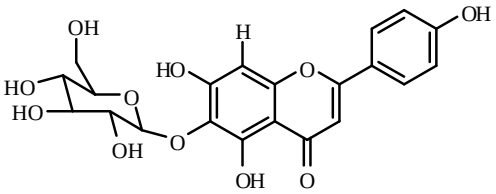

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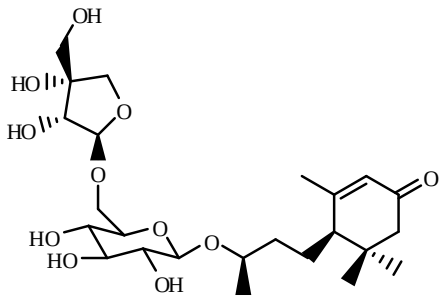

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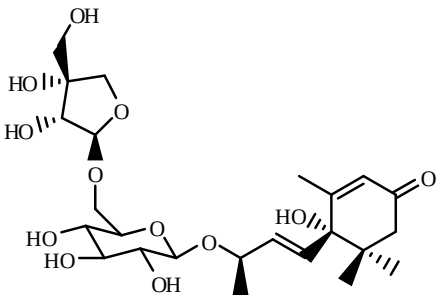
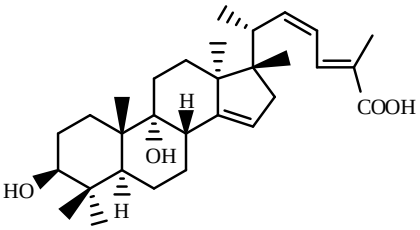


(**18**)

2.2.4 Chemical Constituents Isolated from the Twigs of *G. hombroniana*

Klaiklay et al. (2013) reported triterpenes and other compounds from the twigs of *G. hombroniana*. The compounds include (22*Z*, 24*E*)-3*β*,9*α*-dihydroxy-

17,14-friedolanostan-14,22,24-trien-26-oic acid (**19**), garcihombronane K (**20**), Monoacetate of garcihombronane K (**21**) garcihombronane L (**22**), garcihombronone A (**23**), garcihombronone B (**24**), garcihombronone C (**25**), garcihombronone D (**26**), norathyriol (**27**), gentisein (**28**), 1,3,6,7-tetrahydroxy-8-prenylxanthone (**29**), cheffouxanthone (**30**), bangangxanthone A (**31**), toxyloxanthone B (**32**), 1,3,5,7-tetrahydroxy-2-(3,7-dimethyl-6-hydroxyocta-2-7-dien)xanthone (**33**)*, volkensiflavone (**34**), naringenin 7-*O*-*β*-D-glucuronide (**35**)*, eriodictyol 7-*O*-*β*-D-glucuronide (**36**)*, 4-hydroxybenzoic acid (**37**) and protocatechuic methyl ester (**38**). Saputri and Jantan (2012) also reported 3,5,3',5'-tetrahydroxy-4-methoxybenzophenone (**39**) and 1,7-dihydroxyxanthone (**40**) from the methanol extract of the twigs of *G. hombroniana.*



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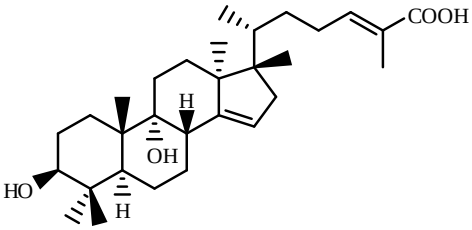

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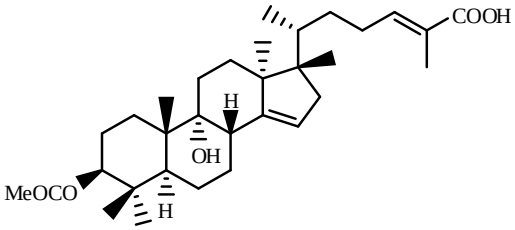

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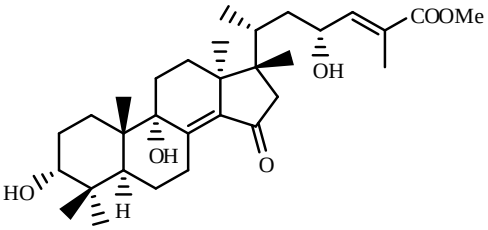

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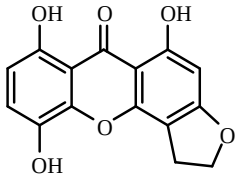

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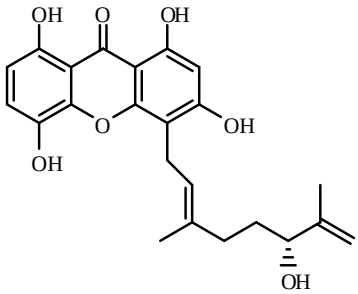

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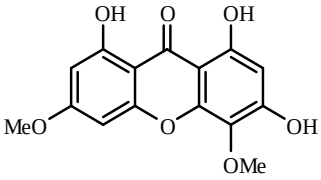

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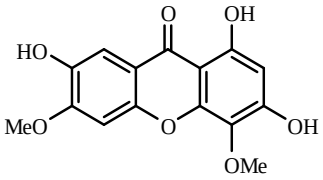

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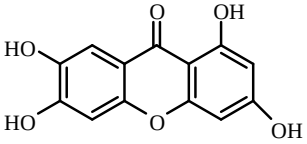

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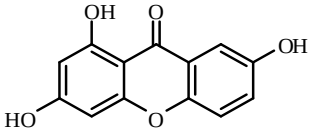

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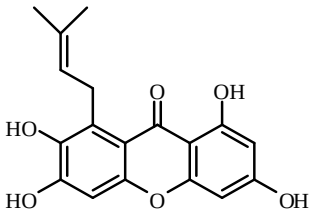

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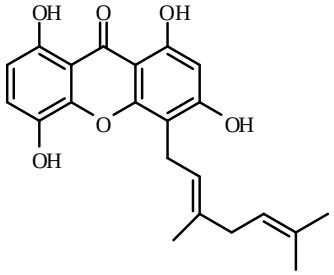

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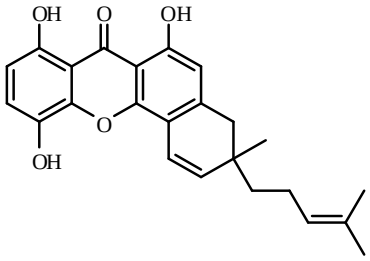

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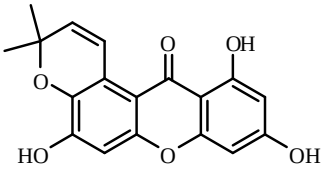

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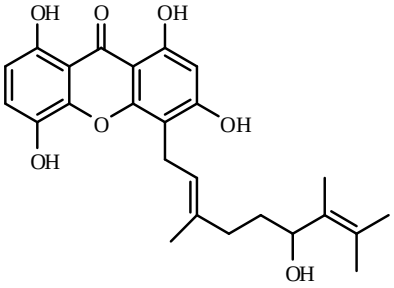

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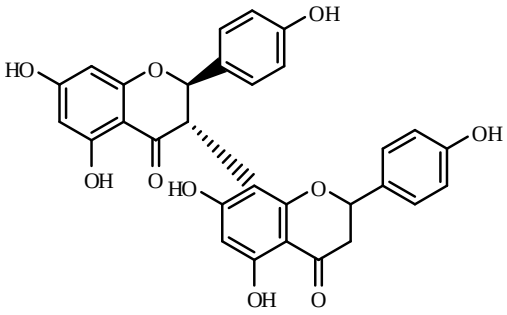

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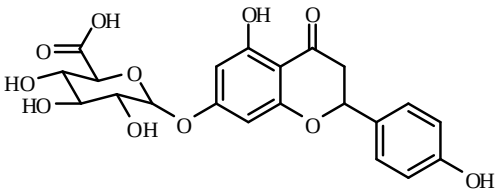

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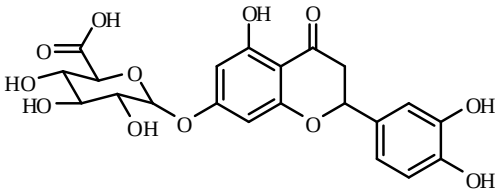

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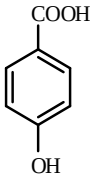

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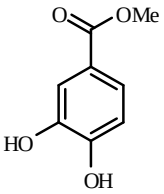

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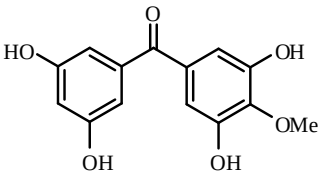

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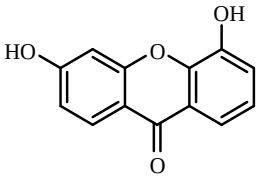

(40)

*Klaiklay PhD Thesis, 2009

2.3 Pharmacological Investigation of *G. hombroniana*

The *Garcinia* species are well known tropical trees used in the folk medicine for the treatment of various diseases and have been reported for their significant pharmacological properties. *G. hombroniana* is reported to have antiplatelet aggregation activities. Saputri and Jantan (2012) reported that the crude methanol extract of the twigs of *G. hombroniana* and the two compounds: (**39**) and (**40**) isolated from it exhibited strong copper mediated LDL antioxidation with IC_{50} of 6.6 and 1.7 μM, respectively. It was further reported that compound (**39**) showed archidonic acid (AA, IC_{50} = 53.6 μM), adenosine diphosphate (ADP, IC_{50} = 125.7

μM) and collagen-induced (IC_{50} = 178.6 μM) antiplatelet aggregation activities while compound (**40**) displayed a selective inhibition against ADP induced aggregation with IC_{50} of 5.7 μM. The crude methanol extract of the twigs of *G. hombroniana* showed antibacterial activities against *S. aureus* and MRSA with the MIC values of 128 and 200 μg/mL, respectively. Compounds (**30**) and (**31**) displayed moderate activities against MRSA with MIC values of 64.0 and 32.0 μg/mL, respectively. In addition, Klaiklay et al. (2013) also reported that compound (**30**) has a good activity against *S. aureus* with a MIC value of 16.0 μg/mL.

2.4 Problem Statement

The biodiversity source in Malaysia is one of the biggest in the world but its potential uses need to be identified and exploited. During the compilation of the economic products of the Malay Peninsula, Burkill (1966) has reported that 1,300 plants were used as folk medicine. The rich biodiversity of Malaysia, the public interest in herbal medicine and the contribution of industries and multinationals in herbal medicine have led to new emphasis of the medicinal plant research. Extensive work on the isolation and identification of chemical compounds from the *Garcinia* species, as well as their biological activities have been reported as many species from this genus are easily available and are known for their medicinal values. Previously the stem wood, twigs, pericarp and leaves of *G. hombroniana* have been investigated for their chemical constituents and biological activities (Klaiklay et al., 2013; Rukachaisirikul et al., 2005a; Saputri & Jantan, 2012). However, to the best of our knowledge, there is no authentic previous study on the bark of *G. hombroniana* either in terms of phytochemical investigation or bioactivities. Taking this into

account, this research work was aimed to isolate, purify and identify biologically active compounds from the bark of *G. hombroniana*.

2.5 Objectives of the Research

The objectives of this research are:

1. To isolate the chemical constituents from dichloromethane and ethyl acetate extracts of the bark of *G. hombroniana*.
2. To characterize and identify the isolated chemical constituents using different spectroscopic techniques such as UV, IR, NMR (1D and 2D) and mass spectrometry (EI-MS and HR/LR-ESI-MS).
3. To evaluate the phenolic and flavonoid contents and to correlate these contents to the antioxidant activities of extracts.
4. To evaluate the antibacterial, anticholinesterase and anticancer activities of the extracts and some selected pure compounds.

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant Material

The plant (*G. hombroniana*) was collected in April 2010 from Penang Botanic Garden, Malaysia. A voucher specimen (PBGK12) has been deposited at the herbarium of this garden.

3.2 Chemicals

All the chemicals and solvents used for extraction, isolation and biological activities were either of analytical or HPLC grade.

3.2.1 Chemicals for Extraction and Isolation

Acetone, AR grade (QRëC, Malaysia); acetone-d_6 for NMR 99.5 atom% D Nitrogen flushed (Merck, Germany); aluminium chloride $AlCl_3.6H_2O$ (Merck, Germany); chloroform $CHCl_3$, AR grade (QRëC, Malaysia); chloroform-d_1, with 0.03% TMS (v/v), 99.8 atom% D, stabilized with silver foil (Merck, Germany); DMSO-d_6 99.8 atom% D (Merck, Germany); ethyl acetate EtOAc, AR grade, (QRëC, Malaysia); ethanol 95% and 99% (QRëC, Malaysia); methanol MeOH, AR grade (QRëC, Malaysia); MeOD-d_4 99.8 atom% D, (ACROS, USA); pyridine C_5D_5N (Merck, Germany); sephadex LH-20 (Sigma-Aldrich, USA); silica gel 60 for column chromatography (0.040-0.063 mm; 230-400 mesh, Merck, Germany); sodium carbonate Na_2CO_3, (Systerm, Malaysia); sodium hydroxide NaOH,

(Systerm, Malaysia); sulfuric acid H_2SO_4, (R & M Chemicals Essex, U.K); TLC aluminium sheets, pre-coated silica gel 60 F_{254}, 20 cm x 20 cm (Merck, Germany).

3.2.2 Chemicals for Biological Activities Evaluation

Acetylthiocholine iodide (ATCI), (Sigma-Aldrich, USA); 2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid (ABTS), (Sigma Aldrich, Germany); buffer pH 3.6 (Sigma-Aldrich, USA); *S*-butyrylthiocholine chloride (BTCCl) (Sigma-Aldrich, USA); cytotoxicity detection kit (Roche Diagnostics, Germany); deionized water; dimethyl sulfoxide DMSO, (Sigma-Aldrich, USA); 5,5-dinitrobenzoic acid (DTNB), (Sigma- Aldrich, USA); 2,2-diphenyl-1-picrylhydrazyl (DPPH), (Sigma Aldrich, Germany); ferric chloride ($FeCl_3.6H_2O$), (Bendosen Laboratory Chemicals); 10% fetal bovine serum (Gibco, USA); Folin-Ciocalteu Reagent (Sigma Aldrich, Germany); gallic acid (Sigma Aldrich, Germany); glioma DBTRG cell lines [American Type Culture Collection (ATCC) Rockville, USA]; human breast cancer MCF-7 cell lines [American Type Culture Collection (ATCC), Rockville, USA]; hydrochloric acid HCl, (R & M Chemicals Essex, U.K); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), (Sigma Aldrich, Germany); Muller-Hinton agar and broth MHA and MHB, (Oxoid, England), *p*-nitrotetrazolium violet (INT), (Sigma-Aldrich, USA); osteosarcoma U2OS cell lines, [American Type Culture Collection (ATCC), Rockville, USA]; potassium persulfate ($K_2S_2O_8$), (Merck, Germany); prostate PC3 cell lines, [American Type Culture Collection (ATCC), Rockville, USA]; physostigmine (Sigma-Aldrich, USA); quercetin (Aldrich Milwukee, WI); rutin (Aldrich Milwukee, WI); 2,4,6-tripyridyl-*s*-triazine TPTZ, (Merck, Germany).

3.3 Instruments Used for Structures Elucidation and Biological Activities

3.3.1 Melting Point

Melting points of the isolated pure compounds were determined by a Stuart Scientific SMP-1 (UK) melting point apparatus.

3.3.2 Infrared (IR) Spectroscopy

IR spectra of the isolated compounds were recorded on a Perkin-Elmer System 2000 FT-IR spectrometer (England, UK). The samples were prepared as potassium bromide (KBr) discs and scanned in a measurement range of 400-4000 cm^{-1}.

3.3.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectra of the isolated pure compounds were recorded on a Bruker Avance 500 spectrometer operated at 500 MHz for ^{1}H-NMR, and 125 MHz for ^{13}C-NMR, respectively. The measurements of the samples were performed in deuterated solvents (chloroform-d_1, dimethyl sulfoxide-d_6, methanol-d_4, acetone-d_6 and pyridine-d_5). The coupling constants (J) were reported in Hertz (Hz).

3.3.4 Direct Probe Mass Spectrometry

Direct probe mass spectrometry through EI-MS and LR/HR-ESI-MS spectra were respectively recorded on an Agilent 5975C MSD and ThermoFinnigan MAT95XL mass spectrometers at the National University of Singapore (NUS), Singapore.

3.3.5 Ultraviolet (UV) Spectroscopy

UV spectra were recorded using a Perkin Elmer, Lambda 25 UV/Vis spectrometer in the region of 200-500 nm. The UV spectra for biflavonoids were measured using different shift reagents according to the method described by Bloor (2001). Stock solutions of the biflavonoids (**EN3**-**EN5**) were prepared by dissolving about 0.1 mg of compounds in 10 mL of methanol and divided each into three parts. The UV spectra were then measured using the following procedure.

i. The UV spectra of the samples prepared in methanol were measured.

ii. The spectra were recorded immediately after the addition of few drops of NaOMe (2.5 g metallic Na/100 mL MeOH) to first part of the stock solutions.

iii. After 5 minutes the NaOMe spectra were recorded again.

iv. The spectra were recorded immediately after the addition of 3 drops of 5% $AlCl_3$ reagent to second part of the stock solutions.

v. The spectra were recorded immediately after the addition of 6 drops of 50% HCl reagent to the solutions of step (iv).

vi. The spectra were measured after the addition and mixing of anhydrous sodium acetate (NaOAc) powder to third part of the stock solutions. The quantity of NaOAc added was such that about 2 mm layer settled to the bottom of the cuvette.

vii. The spectra were recorded immediately after the addition of anhydrous boric acid powder to the solutions used in step (vi).

3.3.6 Circular Dichroism (CD) Spectroscopy

The absolute stereochemistry of **EN5** was determined using Jasco-815 spectrometer at 25 ^{o}C (Scan range λ = 200-350 nm, cell length = 10 mm).

3.3.7 X-Ray Crystallography Analysis

Single crystal X-Ray structure analysis of compound **EN2** was performed on a Bruker SMART APEXII CCD area detector diffractometer at the School of Physics, USM. The X-Ray crystallographic structure was solved and refined using SHELXTL software.

3.3.8 Microplate Reader

A Tecan Infinite 200 Pro Microplate spectrometer was used for the evaluation of the biological assays. Absorbance at different concentrations was measured with the microplate reader.

3.4 Extraction Procedure

The air dried ground bark (5.2 kg) of *G. hombroniana* was sequentially extracted using Soxhlet extractor with *n*-hexane, dichloromethane, chloroform, ethyl acetate and methanol at 40 ^{o}C. The filtered extracts were evaporated to dryness using a rotary evaporator at 40 ^{o}C to give a 30.1 g of *n*-hexane extract, 50.0 g dichloromethane extract, 5.0 g of a chloroform extract, 18.2 g of ethyl acetate extract and 30.1 g of a methanol extract. Another 100 g was extracted by the direct maceration into distilled hot water for 24 h to give aqueous-di extract (Figure 3.1).

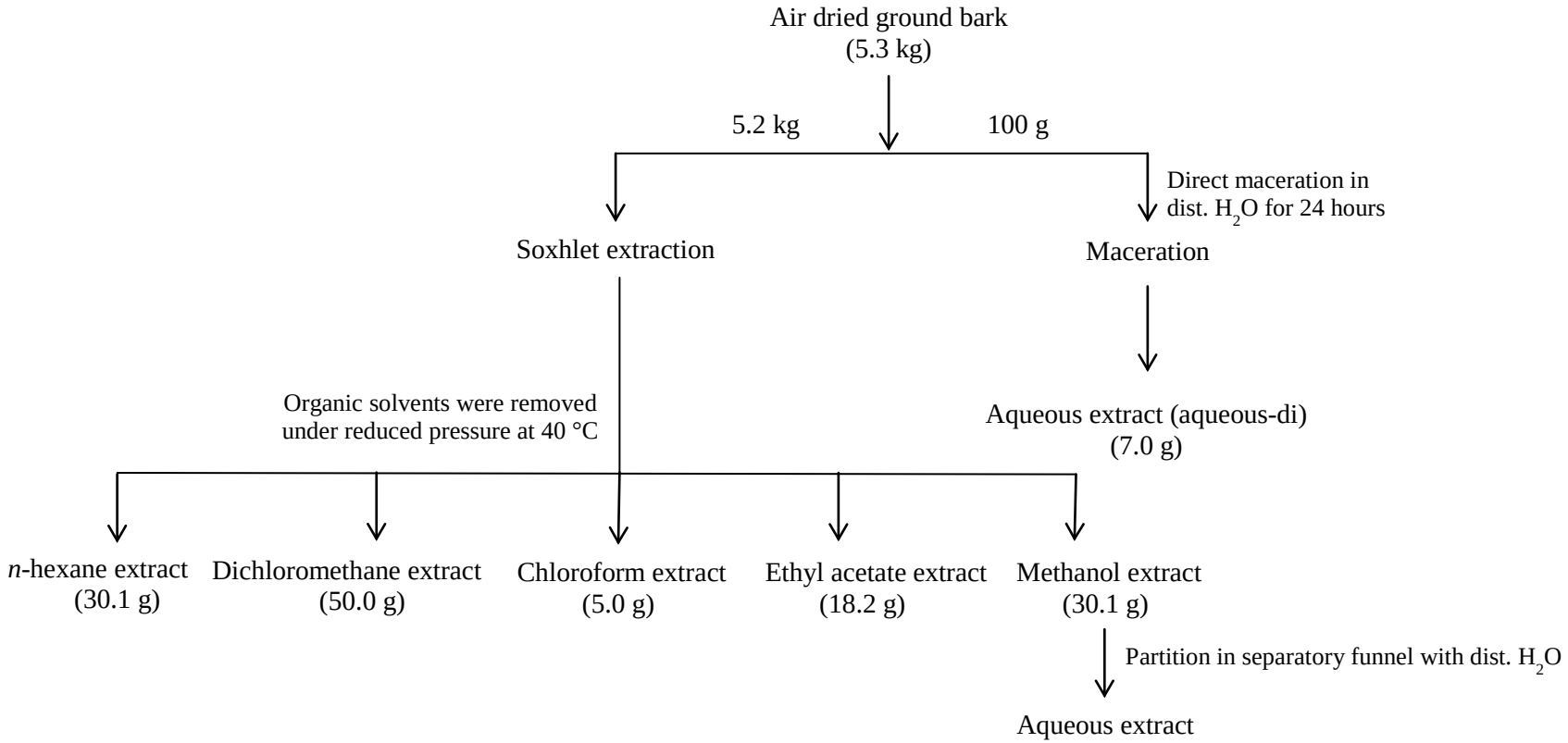

Figure 3.1. Extraction procedure of *G. hombroniana* bark

3.5 Phytochemical Screening

The crude *n*-hexane, dichloromethane, chloroform, ethyl acetate, methanol and aqueous extracts, and the extract (aqueous-di) were screened for the detection of some secondary metabolites such as terpenoids, phytosterols, phenolics, flavonoids, carbohydrates, reducing sugars, alkaloids, phlobatannins and tannins, following the procedures of Indian Pharmacopoeia (1985).

3.5.1 Detection of Carbohydrates and Glycosides

Molish's Test: Few drops of α-naphthol were added to 2 mL filtrate of the crude extracts. After mixing and shaking, 1 mL of sulfuric acid was gradually added along the sides of the test tube and allowed to stand. A violet ring indicated the presence of carbohydrates.

Fehling Solution's Test: 1 mL of Fehling's solution was added to 2 mL filtrate of crude extracts and boiled at 100 ^{o}C. The formation of red precipitate indicated the presence of reducing sugars.

3.5.2 Detection of Proteins and Amino Acids

Biuret Test: A Few drops of 2% $CuSO_4$ solution were added to 5 mL filtrate of the crude extracts. Then 2 mL of 95% ethanol and excess of potassium hydroxide pallets were added. An absence of pink colour indicated an absence of proteins.

Ninhydrin Test: Few drops of ninhydrin solution (5% w/v in acetone) were added to 2 mL filtrate of the crude extracts. Appearance of no purple colour revealed the absence of amino acids.

3.5.3 Detection of Terpenoids (Salkowski's Test)

2 mL chloroform was added to 5 mL filtrate of the crude extracts, and then 3 mL of conc. H_2SO_4 was added slowly along the sides of the test tube. A reddish interface showed the presence of terpenoids.

3.5.4 Detection of Phytosterols (Liebermann-Burchard's Test)

2 mL of acetic anhydride was added to 5 mL filtrate of the crude extracts. Afterwards, 1-3 drops of conc. H_2SO_4 were added by the sides of the test tube. Array of the colour change showed the presence of phytosterols.

3.5.5 Detection of Phenolic Compounds

Ferric Chloride Test: A few drops of 5% ferric chloride solution were added to 2 mL filtrate of the crude extracts. Formation of dark green colour indicated the presence of phenolic compounds.

Lead Acetate Test: 3 mL of 10% lead acetate solution was added to 5 mL filtrate of the crude extracts. A white precipitate indicated the presence of phenolic compounds.

3.5.6 Detection of Flavonoids

NH_4OH Solution Test: 1 mL of 10% NH_4OH solution was added to 5 mL filtrate of the crude extracts. A formation of yellow florescent colour indicated the presence of flavonoids. Furthermore, 5 mL of DMSO was added to it, heated it, and

magnesium chloride and 1 mL of conc. HCl was added. Appearance of red colour showed the presence of flavonoids.

NaOH Solution Test: 4 mL of dil. NaOH solution was added to 2 mL filtrate of the crude extracts. The formation of golden yellow colour revealed the presence of flavonoids.

3.5.7 Detection of Tannins

A few drops of 5% ferric chloride were added to 5 mL filtrate of the crude extracts. A brown colour indicated the presence of tannins.

3.5.8 Detection of Phlobatannins

5 mL of deionized water was added to 5 mL filtrate of the crude extracts. Then a few drops of 1% HCl were added and boiled it at 100 ^{o}C for 10 min. The absence of formation of red precipitate indicated the absence of phlobatannins.

3.5.9 Detection of Alkaloids

A few drops of Wagner's reagent were added to 5 mL filtrate of the crude extracts. Orange precipitate showed the presence of alkaloids.

3.6 Separation Techniques

3.6.1 Thin Layer Chromatography (TLC)

The crude extracts were preliminary examined by thin layer chromatography, followed by the determination of the appropriate solvent systems. The selected

solvent systems were used for the column chromatographic separation of compounds in the crude extracts. The purity of the isolated compounds was also checked with TLC. In this phytochemical investigation, pre-coated TLC plates of silica gel 60 F_{254} (20 x 20) on aluminium sheets were used. The developed plates were visualized with a UV light (Vilber Lournet, multiband UV-254/365 nm) and/or by spraying them with 95% methanolic H_2SO_4 or the Libermann-Burchard's reagent (Horborne, 1998) followed by heating with a heat gun at 100-105 ^oC.

3.6.2 Column Chromatography (CC)

Column chromatography was carried out using silica gel (230-240 mesh, 0.040-0.060 mm) and Sephadex LH-20 (bead size 25-100 μ). Elution was carried out with a gradient solvent system of increasing polarity (*n*-hexane, chloroform, ethyl acetate, acetone and methanol) when silica gel was used as an absorbent. However, 100% methanol was used as an eluting solvent with Sephadex LH-20.

3.7 Isolation and Purification

3.7.1 Dichloromethane (DCM) Extract

The DCM extract (20.0 g) was subjected to silica gel CC. Elution was carried out using *n*-hexane, *n*-hexane-ethyl acetate, ethyl acetate-methanol in a polarity gradient of 10:0, 9:1, 8:2, 7:3, 6:4, 1:1, 4:6, 3:7, 2:8 and 0:10. Eluents were collected in 42 fractions DFA1 to DFA42. Fractions having similar TLC profiles were combined to give nine fractions (DFB1 to DFB9).

Fraction DFB1 (3.8 g) with a greenish white crystalline solid was rechromatographed on silica gel CC, eluted with *n*-hexane and ethyl acetate. Sub-