UNIVERSITI PUTRA MALAYSIA

PHYTOCHEMICAL STUDIES AND BIOLOGICAL ACTIVITIES OF GARCINIA MANGOSTANA L., G. NITIDA PIERRE AND G. BENTHAMIANA (PLANCH. & TRIANA) PIPOLY

IRENE SEE

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By

IRENE SEE

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

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

PHYTOCHEMICAL STUDIES AND BIOLOGICAL ACTIVITIES OF *GARCINIA MANGOSTANA L.*, *G. NITIDA* PIERRE AND *G. BENTHAMIANA* (PLANCH. & TRIANA) PIPOLY

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January 2017

Chair: Gwendoline Ee Cheng Lian, PhD
Faculty: Science

Detailed phytochemical studies on *G. mangostana*, *G. nitida* and *G. benthamiana* have led to the isolation of fourteen compounds, which included four new compounds and ten other compounds. Various chromatographic methods were used in the process of purification of these phytochemical compounds. The structures of these compounds were elucidated by interpreting spectroscopic data obtained from GC-MS, UV, IR, 1D and 2D NMR.

The stem bark extracts of *G. mangostana* furnished ten secondary metabolites, which included three new compounds and seven known compounds. The hexane extract afforded stigmasterol (**109**) and *β*-mangostin (**100**), while the chloroform extract gave cowagarcinone B (**101**). Isolation work on the ethyl acetate extract yielded two new xanthones, mangaxanthone A (**96**) and B (**97**), along with *α*-mangostin (**99**), dulcisxanthone F (**102**), mangostanin (**104**) and mangostenol (**103**). The methanol extract gave one new benzophenone, mangaphenone (**98**). Chromatographic purification of various stem bark extracts of *G. nitida* resulted in three known compounds from the hexane and chloroform extracts which are stigmasterol (**109**), osajaxanthone (**106**) and rubraxanthone (**105**). The hexane extract of *G. benthamiana* furnished one known benzophenone, congestiflorone (**108**) along with one common sterol, stigmasterol (**109**) and a new benzophenone, benthamianone (**107**).

In silico study was carried out and all the compounds were predicted to effectively induce apoptosis of both cell lines through fatty acid synthase (4PIV). This suggested that all the secondary metabolites would be potential candidates for inhibition of MDA-MB-231 and MCF-7 cells.

All the extracts and compounds were subjected to preliminary *in vitro* screening towards MCF-7, MDA-MB-231 and Vero cell lines. Among all the extracts of these *G.* species, the ethyl acetate and methanol extracts of *G. benthamiana* exhibited potent inhibitory effect against MCF-7 and both showed weak cytotoxicities toward Vero cell line. Mangaphenone (**98**) demonstrated high inhibitory activity against MCF-7 cells but moderate inhibitory activity towards MDA-MB-231 cell line but was weak cytotoxic towards Vero cells.

The ethyl acetate and methanol extracts of *G. benthamiana* showed the highest total phenolic content among all the extracts. The methanol extract of *G. nitida* had the strongest scavenging ability against DPPH free radical, which was stronger than BHT. Besides, the methanol extract of *G. benthamiana* demonstrated the most potent reducing ability towards ferric ion while the ethyl acetate extract of *G. nitida* demonstrated a strong inhibitory effect against *β*-carotene bleaching. However, all the extracts of the three *Garcinia* species exhibited weak chelating ability, which was less than 45% chelating power for 500 µg/mL of extract. All the tested compounds exhibited weak antioxidant power. On the other hand, all the *G. benthamiana* extracts except the methanol extract, had weak activity against *Bacillus sublitis* and *Staphylococcus aureus*. All the extracts had no effects against *Staphylococcus epidermidis*, *Escherichia coli* and *Serratia marcencens*.

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

KAJIAN FITOKIMIA DAN AKTIVITI BIOLOGI DARIPADA *GARCINIA MANGOSTANA* L., *G. NITIDA* PIERRE DAN *G. BENTHAMIANA* (PLANCH. & TRIANA) PIPOLY

Oleh

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Januari 2017

Pengerusi: Gwendoline Ee Cheng Lian, PhD
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Kajian fitokimia yang terperinci ke atas pokok *Garcinia mangostana*, *G. nitida* dan *G. benthamiana* menghasilkan empat belas sebatian, termasuk empat sebatian baru dan sepuluh sebatian yang telah diketahui. Pelbagai kaedah kromatografi telah digunakan dalam proses penulenan sebatian fitokimia. Struktur-struktur sebatian ini telah ditentukan melalui penafsiran data spektroskopi yang diperolehi dari GC-MS, UV, IR, 1D dan 2D NMR.

Ekstrak kulit kayu batang *G. mangostana* memberikan sepuluh metabolit sekunder, termasuk tiga sebatian yang baru dan tujuh sebatian yang diketahui. Ekstrak heksana memberikan stigmasterol (**109**) dan *β*-mangostin (**100**) manakala ekstrak kloroform memberikan cowagarcinon B (**101**). Kerja-kerja pengasingan pada ekstrak stil asetat menghasilkan dua xanton yang baru, mangaxanton A (**96**) dan B (**97**), bersama-sama dengan *α*-mangostin (**99**), dulcisxanton F (**102**), mangostanin (**104**) dan mangostenol (**103**). Ekstrak metanol memberikan satu benzofenon yang baru, mangafenon (**98**). Penulenan kromatografik pelbagai kulit kayu batang *G. nitida* menghasilkan tiga sebatian yang diketahui daripada ekstrak heksana dan kloroform, iaitu stigmasterol (**109**), osajaxanton (**106**) dan rubraxanton (**105**). Ekstrak heksana *G. benthamiana* manghasilkan satu benzofenon yang diketahui, congestiflorone (**108**) bersama-sama dengan satu sterol biasa, stigmasterol (**109**) bersama-sama dengan satu benzofenon yang baru, benthamianon (**107**).

Kajian simulasi pengkomputeran telah dijalankan dan semua sebatian dijangka dapat mendorong apoptosis kedua-dua garisan sel ini dengan berkesan melalui synthase asid lemak (4PIV). Ini mencadangkan bahawa semua metabolit sekunder ini akan menjadi calon yang berpotensi untuk perencatan sel MCF-7 dan MDA-MB-231.

Saringan awal *in vitro* terhadap garisan sel MCF-7, MDA-MB-231 dan Vero telah dijalankan ke atas semua ekstrak dan sebatian. Antara semua ekstrak spesies *Garcinia*, ekstrak etil asetat dan metanol *G. benthamiana* mempamerkan kesan perencatan yang kuat terhadap MCF-7 dan kedua-dua ekstrak tersebut lemah terhadap garisan sel Vero. Mangafenon (**98**) menunjukkan aktiviti perencatan yang tinggi terhadap MCF-7 dan aktiviti sederhana terhadap MDA-MB-231 tetapi sitotoksik yang lemah terhadap sel Vero.

Ekstrak etil asetat dan metanol dari *G. benthamiana* menunjukkan kandungan fenolik yang tertinggi. Ekstrack metanol *G. nitida* mempunyai keupayaan memerangkap DPPH radikal yang paling kuat, iaitu lebih kuat daripada BHT. Di samping itu, ekstrak metanol dari *G. benthamiana* membuktikan keupayaan menurunkan ferik ion manakala ekstrak etil asetat dari *G. nitida* menunjukkan kesan pembantutan yang kuat terhadap pelunturan *β*-karotena. Walau bagaimanapun, semua ekstrak dari ketiga-tiga spesies *Garcinia* tersebut mempamerkan keupayaan "chelating" yang lemah, iaitu kurang daripada 45% keupayaan "chelating" untuk 500 µg/mL ekstrak. Semua sebatian yang diuji menunjukkan keupayaan antioksidan yang lemah. Di samping itu, semua ekstrak dari *G. benthamiana* kecuali ekstrak metanol, mempunyai aktiviti yang lemah terhadap *Bacillus sublitis* dan *Staphylococcus aureus*. Semua ekstrak adalah lemah terhadap *Staphylococcus epidermidis*, *Escherichia coli* and *Serratia marcencens.*

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I certify that a Thesis Examination Committee has met on 23 January 2017 to conduct the final examination of Irene See on her thesis entitled "Phytochemical Studies and Biological Activities of *Garcinia mangostana* L., *G. nitida* Pierre and *G. benthamiana* (Planch. & Triana) Pipoly" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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α	Alpha
Vero	African Green Monkey Kidney Epithelial Cell Line
ATCC	American Type Culture Collection
β	Beta
BCB	Beta-carotene Bleaching Activity
K_d	Binding Affinity
BSC	Biosafety Cabinet
Br s	Broad singlet
BuOH	Butanol
^{13}C	Carbon-13
CO_2	Carbon dioxide
cm	Centimetre
δ	Chemical Shift in ppm
$CHCl_3$	Chloroform
CFU	Colony Forming Unit
C	Concentration
CC	Column Chromatography
COSY	Correlation Spectroscopy
J	Coupling Constant in Hz
CPU	Central Processing Unit
°C	Degree Celsius
DMEM	Dulbecco's Modified Eagle Medium

DMSO	Dimethylsulfoxide
DEPT	Distortionless Enhancement by Polarization Transfer
$(CD_3)_2CO$	Deuterated Acetone
$CDCl_3$	Deuterated Chloroform
CD_3OD	Deuterated Methanol
d	Doublet
dd	Doublet of doublet
EIMS	Electron Ionization Mass Spectrometry
ER	Human Estrogen Receptor
EtOH	Ethanol
EtOAc	Ethyl Acetate
EDTA	Ethylenediaminetetraacetic Acid
FAS	Fatty Acid Synthase
MCF-7	Estrogen Responsive Human Breast Adenocarcinoma Cancer
FRAP	Ferric Reducing Antioxidant Potential
FIC	Ferrous Ion Chelating Activity
FBS	Fetal Bovine Serum
FTIR	Fourier Transform Infrared Spectroscopy
GAE	Gallic Acid Equivalent
G.	*Garcinia*
GC-MS	Gas Chromatography-Mass Spectrometry
ΔG	Gibbs Free Energy
g	Gram
GI_{50}	Half Maximal Growth Inhibition Concentration

EC_{50}	Half Maximal Effective Concentration
IC_{50}	Half Maximal Inhibition Concentration
Hz	Hertz
HMQC	Heteronuclear Multiple Quantum Coherence
HMBC	Heteronuclear Multiple Bond Correlation
HRESIMS	High Resolution Electrospray Ionization Mass Spectrometry
T47D	Human Breast Cancer Cell Line
SK-BR3	Human Breast Adenocarcinoma Cell Line
HeLa	Human Cervical Cancer
HT-29	Human Colorectal Adenocarcinoma Cell Line
MRC-5	Human Diploid Embryonic Lung Cell Line
KB	Human HeLa Contaminant Carcinoma Cell Line
A549	Human Lung Carcinoma Cell Line
SHSY5Y	Human Neuroblastoma Cell Line
HL-60	Human Promyelocytic Leukemia Cell Line
NB4	Human Promyelocytic Leukemia Cell Line
PC3	Human Prostate Cancer Cell Line
NCI-H460	Human Non-small Cell Lung Carcinoma Cell Line
IR	Infrared Spectroscopy
kg	Kilogram
Lit.	Literature
L	Litre
Hep G2	Liver Hepatocellular Cancer

MeOH	Methanol
MIC	Minimum Inhibitory Concentration
M	Molar
m/z	Mass per charge
MHz	Megahertz
m.p.	Melting Point
µ	Micro
mL	Millilitre
mm	Millimetre
m	Multiplet
m	Metre
nm	Nanometre
MDA-MB-231	Non-estrogen Responsive Human Breast Adenocarcinoma Cancer Cell Line
NMR	Nuclear Magnetic Resonance
NF-κB	Nuclear Factor-kappa B
1D-NMR	One-Dimensional Nuclear Magnetic Resonance
ppm	Parts Per Million
%	Percentage
PBS	Phosphate Buffered Solution
PTLC	Preparative Thin Layer Chromatography
^{1}H	Proton
rpm	Revolutions Per Minute
RMS	Root Mean Square

RPMI	Roswell Park Memorial Institute Media
s	Singlet
SEC	Size Exclusion Chromatography
SEM	Standard Error of Mean
SRB	Sulfohodamine B
T	Temperature
TMS	Tetramethylsilane
TLC	Thin Layer Chromatography
3D	Three-Dimensional
TPC	Total Phenolic Content
t	Triplet
TNF-α	Tumor Necrosis Factor-alpha
2-NMR	Two-Dimensional Nuclear Magnetic Resonance
UV-Vis	Ultraviolet-Visible Spectroscopy
UATR	Universal Attenuated Total Reflection
λ_{max}	Maximum Wavelength in nm
DPPH	2,2-diphenyl-1-picrylhydrazyl
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

CHAPTER 1

INTRODUCTION

1.1 General introduction

Natural product chemistry involves the chemistry of secondary metabolites from plants, animals, insects, marine organisms and microorganisms. Different classes of compounds can be found from these natural resources such as flavonoids, alkaloids, terpenoids, glycosides and xanthones. Not only are these secondary metabolites important in the search for alternative drugs for diseases, they are also used as flavouring enhancement and pigments in food.

About one third of clinical drugs have been isolated from natural resources (Xu, R., 2012). Previous natural products research have found a number of effective cancer drugs, which are being used in hospitals for treatment of certain cancers. According to National Cancer Institute, doxorubicin hydrochloride was isolated from the bacterium *Streptomyces peucetius var. caesius* and was clinically proven to be an anticancer agent and used to treat a number of cancers such as gastric, ovarian, thyroid and small cell lung cancers (National Cancer Institute, 2014a). Other than that, paclitaxel or taxol which was isolated from the *Taxus brevifolia* tree (National Cancer Institute) is also used in hospitals to treat breast, ovarian and non-small lung cancers (National Cancer Institute, 2014b). Therefore, natural products are still the main source to establish novel or alternative drugs for diseases and cancers.

Breast cancer has become a major concern in Malaysia as it is the number one cancer that affected Malaysians. About 14.5% of Malaysians were diagnosed with breast cancer followed by colorectal (12.1%) and lung cancer (11.8%) (Yuen, 2016). In recent years, about 4, 000 new cases of breast cancer were reported each year and mostly involved women (Yuen, 2016).

There are various treatments available for breast cancer, which include surgery, radiotherapy, chemotherapy, endocrine therapy and aromatase inhibitors (CPG Secretariat, 2010). These treatments can cause various side effects such as appetite loss, diarrhoea, fatigue, hair loss, nausea and vomiting, sexual and fertility problems and others (National Cancer Institute, 2015). Therefore, the search for new effective breast cancer drugs with minimal side effects is very important. Furthermore, Southeast Asia is considered to be the oldest and one of the most biologically diverse in the world according to World Wildlife Fund for Nature of Malaysia. There are many more plants in Malaysia which are yet to be discovered and studied for their phytochemical contents and medicinal properties, such as *G. benthamiana*.

On the other hand, an antioxidant is a substance which is able to decrease the damages caused by oxygen, specifically free radicals (National Cancer Institute). Antioxidants are consumed daily in the human body to counteract with the reactive oxygen species, which might damage the cells in the body and lead to aging and diseases such as heart disease and weak immune system (Mandal, 2013). Besides that, antioxidants are also added into food products in order to slow down the rate of deterioration of the food. For example, ascorbic acid is a well-known dietary supplement to increase the immune system of the human body while butylated hydroxytoluene (BHT) is the FDA approved synthetic antioxidant used as a food additive. However, previous studies have shown that prolong exposures to high doses of BHT can cause liver, lung, thyroid and kidney problems in mice or rats. Although mangosteen have been famous as a dietary supplement in the market, the search for alternative supplements is also important and to reveal the potentials of other plants.

There are two different molecular docking, which included between small ligand and macromolecule protein as well as between proteins. There are several docking softwares available to perform molecular docking, such as AutoDock, FlexX, Gold and ICM (Holtje et al., 2008). These docking softwares have a scoring function respectively to arrange in order of the various compound binding modes. There are different scoring functions, which are made up of empirical, force-field-based and knowledge-based. However, AutoDock Vina is used to perform molecular docking studies in this research in order to predict the most promising compounds isolated from *G. mangostana*, *G. nitida* and *G. benthamiana* against human breast cancers. AutoDock Vina has a scoring function which is force-field-based. Additionally, molecular docking is also used to predict the bound conformations and the binding affinity of these secondary metabolites with the protein of human breast cancers.

1.2 Botany of plant

1.2.1 Clusiaceae family

Clusiaceae family is formally known as Guttiferae and consists of about 47 genera like *Garcinia*, *Mesua*, *Mammea*, *Cratoxylum* and *Calophyllum* (Piccinelli et al., 2005). Plants from this family are mainly found in tropical regions such as Africa, Brazil and Polynesia. The characteristics of the tree of this family are smooth with thin truck, which contain white or yellow latex (Osman and Milan, 2006). Species that belong to the Clusiaceae family are woody plants and are mostly glabrous with unicellular hairs (Kubitzki, 2007). Furthermore, the fruits of this family are often capsular and they will dehiscence through the breaking away from the valves or splitting longitudinally though the septa (Kubitzki, 2007). Other than that, Clusiaceae plants contribute to the economic growth as well. For example, the wood of *Mesua ferrea,* or commonly known as iron wood, has been used as timber (Orwa et al., 2009). *G. mangostana* (mangosteen) fruits have been used as a dietary supplement and consume as fresh fruits.

1.2.2 *Garcinia* genus

Among the genera of the *Clusiaceae* family, the *Garcinia* genus has the largest number of species, which was about 400 species of the polygamous tree. *Garcinia* plants are mainly found in tropical Asia, Africa and Polynesia (Ampofo and Waterman, 1986a). The tree of this species mostly form yellow resin whereas the fruits are edible (Magadula, 2010). Therefore, *Garcinia* trees are also classified as saptrees. This genus is famous for producing a wide range of bioactive secondary metabolites, including polyisoprenylated benzophenones and xanthones (Nilar et al., 2005). For example, *G. atroviridis* has been used in the treatment for cough, dandruff, earache, stomach pain that were related to pregnancy as well as throat irritation (Permana et al., 2001).

1.2.3 *G. mangostana*

G. mangostana is commonly known as mangosteen in Malaysia and is famously known as 'queen of fruits'. Besides that, there are many vernacular names given to mangosteen in Asia country, like *settor*, *mesetor*, *semetah* or *sementah* in Malaysia; *manggis* or *manggustan* in Philippines; *mongkhut* in Cambodia; *mangkhud* in Laos; *dodol* or *mangkhut* in Thailand and *cay mang cut* in Vietnam (Othman and Tindall, 1995).

Mangosteen trees with their straight vertical truck can grow up to 25 m with a diameter of 25-35 cm. This tree is a valuable shade tree due to the symmetrically arranged branches that form a pyramidal-shaped crown (See Figure 1.1). Yellow latex was easily found in the main tissues of the tree. However, no root hair is found on the main and lateral roots and these roots are fragile (Othman and Tindall, 1995).

The leaves of mangosteen are in elliptical shape and comprise petioles of 1.5-2.0 m long. The appearances of the leaves are shiny, thick, leathery and glabrous. The size of the leaves is 15-25 cm long and 7-13 cm wide (Othman and Tindall, 1995). On the other hand, the fruits of mangosteen are edible and possess milky white, soft and moist edible aril (See Figure 1.2) (Ajayi et al., 2007; Hung et al., 2009). This milky white edible aril is delicious, sweet and has a slightly acidic taste (Ajayi et al., 2007; Yu et al., 2007). However, about two third of the weight of the fruit is due the pericarp of the fruit. The pericarp of the fruit is green colour if the fruit is unripe while it shows dark purple to red purple colour when it is ripe. This fruit is round shape with a diameter of 5-7 cm. The non-edible pericarp is 6-10 mm thick (Zadernowski et al., 2009).

The pericarp of mangosteen fruit has been used as traditional medicine in Southest Asia countries to treat wounds, diarrhoea and skin infections (Hung et al., 2009; Jung et al., 2006). This has urged the phytochemists to conduct research to investigate the phytochemical content of this plant and have found that this plant displayed anti-inflammatory, astringent, antibacterial, antitumour, antimicrobial, antioxidative and cytotoxic activities (Ajayi et al., 2007; Jung et al., 2006).

Figure 1.1: Tree and flower of *G. mangostana*

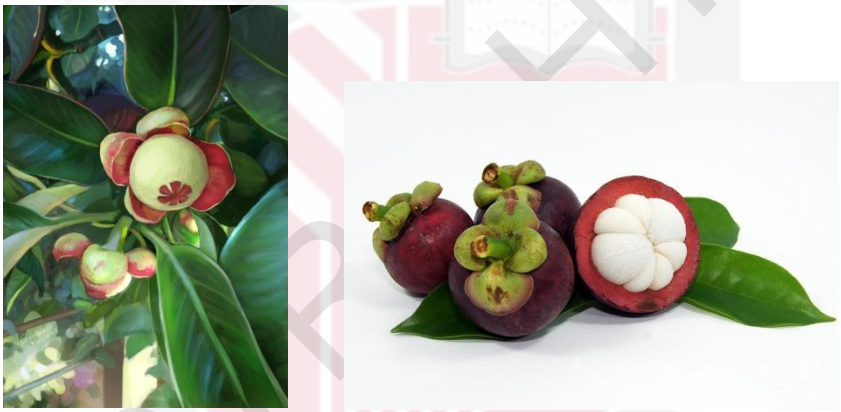

Figure 1.2: The unripe and ripe fruits of *G. mangostana*

1.2.4 *G. nitida*

G. nitida originated from Borneo and it is commonly known as *assam kandis* in Malaysia. It has several vernacular names, which include *assam aur-aur* in Brunei; *assam alui-alui* in Sabah and *kandis* in Sarawak (Lim, 2012). This plant grows in the same climatic and agro-ecological zone as mangosteen. This means that this plant can be readily found in the tropical rain forest, especially in Malaysia. Similar to mangosteen, the tree of *G. nitida* has a dense crown and drooping branches (See Figure 1.3). This plant has large, oval-elliptic to obovate with acuminate tip and glossy green leaves. The leaves have 2-3 cm of long petiole.

The aril part of the fruits of *G. nitida* is edible and often used as an acidulose base in cooking while the dried pericarp of the fruits is commonly used as acidic flavouring (See Figure 1.3) (Lim, 2012).

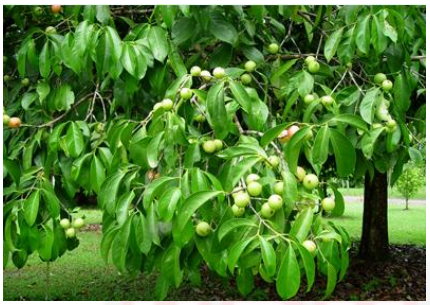
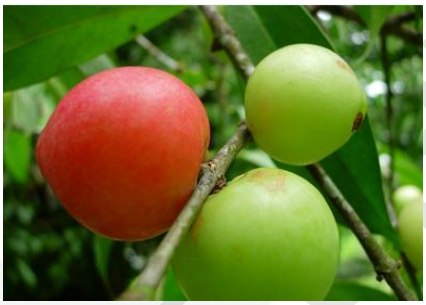

Figure 1.3: Tree and fruits of *G. nitida*

1.2.5 *G. benthamiana*

G. benthamiana is commonly known as bacuri-pari, which means "fruits that falls". This plant was first found in the flooded area of the Amazon forest. Nowadays, it can be found in flooded dead level of small rivers too. Similar to mangosteen tree, this plant has a dense and pyramidal shaped tree as well. The tree can grow up to 20 m tall (See Figure 1.4). The colour of the tree trunk changes from greenish brown when young to brown brownish when mature. The leaves of *G. benthamiana* are oblong-lanceolate and leathery, similar to mangosteen and *G. benthamiana*. However, the fruits are berries with white pericarp pyriform shaped (See Figure 1.5). The aril of the fruits carries 1 or 2 elongated seeds. The shape of the fruits is oblong with 4 to 7 cm long and 3.5 to 4.5 cm wide. The aril of the fruits is edible with the taste of sweet and refreshing (*Garcinia benthamiana*: Family of Clusiaceae, 2016). However, there are no phytochemical reports of this plant.

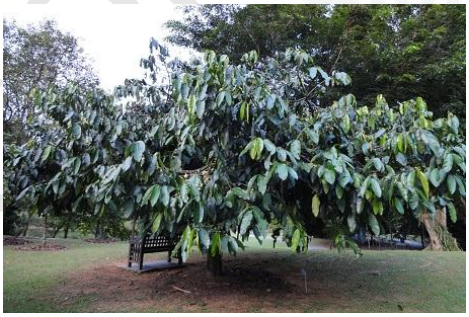


Figure 1.4: Tree and flower of *G. benthamiana*

Figure 1.5: Fruits of *G. benthamiana*

1.3 Problem statement

Current cancer treatment can cause various side effects on cancer patients besides killing the cancer cells. Furthermore, some cancers even build up resistance against cancer drugs after a period of time. Current cancer drugs are not effective enough on curing cancer due to the recurrence of breast cancer in five to ten years. Therefore, the study on three *Garcinia* species would help in the search of new potential breast cancer drug to resolve issues anticipated in the treatment of cancer. Other than that, the search for alternative antioxidant dietary supplement will be carried out in this research too since antioxidant possesses various health benefits.

1.4 Objective of study

This research was designed to explore the phytochemical content of a well-known medicinal plant such as *G. mangostana* as well as plants with less or no phytochemical information such as *G. nitida* and *G. benthamiana*. Besides that, the aim of this research is to discover the biological potential of the extracts and secondary metabolites isolated from these *Garcinia* species and the correlation between these bioactivities. The main objective of this research is to discover new potential anticancer lead compounds. Hence, the specific objectives of this research are:

1. To elucidate the structures of pure compounds isolated from *G. mangostana*, *G. nitida* and *G. benthamiana* through spectroscopic methods

2. To determine the antiproliferative properties of crude extracts and pure compounds *in vitro* against MCF-7 and MDA-MB-231 breast cancer cell lines.

3. To determine the binding ability of pure compounds through molecular docking method.

4. To measure the antioxidant ability of extracts and pure compounds.

5. To evaluate the antimicrobial potential of crude extracts

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