DETERMINATION OF PHYTOCHEMICAL CONTENTS, AND ANTIOXIDANT AND ANTICANCER PROPERTIES OF SELECTED *GARCINIA* SPECIES FROM TENOM, SABAH.

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INSTITUTE FOR TROPICAL BIOLOGY AND CONSERVATION

UNIVERSITI MALAYSIA SABAH

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ABSTRACT

Garcinia mangostana (family of Clusiaceae) has been reported to possess antioxidants, antiproliferative, pro-apoptotic, anti-inflammatory and anti-carcinogenic activities due to its xanthones content in the fruit. The aims of the current study are to determine the phytochemicals content and antioxidant activities, as well as its cytotoxicity effects, on five *Garcinia* species collected in Tenom, Sabah. The selected species are *G. dulcis, G. parvifolia, G. nitida, G. cambogia* and *G. mangostana* var. *mangosta*. The fruits were separated into three parts: peel, flesh and seed, and extracted using two different solvents: 80% methanol and aqueous solvent. Three different parameters were used to determine the antioxidant activities: DPPH free radical scavenging activity, ABTS decolourization assay and FRAP assay. For cytotoxicity activities, three different cancer cell-lines, namely MCF-7 (hormone dependent breast cancer), MDA-MB-231 (non-hormone dependent breast cancer) and HepG2 (liver cancer) were cultured. Samples extracted with 80% methanol were selected and screened by using MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The cell cycle distribution and apoptosis were further evaluated using flow cytometry. Further analysis on the possible compound that contributed to the antioxidant and anticancer activities were analysed using Gas Chromatography – Mass Spectrometry (GC-MS). The results showed that the seed of *G. cambogia* and *G. nitida* as well as the peel of *G. mangostana* possessed the highest phytochemical and antioxidant activities. The fruit parts of *G. dulcis,* peel of *G. mangostana* var. *mangosta* and seed of *G. cambogia* showed positive cytotoxic effects towards all cancer cell lines tested. The lowest IC_{50} value was detected in the seed of *G. dulcis* for MCF-7, MDA-MB-231 and HepG2 with the values of 2.5±0.00, 12.5±3.54 and 7.33±2.52 µg/ml, respectively. The flesh of *G. dulcis* induced cell cycle arrest in HepG2 cancer cell at Sub G_1 (apoptosis) phase in time-dependent manner and the seed of *G. cambogia* induced S phase arrest after 72 h of treatment. Staining with Annexin V-FITC and propidium iodide showed that apoptosis occurred late in the flesh of *G. dulcis* and early in the seed of *G. cambogia* with the total of 41.2% and 51.2% of cells, respectively at 72 h of treatment. The apoptosis of the flesh of *G. dulcis* were accompanied by increased in caspase 3. GC-MS analysis showed that Hydroxy methyl furfural or 5-hydroxymethylfurfural (HMF) was the most abundant compound found in the peel and flesh of *G. dulcis* and seed of *G. cambogia*. The five *Garcinia* species collected in Tenom, Sabah have a promising potential for anticancer remedy as evidenced by cell cycle arrest and induction of apoptosis and can be developed as new nutraceutical and pharmaceutical products in the future.

ABSTRAK

FITOKIMIA, ANTIOKSIDA DAN POTENSI ANTIKANSER DARIPADA SPESIS GARCINIA TERPILIH YANG DI PEROLEHI DARI TENOM, SABAH

Garcinia mangostana (keluarga Clusiacea) dilaporkan mempunyai aktiviti antioksida, anti-proliferatif, pro-apoptotik, anti radang dan anti-karsinogenik yang disebabkan oleh kehadiran xanthone di dalam buah tersebut. Objektif kajian ini adalah untuk mengenalpasti kandungan fitokimia dan aktiviti antioksida serta kesan sitotoksik terhadap lima spesis Garcinia yang dikumpul di Tenom, Sabah iaitu G. dulcis, G. parvifolia, G. nitida, G. cambogia dan G. mangostana var. mangosta. Buah-buahan tersebut dibahagikan kepada tiga bahagian: kulit, isi dan biji dan diekstrak menggunakan dua jenis pelarut: 80% metanol dan akuous. Tiga parameter berbeza digunakan untuk mengukur aktiviti antioksida: penghapusan radikal bebas DPPH, ujian penyahwarnaan ABTS dan ujian FRAP. Untuk aktiviti sitotoksik, tiga sel kanser berbeza digunakan iaitu kanser payu dara (MCF-7, MDA-MB-231) dan kanser hati (HepG2). Kesemua sampel yang diekstrak menggunakan 80% metanol dipilih dan disaring untuk kesan sitotosik dengan menggunakan kaedah MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Analisis kitaran sel dan apoptosis turut dikaji secara lanjut menggunakan aliran sitometri. Analisis lanjut bagi mengenalpasti sebatian yang menyumbang kepada aktiviti antioksida dan anti-kanser dikenalpasti menggunakan analisis GC-MS. Keputusan kajian menunjukkan bahawa bahagian biji G. cambogia dan G. nitida serta bahagian kulit G. mangostana var. mangosta merupakan antara sampel yang mengandungi fitokimia dan aktiviti antioksida yang paling tinggi. Buah G. dulcis, bahagian kulit G. mangostana var. mangosta dan biji G. cambogia menunjukkan kesan sitotoksik yang positif terhadap semua sel kanser yang diuji. Nilai IC_{50} yang paling rendah dikesan pada biji G. dulcis bagi sel kanser MCF-7, MDA-MD-231 dan HepG2, masing-masing dengan nilai 2.5±0.00, 12.5±3.54 and 7.33±2.52 μg/ml. Bahagian isi G. dulcis merangsang kitaran sel rehat dalam sel kanser HepG2 pada kitaran sel fasa Sub G1 (apotosis) secara bergantung kepada masa. Manakala biji G. cambogia merangsang kitaran sel rehat selepas 72 jam rawatan. Pewarnaan dengan Annexin V-FITC dan propidium idode menunjukkan bahawa apoptosis berlaku lambat pada bahagian isi G. dulcis dan awal pada biji G. cambogia dengan jumlah sel masing-masing sebanyak 41.2% dan 51.2% pada 72 jam rawatan. Apoptosis pada isi G. dulcis diikuti dengan peningkatan pada caspase 3. Analisis GC-MS menunjukkan bahawa Hydroxy methyl furfural atau 5-hydroxymethylfurfural (HMF) merupakan sebatian yang paling banyak di temui dalam kulit dan isi G. dulcis dan biji G. cambogia. Spesis Garcinia yang dikumpul di Tenom, Sabah boleh dijadikan pengubat antikanser seperti yang dibuktikan melalui kitaran sel rehat dan apoptosis dan boleh dimajukan sebagai produk nutraseutikal dan farmaseutikal di masa hadapan.

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µg	-	microgram
µl	-	microlitre
h	-	hour
min	-	minute
sec	-	second
mm	-	milimetre
nm	-	nanometre
mM	-	milimolar
g	-	gram
mg	-	miligram
l	-	litre
M	-	Molar
i.e.	-	Example

LIST OF SYMBOLS

%	-	Percentage
°C	-	Degree celcius
>	-	More than
<	-	Less than
≥	-	Equal or more than
≤	-	Equal of less than
~	-	Approximately

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Children are taught by their parents to eat fruits and vegetables without knowing that there is significant effect on health and may reduce the risk of chronic diseases. Chronic diseases such as cancer, cardiovascular disease (CVDs) including coronary heart diseases and hypertension, diabetes and chronic respiratory disease such as asthma are among four main types of common chronic disease which are also known as non-communicable diseases (WHO, 2015). Non-communicable diseases (NCDs) can be defined as a medical condition or disease that have slow progression, non-infectious and cannot be transferred to another human being (Kim and Oh, 2013). It is estimated that 38 million people died each year due to NCDs and up to three quarters of death occurred in low and middle income countries in the year 2012. CVDs accounted for causing the most death worldwide (17.5 million/year) followed by cancers (8.2 million). International Agency for Research Cancer (IARC) projected that the burden of cancer incidence will increase up to 23.6 million by 2030. The most detectable cancers that cause mortality worldwide annually, as of 2012, are lung (1.59 million), liver (746,000), colorectal (694,000) and female breast cancer (522,000) (IARC, 2014).

Higher consumption of fruits and vegetables has been associated in reducing risk of chronic diseases. Chronic diseases are related to aging process that are caused by free radicals such as reactive oxygen species (ROS) which normally are generated as part of the normal biochemical reactions in human body (Zarena and Sankar, 2009). Fruits and vegetables are rich in health-promoting nutrients and phytochemical compounds that can inhibit free radicals in the body. These includes vitamin C and folate which act as antioxidant promoting compound, potassium,

phytochemicals, dietary fibre and plant proteins (Leong and Shui, 2002). The nutrient alone however may not attribute to decrease the risk of chronic disease, but combination of the non-nutrient compound does. Epidemiological research suggested that the consumption of natural antioxidants such as polyphenol-rich food, fresh fruits, vegetables or teas have protective effects against several diseases such as cancer, arthritis as well as in the degenerative process related to aging, including Parkinson's and Alzheimer's diseases (Almeida *et al.,* 2011).

A healthy diet plays a crucial role in the prevention of chronic diseases where the intake of vegetables and fruits may significantly reduce the risk of chronic diseases such as various types of cancer, cardiovascular disease, stroke and diabetes mellitus type II, thus reducing mortality rate (Boeing *et al.,* 2012). A cohort study by Hjartåker *et al.* (2014) reported that consumption of vegetables, fruits and berries showed significantly reduced risk of cancer mortality and 8 – 10% reduced risk of all-causes of mortality as well as 20% reduced risk of stroke mortality when compared to those with low consumption of vegetables, fruits and berries. However, a healthy body comes from a healthy lifestyle. Therefore, it is important to do regular exercise, avoid tobacco use and alcohol intake as well as reduce the calorie and sugar intake in daily meals. The ScienceDaily (2013) reported that experts confirmed that fruits and vegetables consumption reduces the risk of mortality and the effect was much better in people with bad eating and lifestyle habit. Those people who consumed alcohol may have reduction risk around 30 to 40% while 20% reduction for obese people.

Sabah is a part of Borneo (Malaysia-Sabah and Sarawak; Indonesia-Kalimantan and Brunei) that is recognized as one of the biodiversity hotpot. The flora and fauna found in Sabah is a notable natural feature of this state whereby the number of wild plants species is approximately 10,000 (Halim *et al.,* 2012). Due to this matter, Sabah offers a huge diversity of wild edible underutilised fruits that are yet to be explored and commercialized. There are many species that can only be found in Sabah (Borneo) such as Pengolaban (*Litsea garciae*), Dabai (*Canarium odonthophyllum*), Bambangan (*Mangifera pajang*), Tarap (*Artocarpus odoratissimus*), Tampoi (*Baccaurea lanceolata*) and Liposu (*Baccaurea lanceolata*).

These underutilised fruits are believed to have its own health benefits due to its phytochemicals and antioxidants activity (Ali-Hassan *et al.* 2013b, 2013c; Abu-Bakar *et al.,* 2009; Abu-Bakar *et al.,* 2014).

Based on all the evidence, researchers are continuously looking for the best fruits and vegetables with high antioxidant and phytochemicals that could cure and prevent chronic diseases. Therefore, this research is carried out to investigate the potential of five *Garcinia* species collected in Sabah in which mostly are still underutilized, namely Mundu *(G. dulcis)*, Takob-akob *(G. parvifolia)* and Asam kandis *(G. nitida)*. It is hoped that these fruits can be recommended as one of the disease-preventing fruits in future especially in the prevention of cancer.

1.2 Problem Statement

As the prevalence of cancer is increasing, more research on alternative prevention or treatment such as using plants extracts and their bioactive compounds with cancer chemopreventive and chemotherapeutic properties need to be explored. Eventhough there are a lot of advancement in cancer therapy, the using of synthetic drugs are known to have side effects after prolonged usage. Therefore, alternative therapies of curing cancer without any side effects are important to be investigated. Epidemiological study revealed that higher consumption of fruits and vegetables in daily diet has been associated with reducing risk of chronic diseases. This is due to the potential of the fruits to serve as natural antioxidants and antitumour properties which is beneficial for human's health.

The *Garcinia* species mainly *Garcinia mangostana* has been used traditionally to treat several diseases in some countries and displayed diverse health benefit properties including cancer chemopreventive and chemotherapeutic activity. There are a number of species of *Garcinia* available and endemic to Borneo however not many reports on these species are available. The *Garcinia* species selected for this study might display health benefits properties including anticancer properties however. It is advisable to consume natural antioxidants in daily meals and extensive study on the phytochemicals and antioxidants in fruits is highly encouraged to replace the usage of synthetic drugs. Hence, this research was

conducted to identify the potential of selected *Garcinia* fruits to serve as natural cancer chemoprevention and chemotherapy as well as to educate local communities and the public at large on the health benefits of indigenous fruits.

1.3 Research Objectives

The main objective of this study is to determine the phytochemicals, antioxidant and anticancer potential of selected *Garcinia* species (*Garcinia dulcis*, *G. parvifolia*, *G. nitida*, *G. cambogia* and *G. mangostana)* collected in Borneo. While the specific objectives of this study are:

i. To investigate the phytochemicals contents (total phenolic, total flavonoid, total anthocyanin and total carotenoid content) of selected *Garcinia* species.

ii. To determine the antioxidant activity of selected *Garcinia* species using DPPH free radical scavenging, ABTS decolourization and Ferric Reducing / Antioxidant Power (FRAP) assays.

iii. To determine the secondary metabolites in selected *Garcinia* species by using Gas-Chromatogrphy-Mass Spectroscopy (GC-MS).

iv. To study the anti-proliferative activity of selected *Garcinia* species extracts using MTT assay against MCF-7 (hormone-dependent breast cancer), MDA-MB-231 (non-hormone dependent breast cancer) and HepG2 (liver cancer) cell lines *in vitro*.

v. To determine the effects of selected fruit extracts on the cell cycle and apoptosis events in HepG2 cancer cell line.

1.3.1 Justification of Study

The selected *Garcinia* species collected in Borneo namely *G. dulcis*, *G. parvifolia* and *G. nitida* are well-known among people in some areas of Sabah especially Tenom, Keningau and Sipitang particularly among the Murut and Kadazandusun communities of that area. *Garcinia cambogia* is a native species from Indonesia but cultivated in Sri Lanka and Southern India and distributed widely in other tropics including Malaysia while *G. mangostana* is from mangosta variety which can only be found in Tenom Agricultural Research Station (TARS). All of these fruits remain underutilized among the people in other regions of Sabah as well as in Peninsular

Malaysia. Therefore, this study intends to provide scientific data and evidence on the selected underutilized fruits which might have a great potential for health benefits as well as for fruit-based product development in the future.

1.3.2 Hypothesis of Study

The selected *Garcinia* species might contain undiscovered phytochemical content (total phenolic, total flavonoid, total anthocyanins and total carotenoid content), natural antioxidant and anticancer properties that could contribute to health benefits as well as to promote the utilization of these indigenous species.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is currently and still one of the leading causes of death worldwide. In 2012, it was estimated about 14 million of new cancer cases worldwide were reported of which 8.2 million deaths were due to cancer. The number of cases is expected to increase over 70% in two decades (WHO, 2015). Recent studies reported that there is a decline in cancer death rate with 1.8% per year in male and 1.4% per year in female within five years (2006-2010) in the United States (developed country). The combined cancer death rate (deaths per 100,000 population) has been continuously declining these past two decades, from a peak of 215.1 in 1991 to 171.8 in 2010 which indicate 20% decline in total (Siegel, 2014). However, the cases are still growing in economically developing country. According to World Cancer Report, more than 60% of world's total new annual cases occur in Africa, Asia and South America (developing country). These regions account for 70% of the world's cancer deaths (WHO, 2015). Cancer survivals were less in developing countries, most likely because of late stage diagnosis and limited access to timely and standard treatment (Jemal, 2011).

Malignancy or commonly-called as cancer refers to a group of disease characterized by uncontrolled growth and spread of abnormal cells in human body, forming lumps or masses of tissues which are known as tumours and if left untreated may cause death. Invasion is the process where the cells break away from the tumour and penetrate their surroundings. In this form, the tumour is called malignant. The tumour is benign when they do not invade their surroundings (Inoue *et al.,* 1987). In order to achieve full malignancy, transforming characteristics of the cells needs to be developed. This include self-sufficiency in growth signalling and non-limitation of replication potential, become unresponsive to antiproliferative