UNIVERSITI PUTRA MALAYSIA

BIOASSAY-GUIDED ISOLATION, AND IDENTIFICATION OF BIOACTIVE COMPOUNDS FROM GARCINIA ATROVIRIDIS (ASAM GELUGOR)

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By

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'MAY GOD BLESS YOU ALL'

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LIST OF ABBREVIATIONS

ATCC	:	American Type Culture Collection
CD_{50}	:	50% Cytotoxic dose
CFU	:	Colony-forming units
CGM	:	Complete growth medium
$CHCl_3$	:	Chloroform
DMSO	:	Dimethyl sulphoxide
EBV	:	Epstein Barr virus
EtOAc	:	Ethyl acetate
Ext.	:	Extract
FTC	:	Ferric thiocyanate
HCA	:	Hyroxycitric acid
HeLa	:	Human cervical carcinoma cell line
IC_{50}	:	50% Inhibitory concentration
LD_{50}	:	50% Lethal dose
MeOH	:	Methanol
MID	:	Minimum inhibitory dose
MRSA	:	Methicillin-resistant *Staphylococcus aureus*
n-BuOH	:	n-Butanol
NA	:	Nutrient agar
NB	:	Nutrient broth
PDA	:	Potato dextrose agar
PDB	:	Potato dextrose broth
PMA	:	Phorbol 12-myristate 13-acetate
TBA	:	Thiobarbituric acid
TLC	:	Thin layer chromatography

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Master of Science

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September 1998

Chairman: Associate Prof. Dr. Abdul Manaf Ali

Faculty: Food Science and Biotechnology

This study was undertaken to characterise the biological activities of *Garcinia atroviridis*, a food plant and traditional vegetable, as well as to isolate and identify its bioactive constituents Ethnobotanical reports on the use of *G atroviridis* as an anti-infective agent and biopreservative, coupled with the paucity of scientific reports on the biological activities of *G atroviridis* were the basis for selecting this plant as the subject of this study Crude extracts (methanol dimethyl sulfoxide–9 1) of various parts of this plant were screened for antimicrobial (disc diffusion method), cytotoxic (microtitration method), brine shrimp toxic, antitumour-promoting (Epstein Barr virus activation assay) and antioxidant (ferric thiocyanate and thiobarbituric assays) activities The crude extracts exhibited predominantly antibacterial activity with the roots showing the strongest inhibition against the test bacteria at the minimun inhibitory dose (MID) of 15 6 µg/disc Although all the extracts failed to inhibit the growth of most of the test fungi, significant antifungal activity against *Cladosporium herbarum* was exhibited by most notably the fruit extract (MID 100 µg), and the leaf extract (MID 400 µg) None of the extracts were significantly

cytotoxic and lethal towards brine shrimps. The root, leaf, trunk and stem bark extracts (except for the fruits) showed strong antioxidant activity exceeding that of the standard antioxidant, α-tocopherol. Antitumour-promoting activity (> 95% inhibition) was shown by the fruit, leaf, stem and trunk bark extracts.

Subsequently, the fruit extract was subjected to the convenient thin layer chromatography bioautograpy guided isolation against *C. herbarum*. The *C. herbarum* inhibitory constituents were isolated by several steps of separation that involved solvent partitioning, chromatography and recrystallisation to yield two new pure compounds, AG2-1 (MID: 0.4 μg) and AG3 (MID: 0.8 μg) . Both the anti-*Cladosporium* compounds were identified by spectroscopic methods as 1-butyl 5-methyl 3-carbobutoxy-2-hydroxycitrate (AG2-1) and 3-carbobutoxy-3-carbobutoxyhydroxymethyl-3-propiolactone (AG3). However, both compounds were non-cytototoxic and non-inhibitory against all other test microorganisms. Besides anti-*Cladosporium* activity, both compounds showed antitumour-promoting activity at the 50% inhibitory concentration (IC_{50}) values of 70 μM (AG3) and 560 μM (AG2-1) but were inactive in all the antioxidant assays.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia untuk memenuhi keperluan untuk ijazah Master Sains.

PENGASINGAN BERPANDUKAN BIOCERAKINAN, DAN PENGENALPASTIAN SEBATIAN-SEBATIAN BIOAKTIF DARIPADA *GARCINIA ATROVIRIDIS* (ASAM GELUGOR)

Oleh

MUHAMMAD MUKRAM BIN MOHAMED MACKEEN

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Pengerusi: Prof. Madya Dr. Abdul Manaf Ali

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Kajian ini telah dijalankan untuk mencirikan aktiviti-aktiviti biologi buah, daun, akar, kulit ranting dan kulit batang *Garcinia atroviridis*, sejenis tanaman makanan ulam, secara lebih menyeluruh. *Garcinia atroviridis* dipilih sebagai bahan kajian kerana laporan-laporan etnobotani tentang penggunaannya sebagai ejen anti-jangkitan and biopemuliharaan, serta kekurangan laporan saintifik terhadap aktiviti-aktiviti biologi. Ekstrak kasar (metanol:dimetil sulfoksida-9:1) yang disediakan daripada bahagian berlainan pokok telah diuji untuk aktiviti-aktiviti antimikrob (kaedah resapan cakera), sitotoksik (kaedah mikrotitratan), ketoksikan udang pepai, anti-promosi barah (cerakinan pengaktifan virus Epstein Barr) and antioksida (cerakinan ferik tiosianat dan tiobarbiturik). Ekstrak kasar lebih ketara menunjukkan aktiviti antibakteria berbanding aktiviti antikulat. Ekstrak akar telah memberi nilai dos perencatan minimum (MID) yang paling rendah iaitu15.6 µg/cakera. Walaupun semua ekstrak gagal untuk merencat pertumbuhan hampir semua kulat ujian, aktiviti perencatan yang ketara terhadap *Cladosporium herbarum* telah

ditunjukkan oleh terutamanya ekstrak buah (MID: 100 μg), dan ekstrak daun (MID: 400 μg). Aktiviti-aktiviti sitotoksik and ketoksikan udang pepai tidak ditunjukkan oleh semua ekstrak. Ekstrak-ekstrak akar, daun, kulit ranting and kulit batang (kecuali buah) menunjukkan aktiviti antioksida lebih kuat daripada antioksida komersial, α-tokoferol. Aktivit antipromosi barah (>95% perencatan) telah ditunjukkan oleh ekstrak-ekstrak buah, daun, kulit batang dan kulit batang.

Seterusnya, pengasingan berpandukan bioautografi kromatografi lapisan nipis (TLC) terhadap *C. herbarum* telah digunakan untuk memperolehi sebatian-sebatian yang aktif daripada ekstrak buah. Sebatian-sebatian aktif tersebut iaitu AG2-1 (MID: 0.4 μg) dan AG3 (MID: 0.8 μg) berjaya diperolehi setelah menjalankan langkah-langkah pemisahan. Dengan menggunakan kaedah-kaedah spektroskopi, kedua-dua sebatian baru tersebut telah dikenalpasti sebagai 1-butil 5-metil 3-karobutoksi-2-hidroksisitrat (AG2-1) dan 3-karbobutoksi-3-karbobutoksihidroksimetil-3-propiolakton (AG3). Walaupun bersifat anti-*Cladosporium*, kedua-dua sebatian ini tidak sitotoksik serta tidak merencat pertumbuhan mikroorganisma yang lain. Selain aktiviti anti-*Cladosporium*, kedua-dua sebatian telah menunjukkan aktiviti antipromosi barah dengan nilai-nilai kepekatan perencatan 50% (IC_{50}) pada 70 μM (AG3) dan 560 μM (AG2-1) tetapi adalah tidak aktif dalam semua ujian antioksida.

CHAPTER I

INTRODUCTION

Studies into medicinal properties of food plants, i.e. plants consumed as food by humans or used for culinary purposes, is of growing importance. The main reasons for this are firstly, the propagation of the concept of 'functional foods', viz. foods that cure, ameliorate or prevent disease, and secondly, the biologically active components in food plants are presumed to be of low toxicity relative to non-edible, usually toxic, medicinal plants. The chemical constituents of many common food plants have been extensively reported. Consequently, chemical investigations would be expected to yield known constituents and thus, from a chemistry perspective, be of limited significance. However, the biological activities of functional food plants, particularly uncommon species, and their isolated constituents, i.e. food phytochemicals, are still largely unknown.

The flora of Malaysia comprises about 15,000 species of higher plants which includes a group of food plants classified as traditional vegetables, locally known as 'ulam'. There are more than 120 species of 'ulam' belonging to several families, ranging from herbs to trees (Mansor *et al.*, 1988). The roles of 'ulam' as functional food plants or a source of functional food phytochemicals is attractive in the Malaysian context because of its wide consumption among the local people especially the Malay and indigenous communities (Mackeen *et al.*, 1997a). The 'ulam' are

mostly eaten raw as salad, particularly the leaves, or otherwise blanched, sautéed, curried and fried (Bautista *et al*, 1988; Mansor, 1988; Mackeen *et al.*, 1997a). Only a few studies have reported the biological activities of the 'ulam' as a collective group.

Therefore, based on the results of previous studies involving the 'ulam', *Garcinia atroviridis* (local name 'asam gelugor') was chosen as the subject of this study as it was reported to possess strong antimicrobial activity (Grosvenor *et al*, 1995b; Mackeen *et al*, 1997a). Furthermore, ethnobotanical reports of *G. atroviridis* being used in folkloric medicine (Burkill, 1966) and for fish preservation (pers. comm.) also prompted the selection of this plant for further study.

Therefore, the objectives of this study are,

1) to isolate and identify the bioactive compounds of *G. atroviridis*, and
2) to characterise the biological activities of these compounds

CHAPTER II

LITERATURE REVIEW

Overview of *Garcinia* Genus

The Guttiferae or alternatively known as Clusiaceae (Guttiferales Order is a mainly latex-bearing tropical family of about 40 genera and 1000 species (Whitmore, 1983). In Malaya, four genera and 121 species of the Guttiferae are found, namely *Garcinia* (49 spp.), *Calophyllum* (45 spp.), *Mesua* (23 spp.) and *Mammea* (4 spp.), in different habitat. *Garcinia* is an economically important genus of the Guttiferae consisting of about 400 species within palaeotropical regions concentrated mainly in Southeast Asia and secondarily in India and West Africa (Willis, 1973). Species of this genus are typically small to medium dioecious evergreen fruit trees, occasionally shrubs, usually with hard timber and abundant latex.

Garcinia is the largest genus of Guttiferae in Peninsular Malaysia and is important locally as a genus of fruit trees, particularly *G. mangostana*, *G. atroviridis*, *G. prainiana*, *G. dulcis* and *G. cowa* (Corner, 1988). Peninsular Malaysian *Garcinia* species are almost wholly small or medium trees not exceeding 30 m in height or a 120 cm girth and may be found growing from seashores to mountain tops (Whitmore, 1983). About 49 species of *Garcinia* have been recorded in Peninsular Malaysia but the identification of several of them is incomplete especially the group of high mountain species. The 39 species that have been completely identified are listed in Table 1 of which some are endemic.

Table 1: *Garcinia* Species in Peninsular Malaysia

G. atroviridis Griff. Ex T. Anders.
G. bancana (Miq.) Miq.
**G. bancana* var. *curtisii* (Ridley) Whitmore
**G. burkillii* Whitmore
**G. cantleyana* Whitmore
G. cantleyana var. *grandiflora* Whitmore
**G. cataractalis* Whitmore
G. clusiaefolia Ridley
G. costata Hemsley ex King
G. cowa Roxb.
G. cuspidata King
**G. diversifolia* King
G. dulcis (Roxb.) Kurz
**G. dumosa* King
G. eugeniaefolia Wall. ex T. Anders.
G. forbesii King
G. griffithii T. Anders.
**G. hendersoniana* Whitmore
**G. holttumii* Ridley
G. hombroniana Pierre
G. maingayi Hk. f.
G. maingayi var. *stylosa* King
G. malaccensis Hk. f.
G. mangostana L.
G. merguensis Wight
G. minutiflora Ridley
**G. monantha* Ridley
G. montana Ridley
G. murdochii Ridley
**G. murtonii* Whitmore
G. nervosa Miq.
G. nervosa var. *pubescens* King
G. nigrolineata Planch. ex T. Anders.
**G. opaca* King
G. opaca var. *dumosa* Whitmore
G. parvifolia (Miq.) Miq.
G. penangiana Pierre
G. prainiana King
**G. pyrifera* Ridley
G. rostrata (Hassk.) Miq.
G. scortechinii King
**G. uniflora* King
G. urophylla Scortechini ex King
G. xanthochymus Hk. F

(*endemic)

The fruits, latex (gum and resin), timber, leaves and roots of several species are of economic and medicinal value. Ethnopharmacognostic reports have described the use of some species in Malayan folkloric medicine (Table 2). Two species, *G. mangostana* and *G. atroviridis*, are ascribed with salient medicinal properties particularly *G. mangostana* for the efficacy of the bark and dried rind in treating dysentery and diarrhoea as reported throughout Southeast Asia and India (Perry and Metzger, 1980). On the other hand, reports on the use of *G. atroviridis* for medicinal purposes have been restricted to Peninsular Malaysia and Sumatra. In Sumatra, an infusion of the leaves of *G. atroviridis* and *Ananas comosus* is drunk to treat stomach pains brought about by pregnancy (Grosvenor *et al.*, 1995a)

***Garcinia atroviridis* Griff. ex T. Anders**

Botany

G. atroviridis, locally known as 'asam gelugor', is a medium-sized tree growing up to 27 m tall and 70 cm in girth with a deep monopodial crown of dense, slender and drooping branches (Whitmore, 1983; Plate 1). Watery pale-yellow sap is scantily found in the inner bark of the lower bole that is often fluted or sinuous. The leaves, tapered to apex and base, are glossy-leathery, greenish black, big and oblong-shaped usually measuring within 15 x 4 - 25 x 7 cm (Plate 2). The flowers are terminally located with four yellow sepals and four crimson petals (Plate 3). The fruits are big up to 10 cm across, bright yellow, multi-ribbed and thick-rinded with flattened seeds encased in the sour pink-white pulp (Plate 4). Sun-dried thin slices

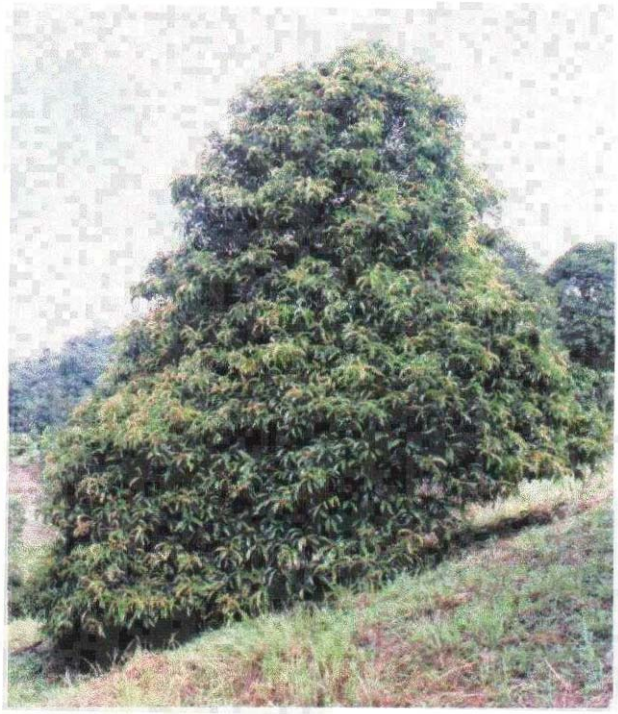

Plate 1: *G. atroviridis* Tree (13 ft)

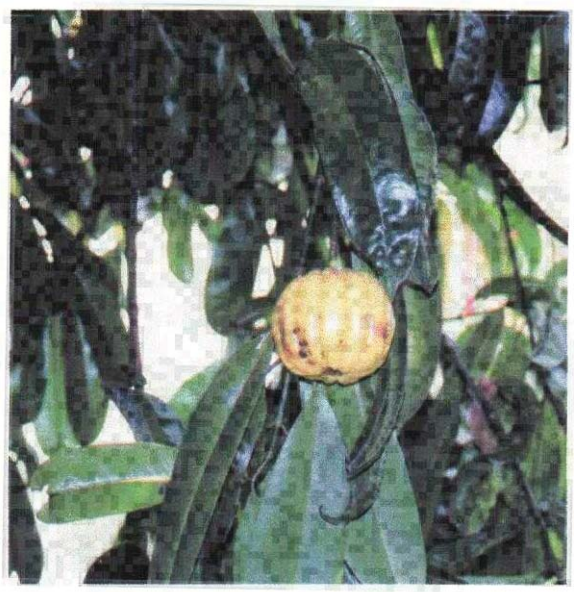

Plate 2: Leaves and a Fruit of *G. atroviridis*

Plate 3: Flowers of *G. atroviridis*

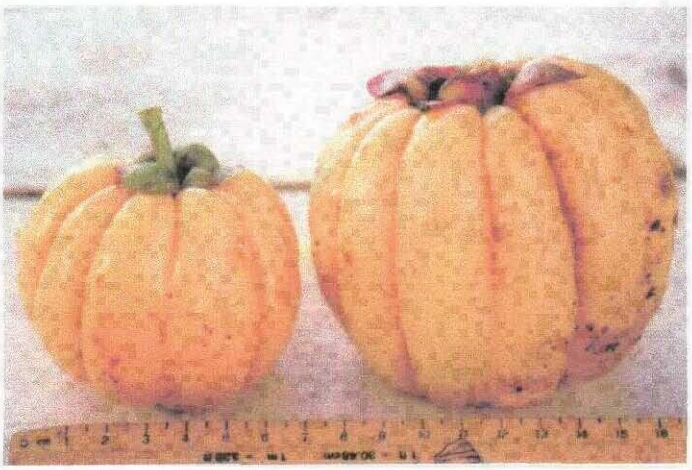

Plate 4: Fresh Fruits of *G. atroviridis*

Plate 5: 'Asam Keping'

of the rind are strongly shrunken and blackish brown. This species grows wild throughout Peninsular Malaysia in the plains of lowland forest and up to 600 m in the hills but is also widely cultivated especially in the northern states.

Econo- and Ethno- botany

Fruits cut into thin slices and sun-dried are locally known as 'asam keping' and are commercially sold (Plate 5). 'Asam keping' is popularly used as a seasoning in curries, sour relish and also for dressing fish (Burkill, 1966; Corner, 1988). The fresh fruits are not consumed raw but are sugar-sweetened for eating. Occasionally, the young leaves are used for culinary purposes and as a traditional vegetable ('ulam'). In the East Coast states of Kelantan and Trengganu, fresh fish are steamed with the leaves of *G. atroviridis* to delay spoilage (pers. comm.). The medicinal value of *G. atroviridis* in folkoric medicine has been previously mentioned (Table 2; Grosvenor *et al.*, 1995a).

Table 2: *Garcinia* Species Used in Folkloric Medicine of Peninsular Malaysia

Species	Medicinal Use (Burkill, 1966; unless mentioned otherwise)
G. atroviridis	1) Decoction of the leaves and roots is used as an ear-drop for ear-ache 2) Vinegar-based lotion of crushed fruit is rubbed over the abdomen after confinement 3) Juice from the leaves is given as a post-partum protectant 4) Raw shoots used to treat throat irritation and cough (Fui, 1992) 5) Crushed shoots are mixed with water and smeared to wet hair for treating dandruff and beautifying hair (Fui, 1992)
G. dulcis	Seeds pounded with vinegar or salt is externally applied to treat glandular swellings
G. scortechinii	Juice of the roots is rubbed on cuts
G. hombroniana	1) Decoction of the roots is used as a post-partum protectant 2) The roots and leaves are prescribed for treating itch
G. mangostana	1) Decoction of the dried rind is used to treat dysentery 2) Decoction of the roots is taken to treat irregular menstruation 3) Infusion of the leaves with unripe bananas and benzoin is applied to circumcision and similar wounds
G. nigrolineata	Lotion prepared from juice of the leaves with salt used to treat running eyes

Biological Activities of *G. atroviridis* Extract

The biological activities of extracts of the *Garcinia* genus are wide-ranging particularly of *G. mangostana* and *G. kola* as exemplified in Table 3 but unfortunately, only a few of these studies involved *G. atroviridis*. From the investigations of the antimicrobial, antinematodal and antitumour-promoter activities of *G. atroviridis*, only antibacterial activity was found to be significant. The methanol-water extract prepared from the leaves and stems of *G. atroviridis* showed exceptionally strong antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* but was inactive against the fungi *Saccharomyces cerevisiae* and *Fusarium oxysporum* (Grosvenor *et al*, 1995b). Similarly, the ethanol-water extract from the fruits of *G. atroviridis* was found to display strong antibacterial activity against *Pseudomonas aeruginosa* by microbiostatic action but moderate activity against *Bacillus cereus* by microbiocidal action (Mackeen *et al*, 1997a). However, either weak or no activity was shown against the bacteria *Bacillus megaterium* and *Escherichia*, and the fungi *Aspergillus ochraceous* and *Cryptococcus neoformans*.

Except for the strong antibacterial activity, the alcohol fruit extract of *G. atroviridis* only weakly inhibited HeLa cells, the phytopathogenic nematode, *Bursaphelencus xylophilus*, and Epstein Barr virus mediated tumour-promotion (Murakami *et al*, 1995; Mackeen *et al*, 1997a; Mackeen *et al*, 1997b).

Table 3: Biological Activities of Extracts of Some *Garcinia* Species

Species	Part	Bioactivity	Reference
G. cowa	Leaves	Antitumour-promoter	Murakami *et al.*, 1995
	Bark	Antimalarial	Likhitwitayawuid *et al.*, 1998a
G. dulcis	Bark	Antimalarial	Likhitwitayawuid *et al.*, 1998b
G. kola	Roots, Seeds	Antimicrobial	Ebana *et al.*, 1991; Madubunyi, 1995
	Seeds	Antihepatotoxic	Braide, 1991
	Seeds	Anti-inflammatory	Braide, 1993
	Seeds	Antiulcerogen	Ibironke *et al.*, 1997
G. livingstonei	Roots	Antifungal	Marston *et al.*, 1993
G. mangostana	Fruit Hulls	Anti-HIV	Chen *et al.*, 1996
	Fruit Hulls	Anti Ca^{2+}-dependent Protein Kinase	Jinsart *et al.*, 1992
	Fruit Hulls	Antioxidant	Yoshikawa *et al.*, 1994