PHYTOCHEMICAL AND CYTOTOXIC STUDIES ON FLAVONOIDS FROM THE FRUIT OF MACARANGA GIGANTEA Muell.- Arg. (EUPHORBIACEAE)

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LIST OF ABBREVIATIONS

Chromatography

TLC	Thin layer chromatography
CC	Column chromatography
PTLC	Preparative Thin Layer Chromatography

Mass spectrometry (MS)

EI-MS	Electron ionization mass spectrometry
m/z	mass/charge
eV	Electron volt

Nuclear magnetic resonance (NMR)

ppm	part per million
J	coupling constant
br	broad
s	singlet
d	doublet
t	triplet
m	multiple
dd	double doublet
COSY	Correlation spectroscopy
DEPT	Distortionless enhancement by polarization
HMQC	Heteronuclear Multiplet Quantum Correlation
HMBC	Hetero Multiplet Bond Correlation

Bioassay

S.E.M.	Standard Error of Mean
ATCC	American Type Culture Collection
DMSO	dimethyl sulphoxide
EBSS	Earle's Balanced Salt Solution
EDTA	ethylenediamine tetraacetic acid
FCS	Fetal Calf Serum
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
MEM	Minimal Essential Medium
MTS	3-(4, 5-dimethythiazol-2-yl)-5-(3- carbomethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium
PBS	phosphate-buffered saline
PMS	phenazine methosulphate
RPMI	Rosewell Park Memorial Institute

KAJIAN FITOKIMIA DAN SITOTOSIK TERHADAP FLAVONOID DARIPADA BUAH *MACARANGA GIGANTEA* Muell.- Arg. (EUPHORBIACEAE)

ABSTRAK

Macaranga gigantea Muell.- Arg. ialah sejenis tumbuhan ubatan yang endemik di Malaysia di mana ia digunakan untuk merawat cirit birit dan disentri. Buah tumbuhan ini, yang dipungut di kampus Universiti Sains Malaysia, Pulau Pinang telah dikeringkan, dijadikan serbuk yang kemudiannya diekstrak dengan MeOH. Komponen kimia ekstrak MeOH diasingkan dan ditulenkan dengan kromatografi turus gel silika dan kromatografi lapisan nipis persediaan. Enam flavanon telah dikenalpasti yang mana tiga merupakan sebatian yang baru, yakni, 5,7,3',5'-tetrahidroksi-6-geranilflavanon (**1**), 5,7,3'-trihidroksi-4'-geranilflavanon (**5**), dan 5,7, 3', 5'-tetrahidroksi-2'-geranilflavanon (**6**). Sebatian-sebatian sedia diketahui yang ditemui termasuklah nymphaeol-B (**2**), nymphaeol-C (**3**) dan bonnanione A (**4**). Kesemua sebatian ini dikenalpasti berdasarkan analisis spektrum menggunakan UV (ultra lembayung), IR (infra merah), EI-MS serta 1-D dan 2D-NMR. Ekstrak methanol dan kesemua sebatian yang diasingkan telah diselidik untuk menilai kesan sitotoksik sebatian tersebut terhadap sel tumor hati (Hep G2) dan payudara (T-47D). Penentuan sitotoksik dilakukan menggunakan kaedah MTS, yakni Penentuan Perkembangan Sel Bukan Radioaktif CellTiter96® A_{queous}. Data dianalisis secara analisa statistik menggunakan kaedah Prisma Grafik. Keputusan menunjukkan bahawa kekuatan aktiviti sitotoksik sebatian tulen yang diasingkan ke atas sel tumor hati (Hep G2) berkurangan mengikuti: (**1**) (EC_{50} = 2.50), (**3**) (EC_{50} = 2.50)

> (**5**) (EC_{50} = 2.60), (**6**) (EC_{50} = 2.60) > (**4**) (EC_{50} = 4.20) > (**2**) (EC_{50} = 6.70); dan terhadap sel tumor payudara (T- 47D) berkurangan mengikuti: (**6**) (EC_{50} = 2.20) > (**2)** (EC_{50} =2.30) > (**4**) (EC_{50} =2.40) > (**1**) (EC_{50} = 2.50), > (**5**) (EC_{50} = 3.50), (**3**) **(**EC_{50} = 3.50).

PHYTOCHEMICAL AND CYTOTOXIC STUDIES ON FLAVONOIDS FROM THE FRUIT OF *MACARANGA GIGANTEA* Muell.- Arg. (EUPHORBIACEAE)

ABSTRACT

Macaranga gigantea Muell.- Arg., a medicinal plant endemic to Malaysia, is used locally to treat diarrhea and dysentery. The fruit of this plant, collected from the campus of Universiti Sains Malaysia, Penang, was dried, powdered and extracted with MeOH. The chemical constituents of the MeOH extract were isolated and purified by silica gel column chromatography and preparative thin layer chromatography. Six flavanones were identified, among which three were novel compounds, namely, 5,7,3', 5'-tetrahydroxy-6-geranylflavanone (**1**), 5,7, 3'-trihydroxy-4'-geranylflavanone (**5**) and 5,7,3',5'-tetrahydroxy-2'-geranylflavanone (**6**). The known compounds found were nymphaeol-B (**2**), nymphaeol-C (**3**) and bonnanione A (**4**). All of these compounds were identified on the basis of spectral analyses using UV, IR, EI-MS, 1-D and 2D-NMR. The methanol extract and all isolated compounds were assayed for their cytotoxic activity toward liver (Hep G2) and breast (T - 47D) solid tumor cell lines. The cytotoxic assay was carried out using MTS method, CellTiter96® A_{queous} Non-Radioactive Cell Proliferation Assay. The data was analyzed by statistical analyses using GraphPad Prism. The results showed that the cytotoxic activity of the pure isolated compounds on liver tumor cells (Hep G2) decreases in the order : (**1**) (EC_{50} = 2.50) , (**3**) (EC_{50} = 2.50) > (**5**) (EC_{50} = 2.60), (**6**) (EC_{50} = 2.60) > (**4**) (EC_{50} = 4.20) > (**2**) (EC_{50} = 6.70); and on breast tumor cells (T - 47D) decreases in the order : (**6**) (EC_{50} = 2.20) > (**2)** (EC_{50} =2.30) > (**4**) (EC_{50} =2.40) > (**1**) (EC_{50} = 2.50) > (**5**) (EC_{50} = 3.50), (**3**) (EC_{50} = 3.50).

CHAPTER ONE

INTRODUCTION

1.0 INTRODUCTION

Natural product chemistry has been a topic of great interest since ancient times, being relevant to the preparation of food stuff, colouring matters, fibers, toxins, medicine. Separation methods for the study of natural products have been developed and, without doubt, have greatly stimulated the development of the refined techniques used today, such as the various analytical and preparative chromatographic methods (column chromatography, GC, TLC, HPLC, paper chromatography). These methods have made it possible for the isolation of extremely small quantities of compounds. Instruments for the structural determination of compounds such as UV, IR, 1D- and 2D-NMR and MS have been developed and refined rapidly (Torssell, 1997).

The Plant Kingdom is an important source of chemical compounds. Some, such as carbohydrates, amino acids and proteins, are classified as primary metabolites, while others, such as alkaloids and phenolics, are classified as secondary metabolites. Secondary metabolites are essential to plant life, many of them providing a defence mechanism against bacterial, viral and fungal attack, analogous to the immune system of animals (Vickery & Vickery, 1981).

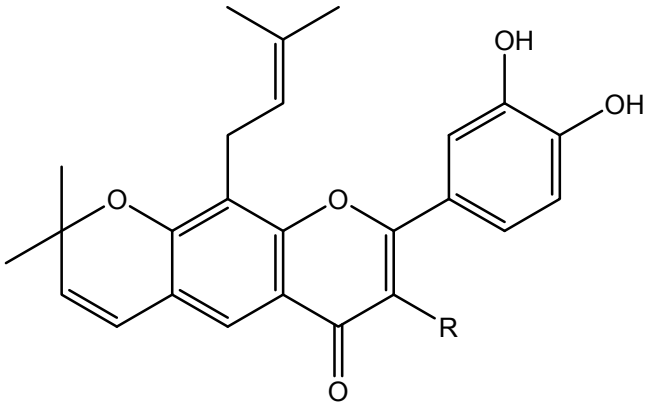
1.1 The genus *Macaranga*

Malaysia, with a tropical climate, is very rich in flora a large number of which possess medicinal properties (Whitmore, 1972). The genus *Macaranga* (Euphorbiaceae) comprises 280 species which are distributed extensively in the tropical and warm regions (Woodland, 2000).

The genus is known for a wide range of mutualistic association with ants, although the degree of interaction between plants and ants may vary from loosely facultative, non-specific myrmecophylic to obligate myrmecophytic associations (Fiala *et al*., 1994). Many *Macaranga* species are characteristic of secondary forests in South Asia, where plants provide food and nesting space for specific ant partners (*Slik et al*., 2003; Vogel *et al*., 2003).

Many species of the genus *Macaranga* have been investigated phytochemically. The activity of crude extracts and chemical constituents isolated have also been assayed for biological activity, in particular, those which are used in traditional medicine.

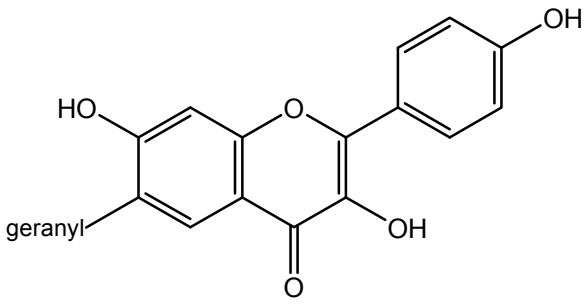
Sultana & Ilyas (1986) isolated two flavonoids, macaflavone I, **1**, and II, **2**, from the acetone extract of the leaves of *Macaranga indica*.



1 R= OH
2 R= OMe

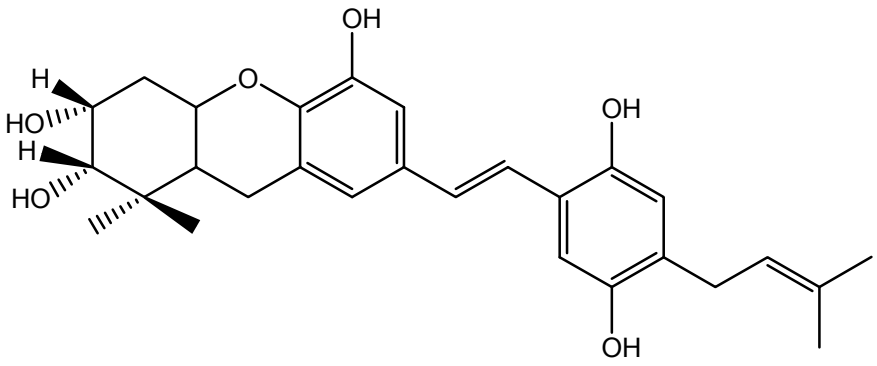
From the methanolic extract of the leaves of *Macaranga vedeliana* Muell.-Arg. used by natives to relieve pains and cure tonsillitis in Lifou (Loyalty Islands, New Caledonia), macarangin, **3**, a geranyl subtituted flavonol, has been isolated. The pharmacological analysis of various extracts of the plant has

demonstrated that the methanolic extract exhibited a hypotensive effect (Hnawia *et al*., 1990).



3

Later, vedelianin, **4**, a prenylated stilbene, has been isolated from the methanolic extract of the leaves of this plant (Thoison *et al.*, 1992).



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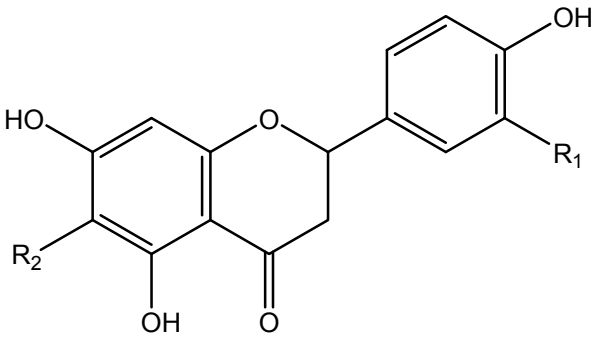
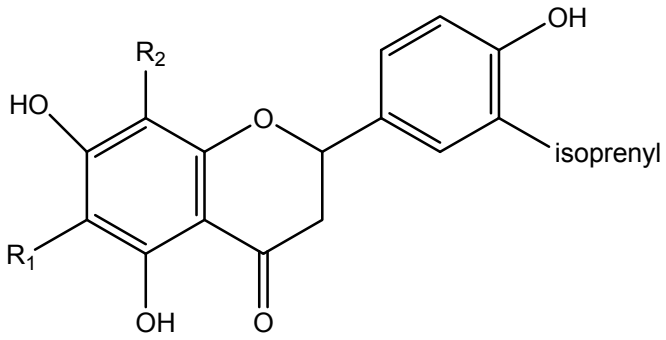
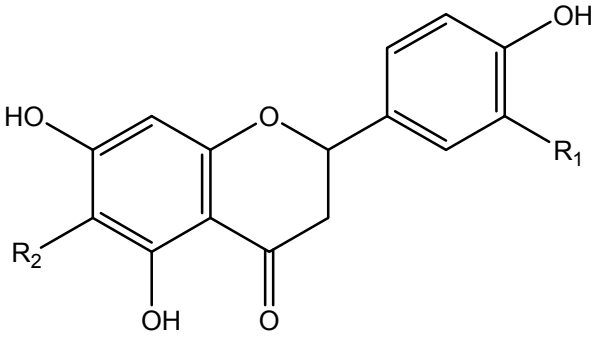
From the CH_2Cl_2 extract of the leaves of *Macaranga pleiostemona* (used by natives in New Guinea to relieve headache), four flavanones, namely, macarangaflavanone A, **5**, B, **6**, euchrestaflavanone A, **7**, and bonannione A, **8**, were isolated. The crude extract and pure compounds isolated from the plant showed antibacterial activity against *Escherichia coli* (ATCC 25932) and *Micrococcus luteus* (ATCC 9341) (Schutz *et al*., 1995).



5 R_1 = geranyl, R_2 = H

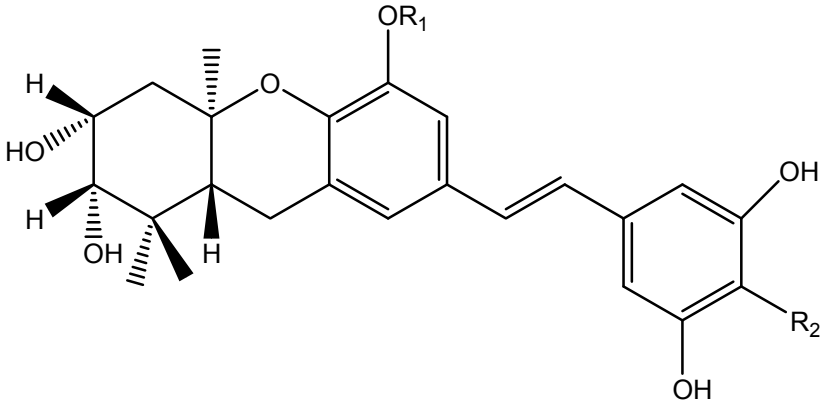


6 R_1= isoprenyl, R_2= H
7 R_1= H, R_2= isoprenyl

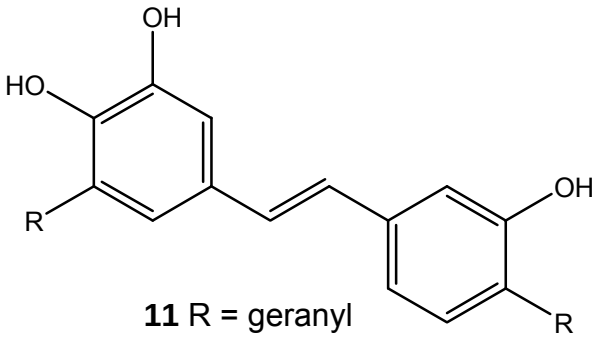


8 R_1 = H, R_2 = geranyl

Three geranyl stilbenes, namely, schweinfurthin A, **9**, B, **10**, and C, **11**, have been isolated from the methanol extract of the leaves of *Macaranga schweinfurthii*. These compounds showed cytotoxic activity (Beutler *et al*., 1998).

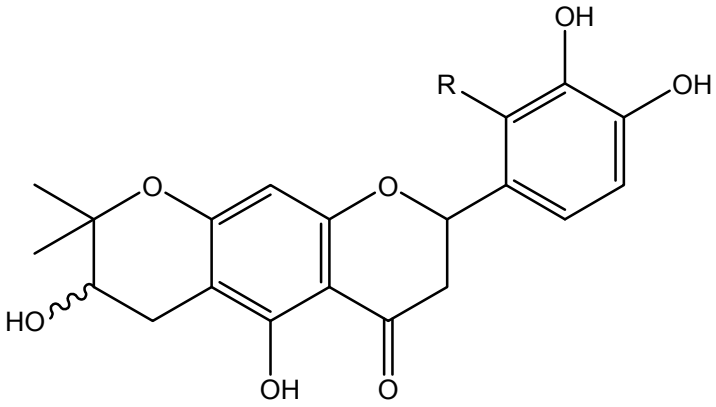


9 R_1= H, R_2 = geranyl
10 R_1 = Me, R_2 = geranyl

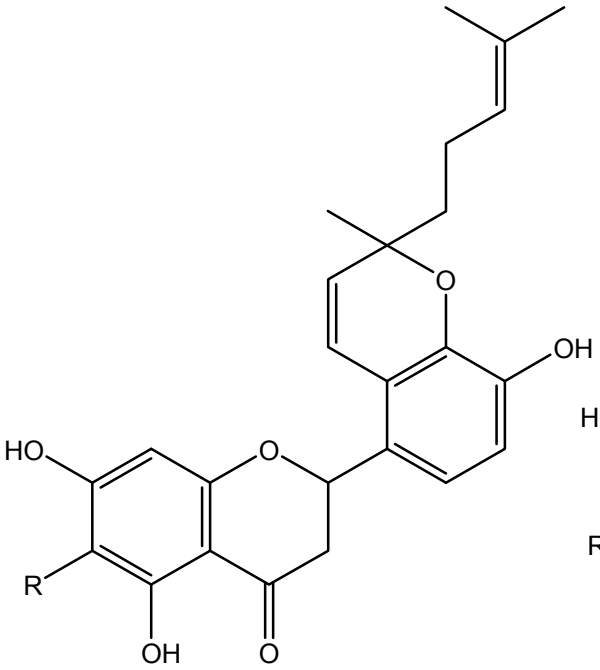


11 R = geranyl

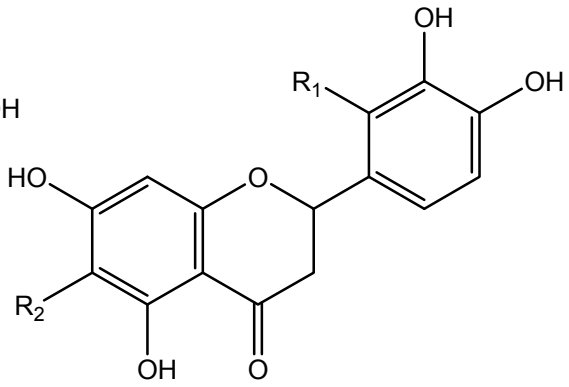
Three prenylflavanones, namely, tanariflavanone A, **12**, B, **13**, and nymphaeol-C, **14**, have been isolated from the methanol extract of fallen leaves of *Macaranga tanarius*. These flavonoids showed phytotoxic activity (Tseng *et al.,* 2001).



12 R = geranyl

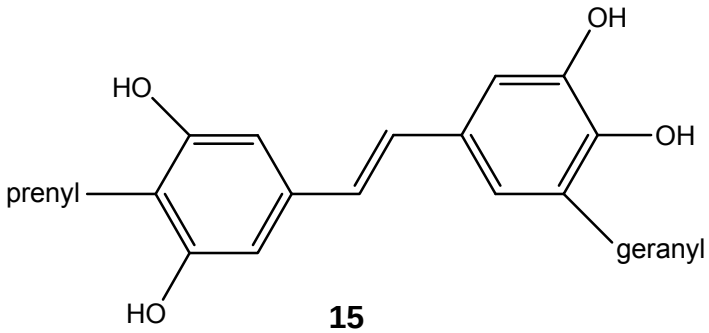


13 R = prenyl



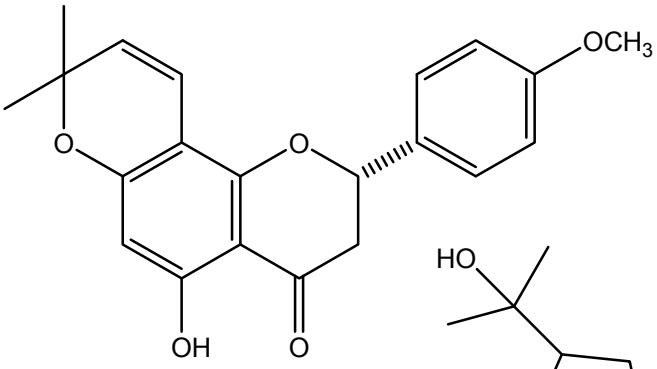
14 R_1 = geranyl
R_2 = prenyl

A cytotoxic prenylated stilbene, mappain, **15**, has been isolated from the leaves of *Macaranga mappa* (Van der Kaaden *et al*., 2001).



15

From leaves of *Macaranga conifera*, two prenylated flavonoid derivatives, 5-hydroxy-4'-methoxy-2",2"-dimethylpyrano-(7,8:6",5")flavanone, **16**, and 5,4'-dihydroxy-[2"-(1-hydroxy-1-methylethyl)dihydrofurano]-(7,8:5",4")flavanone, **17**, together with 5,7-dihydroxy-4'-methoxy-8-(3-methylbut-2-enyl)flavanone, **18**, lonchocarpol A, **19**, sophoraflavanone B, **20**, 5,7-dihydroxy-4'-methoxy-8-(2-hydroxy-3-methylbut-3-enyl)flavanone, **21**, tomentosanol D, **22**, lupinifolinol, **23**, and isolicoflavonol, **24**, have been isolated (Jang *et al*., 2002).



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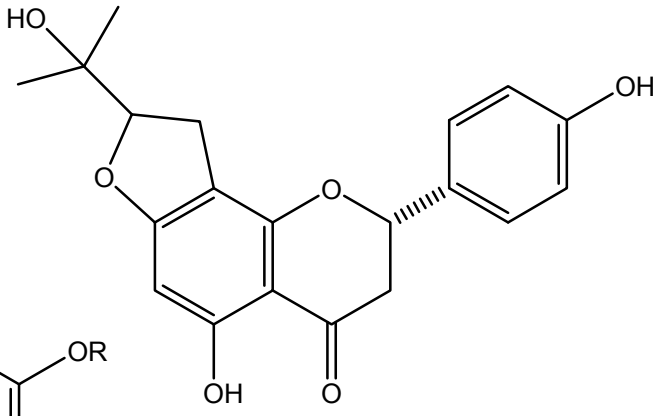

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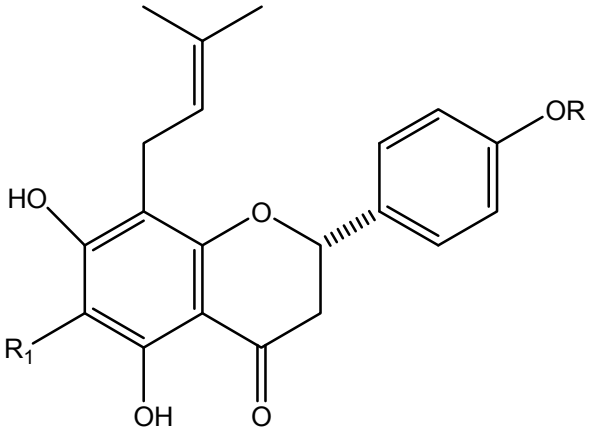
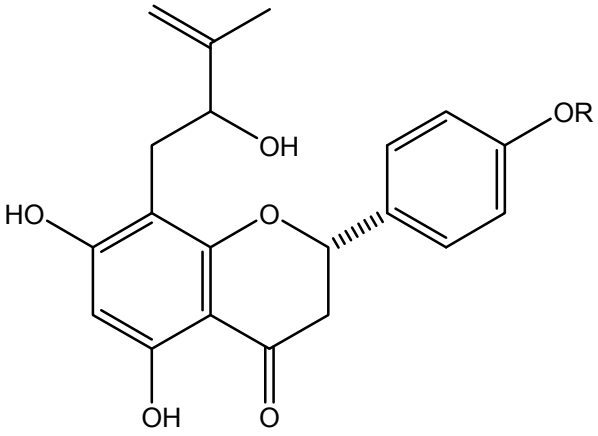
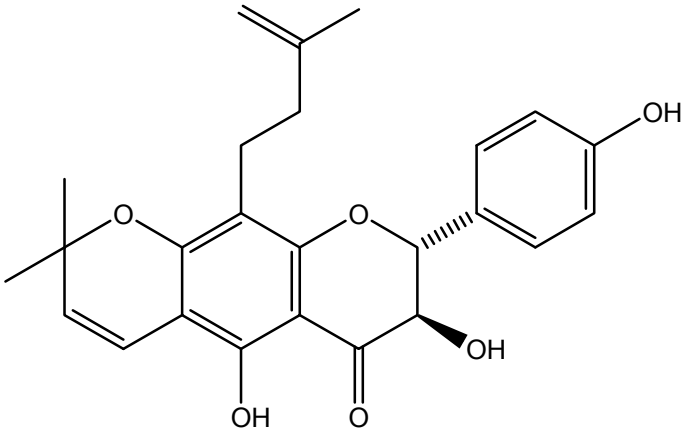


18 R= CH_3, R_1= H
19 R= H, R_1= prenyl
20 R, R_1= H



21 R = CH_3
22 R = H



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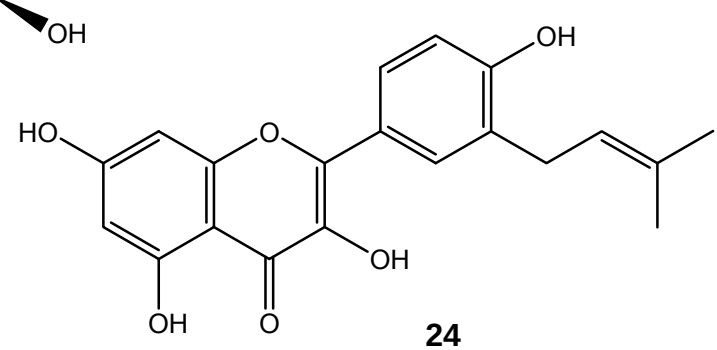
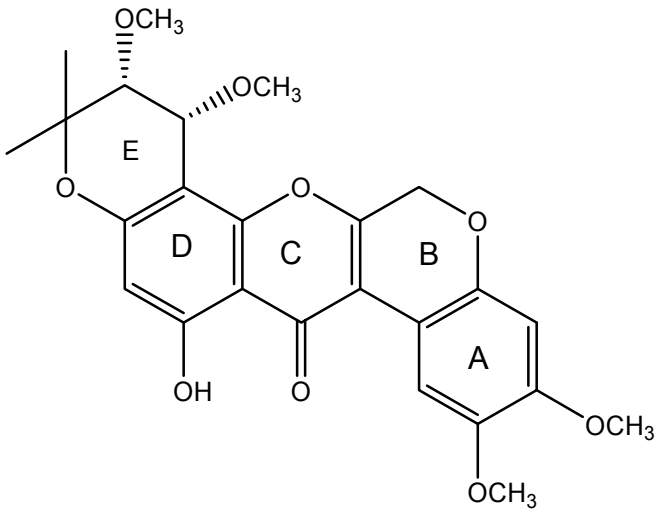


24

From leaves of *Macaranga triloba* the flavonoid 4, 5-dihydro-5'α-hydroxy-4'α-methoxy-6a, 12a-dehydro-α-toxicarol, **25**, was isolated (Jang *et al*., 2004).



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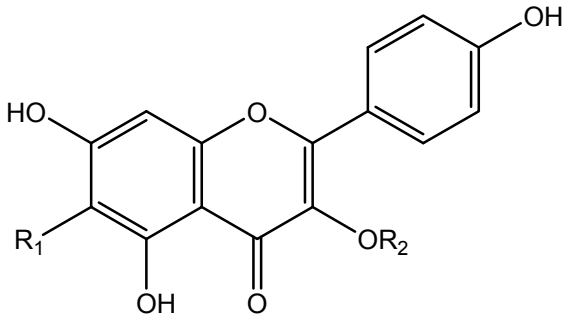
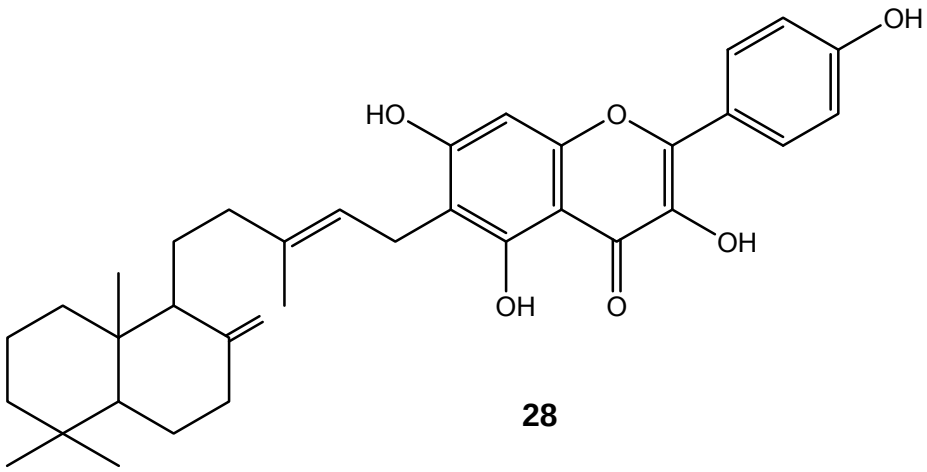
The leaves of *M. triloba* are used to treat stomachache and skin itches in Indonesia and Malaysia (Ahmed and Holdsworth, 1994; Grosvenor *et al*., 1995)

From leaves of *M. denticulata* the diterpenylated and prenylated flavonoids, 3-*O*-methyl-macarangin, **26**, macarangin, **27**, denticulaflavonol, **28**, and sophoraflavanone B, **29**, were isolated (Sutthivaiyakit *et al.*, 2002).



26 R_1 = geranyl, R_2 = Me
27 R_1 = geranyl, R_2 = H



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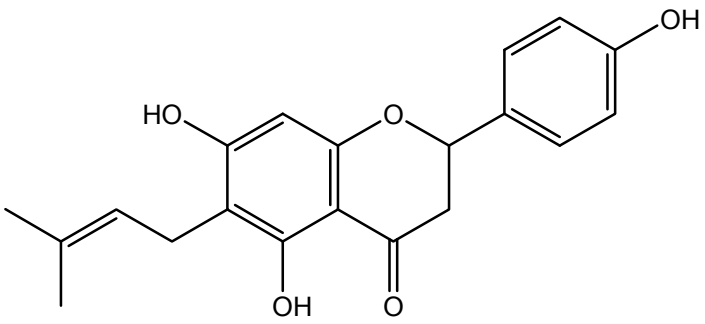


29

Analysis of the chemical composition of *M. gigantea* leaves showed that they contain some volatile compounds, fatty acid derivatives, benzenoids, monoterpenoids and, sesquiterpenoids (Jurgens *et al*., 2006). From the stems of *M. tanarius* were isolated the diterpene ketol, macarangonol (Hui *et al*., 1971), and terpenoids and steroids (Hui *et al.,* 1975). Macaranins, macarinins and hydrolysable tannins were isolated from leaves of *M. sinensis* (Lin *et al*., 1990). From the heartwood of *M. peltata* were isolated bergenin and *O*-methyl ethers of bergenin and the gum-powders are used in Indian medicine for treatment of venereal disease (Ramaiah *et al*., 1979).

Another species, *M*. *kilimandscharica* Pax (leaves and stems), are traditionally used for afterpains (post-partum cramps) (Cos *et al*., 2002). The decoction of *M. griffithiana* and *M*. *hullettii* root is taken orally for fever and stomach discomfort, respectively (Burkill, 1935). *M. hypoleuca* Muell.-Arg.

decoction is reportedly used as febrifuge, expectorant, and anti-spasmodic (Burkill, 1935).

Another genus, *M. populifolia* Muell.-Arg. is administered after childbirth (Burkill, 1935). The decoction of *M. gigantea* root-bark is taken by the Malay community for diarrhea and dysentery (Burkill, 1935).

The leaves, bark and fruits of *M. gigantea* are astringent, containing a high portion of tannins and are much used in traditional medicine to treat dysentery and diarrhoea (Perry, 1980; Mat-Salleh, K, 1997). As there was no previous phytochemical report of *M. gigantea* except one on the leaf volatiles (Jurgens *et al*., 2006), the present study was carried out to isolate constituents from the fruit and to assay these for biological activity.

1.2 Plant morphology

Many of these are small trees, common in secondary jungle. A few occur in the shade of high forest, but the majority, needing a large amount of sunlight, cannot tolerate the gloom. Like other thicket-plants, they must have lived a precarious existence at the edge of rivers, by the coast and on landslips. But, with the opening of the country, the *Macarangas* have spread forth and became one of our notable kinds of wayside trees. *Macarangas* are remarkably alike in general feature, but their leaves differ so much in shape and attachment to the stalk that nearly every kind can be recognized at first glance. They are quick growing, soft-wooded, evergreen trees reaching a height of 20 m. Their crowns are open and uneven or, if well-developed, rather compact and rounded and made up of several large limbs; but, though the leaves are large, they cast little shade on account of their lift. The bark is pale grey or pinkish, smooth or rough

with lenticels, always lined transversely with the leaf-scars, pink to reddish brown internally, tough and easily stripping and it is generally astringent from the tannin-bodies that it contains. On account of the astringent bark and gum, the species find various uses in native medicine. In several kinds the twigs and undersides of the leaves are glaucous that is, covered with a thin layer of bluish-white wax. Many have little stalked glands along the edges of the leaf, very conspicuous as purple blobs in young leaves but shrivelling in to tiny teeth or points on the mature leaves. A few have a pair of flat glands at the blade. On the fruits and on the undersides of the leaves there may be hundreds of tiny dot-like yellow, brown or black glands, for which reason the fruits of some species look as if they have been powdered with golden dust. Peltate leaves are characteristic of several kinds. The leaves of saplings are often purple beneath, and they are generally larger and more deeply lobed or toothed than in the adult plants. It seems, too, that in some the blade of the sapling is peltate or lobed whereas that of the adult is not. The young leaves of mature plants are purple, in some cases shading pink or brown, often richly purple beneath, the old leaves become intensely yellow (Corner,1952). *Macaranga gigantea* Muell.-Arg. (local name : Mahang) is a tree 12 m tall with pubescent branches. Leaves are very large. Indeed, this *Macaranga* has bigger leaves than other Malaysian trees. Those of saplings may be a metre across, as big as a shield, with stalks 1-0.5 m long. Fruits are 8 cm wide, two-shoulder, and occur in bunches (see Plates1-3) (Ridley, 1967).

Plate 1.1 Fruits of *Macaranga gigantea*

Plate 1.2 Tree of *Macaranga gigantea*

Plate 1.3 Leaves and fruits of *Macaranga gigantea*

1. 3 Flavonoids

Flavonoids are polyphenolic compounds isolated from a wide variety of plants, with over 4000 individual compounds known. Apart from catechins and proanthocyanidins, they consist mainly of glycosides of flavonols, flavones, flavanones, isoflavanones, and anthocyanidins. Individual differences arise in the various hydroxylation, methoxylation, glycosylation, and acylation pattern. A single plant may contain different flavonoids, and their distribution within a plant family is useful for classifying that family. Flavonoids play different roles in the ecology of plants. Because of their attractive colours, flavonols, flavones, and anthocyanidins are likely to be a visual signal for pollinating insects. Catechins and other flavonols possess astringent characteristic and they act as feeding repellants, while isoflavones are important protective phytoalexins. Owing to their presence in edible plants as well as foods and beverages derived from plants, flavonoids are important constituents of the nonenergetic part of the human diet. Also, a dozen flavonoid-containing species are known and have long been used in traditional medicine. During the past two decades, an increased effort in pharmacognosy has led to the validation of a number of these phytomedicines for the long-term treatment of mild and chronic diseases or to attain and maintain a condition of well-being. Among the numerous substances identified in medicinal plants, flavonoids represent one of the most interesting groups of biologically active compounds. Approximately 40 species are reported to have been used as phytomedicines because of their flavonoid content, and the list is growing very rapidly. Flavonoid preparations have long been used in medical practice to treat disorder of peripheral circulation, to lower blood pressure. Numerous phytomedicines containing flavonoids are marketed

in different countries as anti-inflammatory, anti-spasmodic, anti-allergic, and anti-viral remedies. Many of the alleged effects of pharmacological dose of flavonoids are linked to their known functions as strong antioxidants, free-radical scavengers, and metal chelators and their interaction with enzymes, adenosine receptors, and biomembranes (Rice-Evans & Packer, 1998). The flavonoids are colouring substances contributing to the beauty and splendour of flowers and fruits. The occurrence of this large class of oxygen heterocycles is restricted to higher plants and ferns. Mosses contain a few flavonoid types but they are absent in algae, fungi and bacteria. Biologically, flavonoids play a major role in relation to insects pollinating or feeding on plants, but some flavonoids have a bitter taste. The flavonoids are structurally characterized as having two hydroxylated aromatic rings, A and B, joined by a three carbon fragment. One hydroxyl group is often linked to a sugar (Torssell, 1997). Some flavonoids have subtituents (geranyl and prenyl) in the A- and B- ring (Yakushijin *et al*., 1980). In general, flavonoids are divided into six classes, namely, flavones, flavonols, isoflavones, flavanones, dihydroflavonols, aurones and chalcones (Fig.1.1) (Mabry *et al*., 1970).

Flavonoids have functions in nature such as serving as ultraviolet filters. Flavonoids are required for the germination of pollen grains and for successful pollen tube growth, as messengers, oviposition stimulants, defensive agents against insect and fungal attack, and as allelopathic agents and phytoalexins.

A wide variety of secondary plant products, including flavonoids, have been identified as defensive agents that protect plants against attack by a number of other organisms. Flavonoids also are used by humans, as antiviral agents, being found to be active against a variety of animal and plant viruses.

Many flavonoids have also been found to have antibacterial and antifungal properties. There are reports in the literature regarding the use of some flavonoids as antioxidants in food (Bohm, 1998).

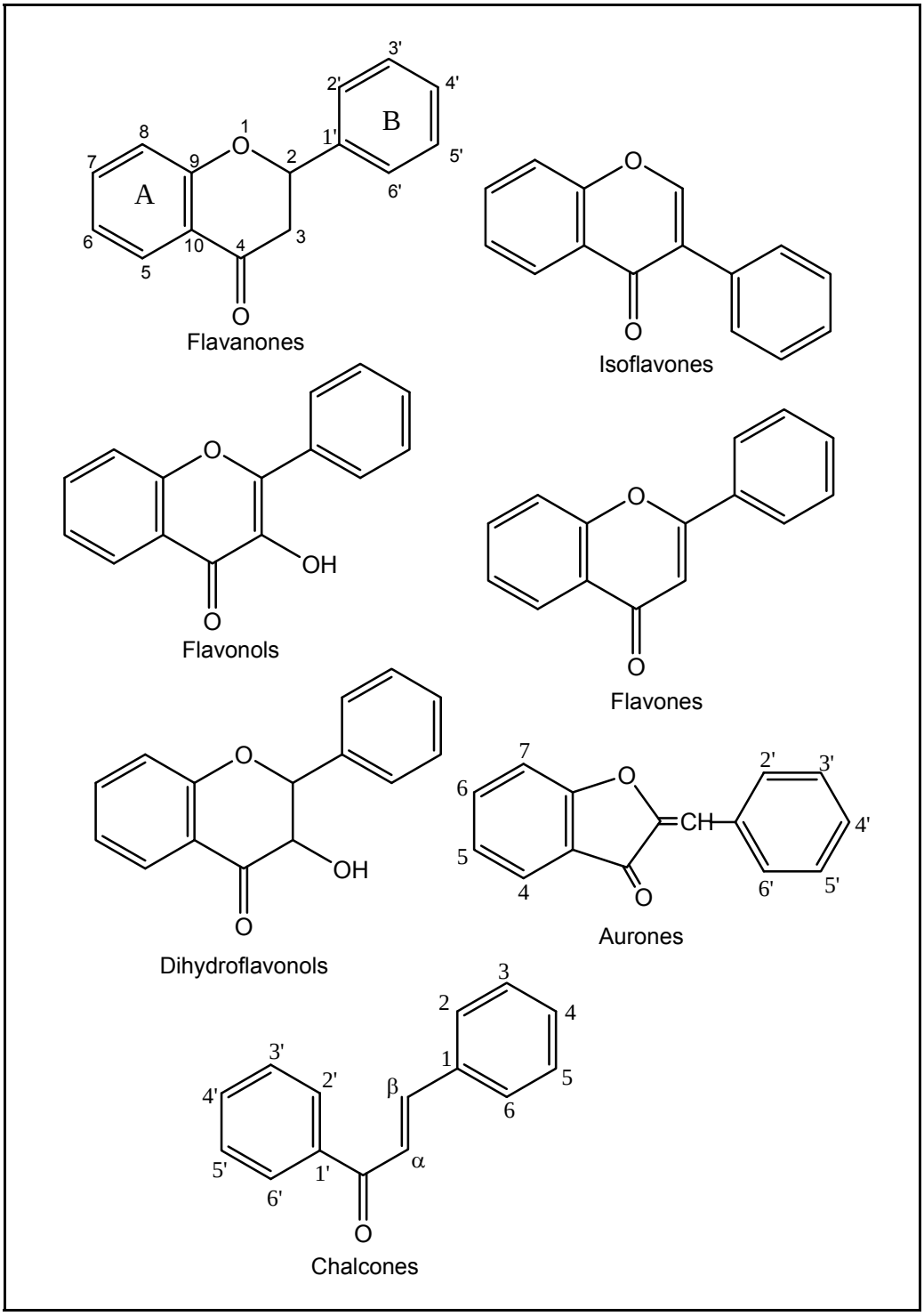
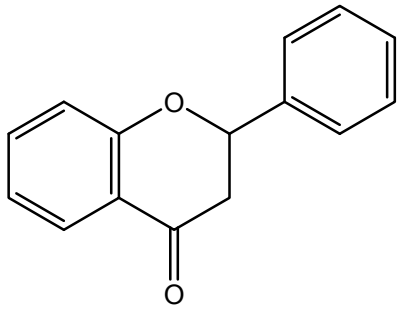


Fig.1.1 Structures and numbering of flavonoids.

1.4 Flavanones

Flavanones are based upon the structure 2-phenylbenzopyran-4-one, **30**, which is flavanone itself.



30

The parent compound is not known to be naturally occurring. The numbering system of the flavanone nucleus is similar to that in most other flavonoid series. Flavanones are isomeric with chalcones from which they can be obtained synthetically and from which they arise biosynthetically. Flavanones have a centre of asymmetry at C-2 so that naturally-occurring members are often optically active. The absolute configuration of a number of these compounds has also been established. It is of historical interest that the isolation of optically active flavanones provided a strong argument that these compounds are natural. Flavanones are interesting compounds since they are obligate intermediate in flavonoid biosynthesis. Flavanones can be dehydrogenated to yield flavones or can under go hydroxylation at position 3 to yield dihydroflavonols (3-hydroxyflavanones) (Harborne *et al*., 1975). Chemically, these compounds display a number of structural features which are interesting and /or are unique amongst flavonoids. The commercial significance of flavanones has been discussed by a number of authors (Herget, 1962).

Flavanones have fungistatic or toxic properties and are important as factors in wood preservation (Herget, 1962). The stereochemistry of flavanones has been reviewed in the literature (Mahesh & Seshadri, 1955; Whalley, 1956; Whalley, 1962; Clark-Lewis, 1962). Literature reported that naturally occurring flavanones were laevorotatory and thus all belonged to the same configuration series (Erdtman, 1956). The most current stereochemical assignment is based upon optical rotary dispersion data and comparison of the resultant curve to that of a flavanone whose absolute configuration is known. The significant ^{1}H-NMR spectrum of compounds are protons at C-2 and C-3. The proton at C-2 appears as a double doublet (J_{trans} = 11 Hz, J_{cis} = 5 Hz) centered at 5.2 ppm. The protons at C-3 each gives rise to double doublets due to spin-spin interaction with each other and with H-2 (J = 17 Hz, 2.7 HZ). The peaks centered at about 2.8 ppm (Harborne *et al*., 1975). Flavanones lack conjugation between the A- and B-rings. UV spectra of flavanones exhibit a low intensity band I absorption which often appears as shoulder to the band II peak. The spectra of these compounds are largely unaffected by changes in the oxygenation and substitution patterns in the B-ring. However, increased oxygenation in the A-ring leads to a bathochromic shift in the band II absorption. Flavanones exhibit its absorption maxima (band II) in the region 270-295 nm (Harborne *et al*., 1975). ^{13}C-NMR spectra of flavanones reported in the literature show that the chemical shift of C-2 is at about 78.4 and C-3 at about 42.3 ppm (Agrawal & Rastogi, 1981).

In the mass spectra of flavanones the molecular ions typically fragmentize by a retro-Diels-Alder (RDA) reaction (pathway-I) to yield ions which correspond to the same $A_1^{+\cdot}$ and $[A_1 + H]^{+}$ ions observed for flavones

(Fig.1.2). However, the most important B-ring ion (designated as B_3 $+\cdot$) from pathway -I contains an ethylene group. This ion is present together with other B-ring fragments even when the B-ring is in the quinonoid form. The intensities of the A- and B -ring fragments from flavanones depend upon the substitution patterns of the two rings (Harborne *et al*., 1975).

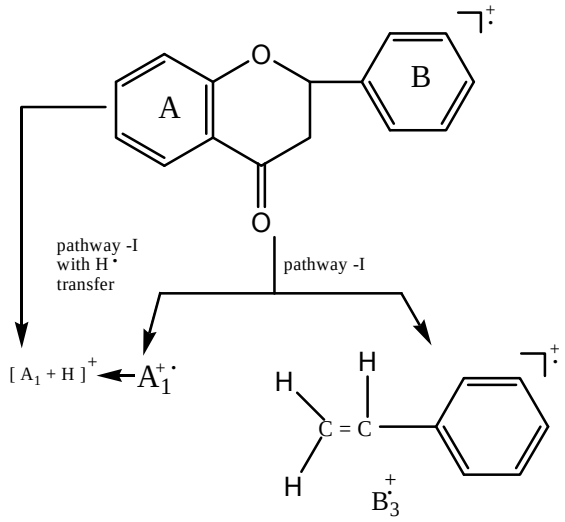


Fig. 1. 2 Diagnostic mass spectral fragmentation pathways for flavanones

1.5 Objectives of the present study

The objectives of the present work were:

1. To isolate and to determine the structures of the chemical constituents isolated from the fruit of *M. gigantea* grown in Penang, Malaysia.
2. To assay the cytotoxic activity of the crude extract and isolated compounds on Hep G2 and T- 47D cell lines.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Collection of plant material

Macaranga gigantea fruits were collected from the campus of Universiti Sains Malaysia, Pulau Pinang, Malaysia, in January 2003. A voucher specimen (No.10833) was deposited at the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia.

2.2 Materials and methods for cytotoxic activity study

2.2.1 Materials

The materials used were purchased from the suppliers as shown in Table 2.1

Table 2.1 Materials used and their suppliers

Materials	Suppliers or manufacturers
RPMI 1640 tissue culture medium, MEM/EBSS tissue culture medium, fetal calf serum, L-glutamine, penicillin-streptomycin solution, sodium pyruvate, non-essential amino acids	Hyclone, USA
Trypan blue, doxorubicin chloride, bovine insuline	Sigma Aldrich, USA
Hep G2 cell line and T-47D cell line	American Type Culture Collection, USA
Dimethyl sulfoxide (DMSO) and PBS tablets	Amresco, USA
CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay	Promega, USA
Sterile disposable syringes 20ml/50ml	Becton-Dickson, Singapore
Avon Kwill filling tubes	SIMS Industries and Medical System, UK
Sterile centrifuge tubes (15ml and 50ml)	Corning, USA

2.2.2 Preparation of ceramics, glasswares and plasticwares

All ceramics, glasswares and plasticwares (flasks, pipette tips, microcentrifuge tubes *etc*.) were autoclaved for 30 minutes at 121°C and a pressure of 97.5 kPa.

2.2.3 Preparation of stock solutions of crude extracts and isolated flavanones

Stock solutions of the pure compounds and crude methanol extract were diluted serially with DMSO in four different working concentrations. Doxorubicin HCl, which was used as a positive control, was prepared in eight different concentrations (µg/ml in H_2O).

2.2.4 Cell culture

All cell lines were obtained from American Type Culture Collection (ATCC, USA). The Hep G2 cell lines were derived from the hepatocellular carcinoma cells. The T-47D cell line was derived from the human breast carcinoma cells.

2.2. 4.1 Thawing cells

The ampoules containing the cryopreserved cells were removed from the liquid nitrogen container and placed in 37°C water bath immediately until the content thawed. The ampoules were then cleaned with 70% (v/v) ethanol in the Biohazard Safety Cabinet. The content was then transferred into a centrifuge tube using a syringe containing 10 ml complete medium and centrifuged at 1000 rpm (Kubota 2010, Japan). The pellet was then resuspended in an appropriate volume of complete medium and then transferred into the T25 flask.

The cells were subsequently incubated in the incubator supplemented with 5 % (v/v) CO_2 at 37°C. The medium was then changed after overnight.

2.2.4.2 Maintenance of cells in culture

Hep G2 cells were maintained in MEM/EBSS and T-47D cells were maintained in RPMI 1640, as recommended by ATCC. All the media used to culture cells were supplemented with 10 % (v/v) fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin solution. Additional supplements added, used to maintain a particular cell type, is shown in Table 2.2.

Table 2.2 Cell lines and growth medium used in this study

Cell lines	ATCC No.	Tissue	Organism	Complete growth medium requirement
T-47D	HTB-133	Ductal carcinoma, mammary gland	Homo sapiens (Human)	RPMI 1640, 10% (v/v) FCS, 0.01mg/ml bovine insulin, 1mM sodium pyruvate
Hep G2	HB-8065	Hepatocellular carcinoma	Homo sapiens (Human)	10 mM HEPES, 2 mM L-glutamine, 100 U/ml and 100 mg/ml Penicillin-streptomycin solution, 0.1m M non-essential amino acid

2.2.4.3 Subculturing of cells

Confluent cells were briefly rinsed with PBS to remove all traces of serum which contained trypsin inhibitors and subsequently, an approximately 2.0 ml of 0.05 % (w/v) trypsin EDTA solution was added to the T25 flask and the cells were observed under an inverted phase contrast microscope (Leitz Wetzler, Germany) until cells were detached from the surface of the flask

(usually within 5 to 15 minutes). The cells were then transferred to a centrifuge tube and centrifuged at 1000 rpm (Kubota 2010, Japan) for 5 minutes. Following centrifugation, the pellet was resuspended in medium containing 10 % (v/v) FCS and cells were then plated into fresh tissue culture flask at ratio recommended by ATCC and depending on cell usage.

2.2.4.4 Treatment of cells for cytotoxicity assay

Near confluent cultures of cell were harvested with 0.05 % (w/v) trypsin-EDTA. The cells were then centrifuged, pellet resuspended with complete medium with 10 % (v/v) FCS and plated onto 96 well plates (Costar, USA) at cell density of approximately 6000 cells/well. Cell viability was routinely determined using trypan blue exclusion (see 2.2.4.5) to make sure cell viability was always in excess of 95%. The cells were then allowed to attach and incubated at 37 $^{\circ}$C in the CO_2 incubator for a further 24-48 h. When the cells reached 80-90 % confluence, the medium was removed and replaced with medium containing only 0.5 % (v/v) FCS. The cells were then incubated for a further 4 h. The reason for this was for the cells to achieve quiescent. The cells were then treated with different concentrations of the extract and isolated pure compounds (see 2.2.3). Control cells were cultured in 0.5 % (v/v) FCS - containing medium alone. The crude extract and pure isolated compounds were dissolved and diluted in 99.9 % (v/v) DMSO and the final concentration of DMSO in every test well was not more than 1 % (v/v). The cells were then incubated for 72 h. Doxorubicin HCl was used as a positive control. Cytotoxicity assays were carried out using MTS assay (see 2.2.4.6)

2.2.4.5 Trypan blue exclusion test

Trypan blue exclusion test is carried out routinely to determine the viability of subcultured cells. Cell suspension used to assess cell viability was adjusted to between 10^5-10^7 cells/ml. A sample of the suspension (10 µl) was mixed with an equal volume of 0.4 % (v/v) trypan blue and left for about 5 min at 37 °C. The sample was then placed on the haemocytometer and viewed under an inverted phase contrast microscope. The percentage of dead cells was estimated from the number of stained cells.

2.2.4.6 MTS tetrazolium assay

MTS assay was carried out using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, USA) as described by the manufacturer. The assay is composed of solutions of a novel tetrazolium compound known as 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) and an electron coupling reagent phenazin methosulphate (PMS). In metabolically active cells, MTS will be converted into aqueous soluble formazan by dehydrogenase enzyme in the mitochondria (Fig. 2.1). The absorbance of the formazan at 490 nm could be measured directly. The quantity of formazan product as measured by the amount of absorbance was directly proportional the number of living cells in culture. Briefly, after 72 h incubation (see 2.2.4.4), 20 µl of the combination solution of MTS and PMS was pipetted into each test and control well. The plates were then incubated for 1 - 4 h at 37 °C in a humidified 5 % (v/v) CO_2 incubator. Subsequently, absorbance was read at 490 nm using V_{max} Kinetic Micro Plate Reader (Molecular Devices, USA).

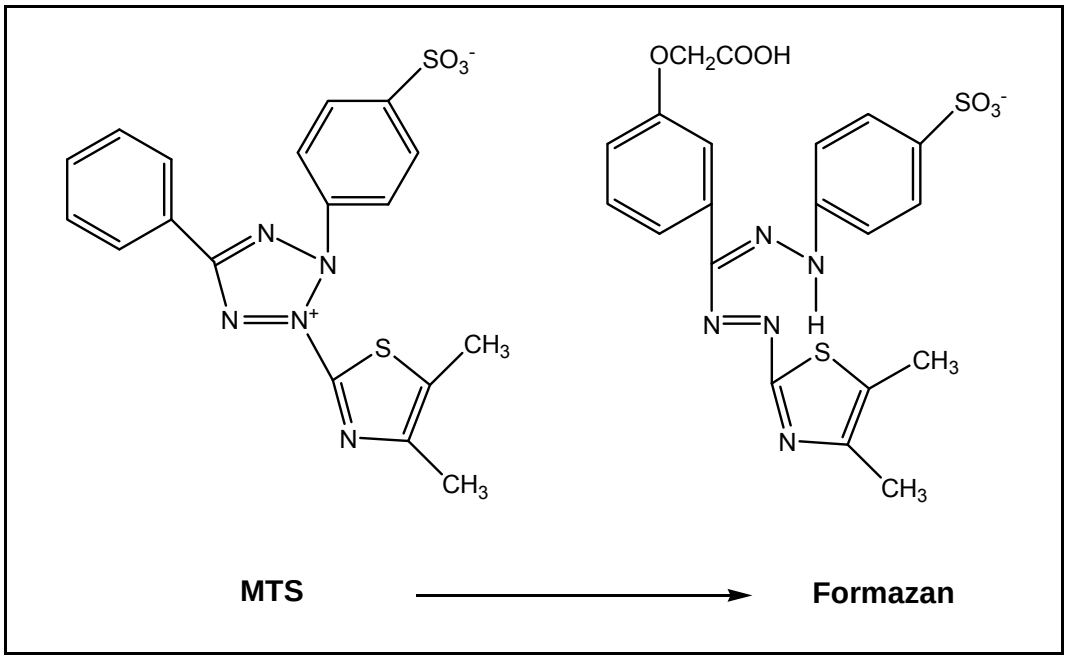


Fig. 2.1 Structure of MTS tetrazolium and its formazan product

2.3 Determination of cytotoxic activities of methanol extract and isolated flavanones from fruit of *Macaranga gigantea*

2.3.1 Experimental design

The extract and pure compounds used this study are shown in Table 2.3. Two cell lines were used for the cytotoxic study using MTS assay (Section 2.4.4.6).The cell lines used were Hep G2 and T- 47D, each representing known cells originating from human hepatocarcinoma and breast carcinoma cells, respectively. The cells lines and growth medium used are listed in Table 2.2. The cells were prepared prior to treatment with fruit extract and pure isolated compounds as described (Section 2.2.4.4). Cytotoxicity assays were carried out after 72 h of incubation.