STUDIES ON STANDARDIZATION OF *FICUS DELTOIDEA* JACK AND *ORTHOSIPHON STAMINEUS* BENTH. LEAF EXTRACTS FOR SELECTED ANTI-HYPERTENSIVE AND ANTI-ANGIOGENIC EFFECTS

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LIST OF ABBREVIATION

AAS	Atomic Absorption Spectroscopy
ACE	Angiotensin Converting Enzyme
$AlCl_3$	Aluminum chloride
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of Variance
As	Arsenic
AST	Aspartate aminotransferase
AT-I	Angiotensin I
AT-II	Angiotensin II
ATR	Attenuated Total Reflection
ATRs	Angiotensin Receptors
bFGF	Basic Fibroblast Growth Factor
BSA	Bovin Serum Albumin
BSTFA	N,*O*-Bis (trimethylsilyl)trifluoroacetamide
CAM	Chick chorioallantoic membrane
Cd	Cadmium
CET	Cetrimide
CO_2	Carbon dioxide
DBP	Diastolic Blood Pressure
DMEM	Dulbecco's Modified Eagle Medium
DMF	N,N-Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1- picrylhydrazil
ECGS	Endothelial Cell Growth Supplement

ECM	Endothelial Cell Medium
ELISA	Enzyme Linked Immunosorbent Assay
EUP	Eupatorin
eV	Electron Volt
FRAP	Ferric Reducing Antioxidant Power
FT-IR	Fourier Transform Infrared
FT-NIR	Fourier Transform Near-Infrared
G	Gram
GC-MS	Gas Chromatography Mass Spectrometry
HA	Hippuric acid
HCT 116	Human colon carcinoma cell line
HDL	High Density Lipoprotein
Hg	Mercury
HHL	Hippuryl-histidyl-leucine
HIF	Hypoxia-inducible factors
HI FBS	Heat Inactivated Foetal Bovine Serum
HL	Histidyl-leucine
HMP	Herbal Medicinal Products
HNO_3	Nitric acid
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
HUVEC	Human Umbilical Vein Endothelial Cell line
IL-8	Interleukin-8
LOD	Limit of Detection
LOQ	Limit of Quantification
LPS	Lipopolysaccharide
M	Molar
MCF7	Hormone-dependent breast carcinoma cell line

MDA-MB-231	Human hormone resistant breast cancer cell line
Mg	Milligram
MIC	Minimum Inhibitory Concentration
Min	Minutes
MLT	Microbial Limit Test
Mm	Millimeter
mM	Milimolar
MRC	Methylripariochromene
mRNA	Messenger ribonucleic acid
MSA	Mannitol Salt Agar
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
mU	Mili Unit
mV	Milli Volt
N	Normal
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
Ng	Nanogram
Nm	Nanometer
NO	Nitric oxide
OPA	*O*-Phthaladehyde
Pb	Lead
PBS	Phosphate Buffered Saline
Ppm	Part Per Million
PS	Penicillin/Streptomycin
RA	Rosmarinic acid
RAAS	Rennin-Angiotensin-Aldosterone-System
Rpm	Round Per Minutes
RSD	Relative Standard Deviation

SBP	Systolic Blood Pressure
SCD	Soybean-Casein Digest agar
SD	Standard Deviation
SDA	Sabouraud Dextrose Agar
SEN	Sinensetin
SGOT	Glutamate Oxaloacetate Transaminase
SGPT	Glutamate Pyruvic Transaminase
SHRSP	Subcutaneous administration in conscious stroke-prone
TAA	Thioacetamide
TLC	Thin Layer Chromatography
TMF	3'-Hydroxy-5,6,7,4'-tetramethoxyflavone
TMSC	Trimethylchlorosilane
TNF-a	Tumor Necrosis Factor Alpha
TPA	12-*O*-Tetradecanoylphorbol-13-acetate
UV/Vis	Ultra Violet and Visible
VEGF	Vascular Endothelial Growth Factor
XLD	Xylose Lysine Deoxycholate agar
µL	Microlitre
µm	Micrometre

Kajian Pemiawaian Ekstrak daun *Ficus deltoidea* Jack dan *Orthosiphon stamineus* Benth. untuk Kesan Anti-hipertensi dan Anti-angiogenik yang Terpilih

ABSTRAK

Di Malaysia, *Ficus deltoidea* (FD) dan *Orthosiphon stamineus* (OS) telah digunakan untuk mengubati pelbagai jenis penyakit berkaitan kencing manis, tekanan darah tinggi keradangan angiogenesis. Oleh itu, kajian ini bertujuan menjana data dan bukti mengenai pempiawaian dua jenis tumbuhan ubatan yang telah dipilih iaitu FD dan *Orthosiphon stamineus* OS.

Kajian ini bertujuan menjana data dan bukti mengenai tekanan darah tinggi dan angiogenesis memandangan kekurangan maklumat yang berkaitan., dua jenis tumbuhan ubatan telah dipilih iaitu *Ficus deltoidea* var. *deltoidae* (FD) dan *Orthosiphon stamineus* (OS).

Dalam kajian ini, analisis gravimetrik dan ujian had mikrob (MLT) telah dijalankan ke atas serbuk daun kering untuk pemeriksaan kualiti dan keselamatan bahan mentah. Kedua-dua daun FD dan OS didapati memenuhi syarat kualiti dari segi sifat fizikokimia dan pencemaran mikrob. Penyaringan fitokimia awal ekstrak metanol dan ekstrak air daun FD dan OS menunjukkan kehadiran sebatian fenolik, asid amino, flavonoid, terpenoid dan saponin. Ekstrak OS juga menunjukkan kehadiran sterol.

Profil kimia yang melibatkan analisis kualitatif dengan teknik spektroskopi (UV, IR, NIR) dan kromatografi (HPTLC dan GC-MS) dan analisis kuantitatif

dengan teknik kromatografi (HPTLC dan HPLC) telah dijalankan untuk pemiawaian. Viteksin, isoviteksin, L-sitrulina, L-arginina and asid ursolik telah digunakan sebagai penanda kimia untuk ujian analitikal FD manakala asid rosmarinik, 3-hidroksi-5,6,7,4-metoksiflavon, sinensetin, eupatorin, asid oleanolik dan asid ursolik telah dipilih sebagai penanda kimia untuk OS berdasarkan nilai perubatan dan tahap. Julat kepekatan flavonoid dalan ekstrak FD ialah antara 5.67 - 18.76 mg/g untuk viteksin dan 1.06 - 9.50 mg/g untuk isoviteksin. Kepekatan untuk L-sitrulina and L-arginina dalam ekstrak FD ialah antara 7.45 - 8.53 mg/g untuk L-sitrulina dan 5.36 – 8.44 mg/g untuk L-arginina. Paras asid ursolik dalam ekstrak FD antara 2.46 – 4.27 mg/g. Asid rosmarinik, konstituen utama ekstrak air OS ialah antara 56.77 mg/g dalam ekstrak metanol dan 15.92 mg/g dalam ekstrak air. Kepekatan flavon dalam ekstrak OS ialah antara 5.61 – 33.16 mg/g untuk sinensetin, 0.35 – 10.48 mg/g untuk 3'-hidroksi-5,6,7,4'-tetrametoksiflavon dan 7.73 – 177.91 mg/g untuk eupatorin. Paras asid oleanolik dan asid ursolik dalam ekstrak OS adalah di dalam julat 8.70 – 13.22 mg/g dan 2.16 – 33.43 mg/g masing-masing. Metabolit primer dan sekunder untuk ekstrak FD dan OS telah diuji untuk kandungan jumlah flavonoid, polifenolik, tannin, protein, polisakarida dan glikosaponin. Ekstrak metanol OS mengandungi kandungan flavonoid (336.58 mg/g) tertinggi manakala ekstrak air tumbuhan ini mengandungi jumlah polifenolik (157.00 mg/g) dan polisakarida (10.12%) tertinggi. Ekstrak metanol FD menunjukkan kandungan tannin (58.10 mg/g) tertinggi.

Kesan *in vitro* anti-hipertensi ekstrak metanol dan air FD dan OS telah disaring menggunakan assai ACE-I berasaskan enzim dan kuantifikasi kepekatan ACE dalam laisat sel-sel endotelium vaskular pusat (HUVEC) selepas dirawat dengan ekstrak. Ekstrak FD dan OS menunjukkan aktiviti ACE-I yang ketara dalam kedua-dua assai. Dalam assai enzim, ekstrak metanol FD menunjukkan perencatan yang tertinggi

sebanyak 96.00% dan nilai IC_{50} yang terendah (19.15 µg/mL) dan kedua-dua ekstrak metanol FD dan OS menunjukkan perencatan yang tinggi ke atas ACE dalam laisat HUVEC (73.51% dan 73.46% perencatan). Kesan anti-angiogenik ekstrak metanol dan air untuk FD dan OS telah disiasat menggunakan assai *in vivo* membran korioalantoik embrio ayam. Keputusan menunjukkan semua ekstrak mempunyai aktiviti anti-angiogenik yang ketara; bagaimanapun ekstrak metanol FD telah memesongkan rangkaian vaskulatur tertinggi pada kepekatan 100 µg/mL. Kesan sitotoksik ekstrak metanol dan ekstrak air FD dan OS telah dinilai menggunakan assai MTT terhadap dua sel kanser manusia iaitu karsinoma kolorektum (HCT 116) dan kanser payudara sensitif hormon (MCF 7). Di samping itu, ekstrak juga diuji ke atas sel normal manusia (HUVECs). Kedua-dua ekstrak FD dan OS menunjukkan activiti sitotoksik yang sederhana terhadap sel-sel (IC_{50} > 20 µg/mL) dan kesan sitotoksik yang lemah ke atas sel HUVECs, yang mencadangkan bahawa potensi anti-kanser daun FD dan OS mungkin bukan dari kesan sitotoksiknya tetapi mungkin disebabkan oleh perencatan angiogenesis.

Kesimpulan, kajian ini membuktikan aktiviti anti-hipertensi dan anti-angiogenik, kualiti dan keselamatan ekstrak terpiawai FD dan OS. Kaedah HPTLC dan HPLC dari pada kajian ini boleh digunakan untuk pemiawaian dan analisis lazim kedua-dua bahan herba FD dan OS.

Studies on Standardization of *Ficus deltoidea* Jack and *Orthosiphon stamineus* Benth. Leaf Extracts for Selected Anti-hypertensive and Anti-angiogenic Effects

ABSTRACT

Ficus deltoidea (FD) and *Orthosiphon stamineus* (OS) were used traditionally for treatment of a wide range of diseases related to diabetes, hypertension, inflammation and angiogenesis related diseases in Malaysia. Therefore, this study was aimed to generate data and evidence about standardization of two medicinal plants namely *Ficus deltoidea* (FD) and *Orthosiphon stamineus* (OS) for anti-hypertensive and anti-angiogenesic effects, since there is lack of sufficient scientific data in this regard.

In this study, gravimetric analysis and microbial limit test (MLT) were applied on the dried powder of the leaves to examine the quality and safety of the raw material. Both FD and OS leaves were found to be qualified in terms of physicochemical properties as well as microbial contamination. Preliminary phytochemical screening on methanol and water extracts of FD and OS leaves showed the presence of phenolics, amino acids, flavonoids, terpenoids and saponins in FD and OS extracts. OS extracts also showed the presence of sterols.

The chemical profile involving qualitative analysis by spectroscopic (UV, IR, NIR) and chromatographic techniques (HPTLC and GC-MS) and quantitative analysis by chromatographic techniques (HPTLC and HPLC) were carried out for standardization. Vitexin, isovitexin, L-citrulline, L-arginine and ursolic acid were used as markers for the analytical study of FD while, rosmarininc acid, 3-hydroxy-5,6,7,4-methoxyflavone, sinensetin, eupatorin, oleanolic acid and ursolic acid were

selected as markers for OS based on their medicinal values and their quantifiable levels. The concentration range of flavonoids varied in FD extracts from 5.67 - 18.76 mg/g for vitexin and 1.06 - 9.50 mg/g for isovitexin. The concentration of L-citrulline and L-arginine in FD extracts varied from 7.45 - 8.53 mg/g for L-citrulline and 5.36 – 8.44 mg/g for L-arginine. The level of ursolic acid in FD extracts varied from 2.46 – 4.27 mg/g. Rosmarinic acid, the principal constituent of OS water extract ranges from 56.77 mg/g in methanol and 15.92 mg/g in water extract. The concentration range of the flavones in OS extracts varied from 5.61 – 33.16 mg/g for sinensetin, 0.35 – 10.48 mg/g for 3'-hydroxy-5,6,7,4'-tetramethoxyflavone and, 7.73 – 177.91 mg/g for eupatorin. The level of oleanolic acid and ursolic acid in OS extracts was in the range of 8.70 – 13.22 mg/g and 2.16 – 33.43 mg/g, respectively. Primary and secondary metabolites of FD and OS extracts were analysed for their total flavonoids, polyphenols, tannins, proteins, polysaccharides and glycosaponins. Methanolic extract of OS contained the highest flavonoid (336.58 mg/g) content, while the water extract of this plant consisted of the highest amount of polyphenolic (157.00 mg/g) and polysaccharides (10.12%). Methanol extract of FD shows the highest amount of tannins (58.10 mg/g).

Methanolic and water extracts of FD and OS were screened for their *in vitro* antihypertensive effect using enzymatic-based ACE-I assay and quantifying ACE concentration in umbilical vascular endothelial cells (HUVEC) lysates after treating with the extracts. FD and OS extracts showed significant ACE-I activity in both assays. In the enzymatic assay, FD methanolic extract exhibited the highest inhibition of 96.00% and lowest IC_{50} value of 19.15 µg/mL and both FD and OS methanol extracts showed high inhibition on ACE expression in HUVEC lysates (73.51% and 73.46% inhibition). Antiangiogenic effects of FD and OS methanol and

water were investigated using *in vivo* chick chorioallantoic membrane assay. The results showed that all extracts had significant antiangiogenic activity; however FD methanolic extract at the concentration of 100 µg/mL distorted the highest vasculature architecture. Cytotoxic effects of FD and OS methanol and water extracts were assessed using MTT assay against two human cancer cell lines namely; colorectal carcinoma (HCT 116) and hormone sensitive breast cancer (MCF 7). In addition, the extracts were also tested against normal human cell line, (HUVECs). Both FD and OS extracts showed modest cytotoxic activity against cancer cells (IC_{50} > 20 µg/mL) and weak cytotoxic effects against HUVECs cells, suggesting that anticancer potential of the leaves of FD and OS might not be due to its cytotoxic effect but may be due to the inhibition of angiogenesis.

In conclusion, the current study provides evidence on antihypertensive and antiangiogenic activities, quality and safety of the standardized FD and OS extracts. The developed quantitative HPTLC and HPLC methods for this study can be used for standardization and routine analysis of both FD and OS herbal materials.

CHAPTER 1
INTRODUCTION

1.1 Significance of Medicinal Plants

Herbs and spices have been used by humans as food source and to treat ailments for generations. Herbal remedies and herbal products have been used over 4,000 years for partial treatment ailments and diseases. Medicinal herbs were the primary health care agents for many centuries before the development of modern medicines. Medicinal plants have played a key role in world's health. In spite of the great advances observed in modern medicine in the recent decades, plants still make an important contribution to health. The consumption of plant-based medicines and other botanicals has increased manifold in recent years. In spite of many developments in synthetic chemistry, plants are still used as sources for new drugs due to the influence of cultural in the use of traditional medicine and also the potential of the plants. Several well-known medicines were derived from plants such as morphine, digitoxin, taxol, colchincine, and L-hyoscyamine. Herbal and other traditional medicines have been used by more that 80% of the world population in developing countries as the primary health care (Hussain *et al*., 2009). Encouraging and promoting the use of traditional and herbal remedies are recommended by World Health Organization (WHO) in the National Health Care Program (Hussain *et al*., 2009).

Herb is defined as a plant or plant part used for its aromatic, savoury, medicinal or cosmetic properties. Generally, the whole plant or plant parts are used singly or in combination with more than one plant for the purpose of treatment. According to the WHO, herbal medicines including plant or plant materials in the crude or processed

state as active ingredients may contain excipients. Combinations with chemically defined active substances or isolated constituents are not considered herbal medicines (WHO Technical Report, 1996). Similarly, the European Medicinal Evaluation Agency (EMEA) defines herbal medicinal products as the medicinal products containing exclusively herbal drugs or herbal drug preparations.

Many plants and plant-based products are being used as folk remedies in the form of fresh or dried plant materials, extracts, tinctures, fatty or essential oils, expressed juices, processed resins or gums, and so forth prepared from herbal drugs, preparation whose production involves a fractionation or concentration processes. Cultivation and utilization of medicinal plants have been increased in spite of many developments in synthetic chemistry. A single plant contains a large number of organic chemicals which may include fatty acids, sterols, alkaloids, glycosides, saponins, tannins and terpenes. The concentration of these substances depends on various factors related to species variation, seedling growing condition and production of the herbal products. These chemical compounds can be used as starting material for synthesis of novel drugs. More than 250,000 species of plants have been identified but only small percentage of them has been investigated for bioactive compounds indicating the potential of plants as a source of new drugs (Prance, 1997).

1.2 Efficacy, Safety and Quality Control in the Standardization of Herbal Medicine

Botanicals herbal preparations are medicinal preparations, containing a single plant or a mixture of two or more different types of medicinal plants, but the problem is to determine which components are biologically active. Pharmacological activity

and the uniformity in herbal products are based on changing chemical constituents which process the activity and environmental factors of the herbs. To overcome this situation, consistent products can be created through standardization.

Standardization is a process to maintain consistency in claimed efficacy of a formulation and its batch to batch reproducibility. Standardization is an important step to know the active constituents are known. However, for many herbs the active constituents are not known. In these cases, products may be standardized on the content of certain ‘markers’ compounds (chemical characteristic of the herb, or present in large quantities). Despite many proven benefits, natural products could not get wider acceptance in the main stream of pharmaceuticals due to lack of standardization. In order to bring these remedies into the mainstream pharmaceutical market, solid scientific evidence is needed to support the efficacy claims of these products (Barnes, 2003).

Herbal products are usually perceived safe because of their long-standing use in various cultures. However, there are case reports of series of adverse events after administration of herbal products. Unknown effects of some of the medicinal herbs have been observed for instance allergic reactions, direct toxic effect especially due to heavy metal poisoning, adverse effects related to herb desired for pharmacological action against possible mutagenic effects and drug contamination. If herbal medicines are not properly assessed, they can have a serious risk of adverse effects and drug-herb and drug-food interactions. Hence, the first priority in herbal research is assessment of the safety of herbal products. There are various approaches to evaluate the safety of herbal medicines. The toxic effects of herbal preparation may be attributed to inherent toxicity of plant constituents, ingredients, manufacturing

malpractice and contamination (Mosihuzzaman and Choudhary, 2008). Therefore, the quality control and standardization of raw material and the herbal preparation themselves should be permanently carried out. The WHO has conducted a number of International Pharmacopoeia which comprises a collection of recommended procedures for analysis and specifications on quality control methods for medicinal plant materials. Examples include macroscopic and microscopic examination, determination of total ash, acid-insoluble ash and water-soluble ash, determination of pesticide residue, determination of swelling and foaming index, limit test for heavy metals, limit test for microorganisms, and test for determination of of extractable matter, water and volatile matter (WHO, 1977).

It is important to note that several factors can greatly affect the quality control and consequently the therapeutic value of herbal medicines such as the use of fresh plants, light exposure, temperature, nutrients, water availability, period and time of collection, method of collecting, drying, packing, storage, transportation of raw material, age, part of the plant collected and other factors. Apart from these variable factors, method of extraction and contamination with microorganism, heavy metals, pesticides and etc, can also interfere with the quality, safety and efficacy of herbal drugs (Calixto, 2000).

The information on safety and efficacy of herbs or plant medicine are limited in a number of ways such as *in vitro* and *in vivo* studies, drug interactions, effects in special populations and toxic reaction (O'Hara *et al*., 1998). The Food and Drug Administration (FDA) has categorized about 250 herbs as "generally recognized safe" (GRAS) is chamomile, echinacea, feverfew, garlic, ginkgo and ginseng based on their long-term traditional use without significant side effects (O'Hara *et al*.,

1998). A number of surveys about regulatory issues regarding herbal products have been conducted by WHO (Ernst, 2002). A global survey accomplished in 2005 into 141 countries which started establishing regulatory authorities for the assessment of safety and efficacy of herbal medicines. The WHO also specified guidelines to improve consumer information, good agriculture, pharmacovigilance and collection practices for herbal medicines (Ko, 1998; Woolf *et al*., 1994; Vanherweghem *et al*., 1993).

Herbal medicinal products (HMPs) consist of hundreds of constituents and some of them may be very toxic such as digitalis, pyrrolizidine alkaloids, ephedrine and phorbal esters. Establishing appropriate strategies for the analysis of quality, safety, efficacy, and the process of standardization of HMPs is always challenging due to their complexity of constituents, unknown chemicals and variation in chemical composition, heterogeneity of plant species, plant part, growth conditions, seasonal cycle, climate, age of the plant and inadequacy or unavailability of standards and analytical methods. With advancments in analytical methods and knowledge, standardized, safe and high quality HMPs can be manufactured successfully.

In this study, two types of local medicinal herbs have been selected, namely *Ficus deltoidea* (FD) locally known as '*mas cotek* or *secotek emas*' and *Orthosiphon stamineus* (OS) locally known as *misai kucing* for antihypertensive and anti-angiogenic activities. *Ficus deltoidea* Jack (Moraceae) is one of the native plants widely distributed in several countries in Southeast Asia. Different parts of the plant have been used traditionally for treatment of various kinds of illnesses. Decoction of the *F. deltoidea* leaves is used to improve blood circulation and to treat diabetes,

high blood pressure, heart problems, gout, diarrhea, inflammation, analgesic conditions, pneumonia, and skin infections. In Sabah, the leaves have been used as herbal tea for anti-aging and youthful appearance (Fasihudin and Hasmah, 1991). *Orthosiphon stamineus* Benth. (Lamiaceae) is a medicial plant grown in South East Asia and currently cultivated in Indonesia and Malaysia. In Malaysia the leaves of this plant (*misai kucing*) have been used traditionally in treating angiogenesis related diseases, urinary lithiasis, edema, inflammation, eruptive fever, influenza, hepatitis, jaundice, rheumatism, diabetes and hypertension (Wangner, 1982).

1.3 Justification of the Research

Currently *F. deltoidea* and *O. stamineus*, have the potential for different pharmacological properties. Although a number of products manufactured from *F. deltoidea* and *O. stamineus* are available in the market, there is still lack of information in terms of chemical components related to pharmacological properties such as antihypertensive and antiangiogenic activities, quality and safety of the plants. The basis for the traditional use of these herbs in hypertension and angiogenesis has not yet been scientifically verified to the best of the researcher's knowledge.

1.4 Objectives of the Study

The objectives of this study are as the following:

1) To analyze and characterize *F. deltoidea* and *O. stamineus* raw materials
2) To develop and validate analytical methods for chemical profiling and standardization of *F. deltoidea* and *O. stamineus* leaf extracts using suitable marker compounds.

3) To evaluate the standardized extracts of leaves of *F. deltoidea* and *O. stamineus* for *in vitro* anti-hypertensive activities.
4) To study the antiangiogenic and cytotoxic properties of *F. deltoidea* and *O. stamineus* standardized leaf extracts.

CHAPTER 2
LITERATURE REVIEW

2.1 *Ficus deltoidea*

Ficus deltoidea Jack or *mas cotek* is a plant derived from Moraceae family. It is one of the highly variable species, native and widely distributed in several countries in Southeast Asia (Burkill, 1996; Corner, 1965). The species is popular as a traditional medicinal plant in villages and nowadays is used in the modern pharmaceutical market. The enormous variability of *F. deltoidea* creates a complex morphological variation within the species (Asiah *et al*., 2011). Identification of varieties within the species is very confusing because features of one variety is always overlapping with those of the other varieties, and oppositely, features of the same individual plant sometimes display the different states of characters (Asiah *et al*., 2011).

2.1.1 Taxonomy

Family	Moraceae
Genus	*Ficus*
Species	*Deltoidea*
Scientific name	*Ficus deltoidea* Jack
Synonyms	*Ficus diversifolia* Blume
Common name	Mistletoe fig

(Starr *et al*., 2003)

The family of Moraceae comprises of 53 genera and 1400 species, distributed in tropical and subtropical zones (Wagner *et al*., 1999; Starr *et al*., 2003). The

Moraceae family are composed of trees, shrubs, subshrubs, less often herbs. The trees and shrubs terrestrial are usually abscise and sometimes armed with thorns or prickle (Berg, 2001). The largest genus is *Ficus* with 759 species and occupies many habitats from lowland swamps to mountain rainforest and semi-arid areas. They have a wide range of life forms from lianescent climbers to hemiepiphytes and forest canopy trees (Clement and Weiblen, 2009).

Ficus deltoidea Jack is an evergreen shrub or a small tree up to 2 m tall, usually bushy and sometimes epiphytic with aerial roots, often begins its life as an epiphyte but is not a banyan. The plant loves warmth and humidity, and produces picturesque aerial roots under such conditions. (Riffle, 1998). Leaves of this plant are broadly spoon-shaped to obovate, leathery, 2-8 × 1.5-7.5 cm long and have blade thin to thick, bright green and rust-red to olive brown beneath. *F. deltoidea* can be divided into two different groups (female and male) based on the leaf shapes. Different appearance can be observed between the male and female plants. The male plant leaves are small in size, long and thin, with red spots on the lower surface of leaves. While the female plant leaves are bigger, thicker and have oval or round shape (Kiong *et al*., 2007). The spots on the lower surface of the leaves are black in colour instead of red (Osman, 2004 as cited by Kiong *et al*., 2007)

In the whole genus the leaf shape is the most variable and ranges from elliptical or lanceolate to obovate or spatulate that, the two latter shapes are more common than the formers two. The spatulate-shaped leaf form is only regularly commercialised and cultivated. The leaves are 7.6 cm long, thick, dark green and leathery, almost succulent, and the ends of the spatulate-shaped leaves usually contain a shallow notch (Starr *et al*., 2003). The underside of the leaf is midrib

forked and generally there is a black spot (gland) at the fork and on the upper side of the leaves, yellow spots can be observed all over the surface. The fig shapes vary from spherical to round: peduncle 3-15 × 1-2 mm: basal bract 1 mm: body 5-7 mm wide and with 1.5 cm across. Figs ripen from dull yellow to orange and red and are freely produced in pairs (Brickell and Zuk, 1997).

In Peninsular Malaysia, East Malaysia and Kalimantan, *F. deltoidea* plant is known as *mas cotek, secotek emas, telinga kera, sempit-sempit* and *agoluran*. In Indonesia, it is known as *tabat barito, ara jelatih, ara tunggal, api-api telinga gajah* and *api-api telinga kera* (Musa, 2006).

In Malaysia the local name of this plant refers to the golden dot (*emas* or *mas*) which can be observed on the upper layer of the leaves. Also the shape of the leaves is slightly same as the ear of '*beruk* or *kera*' (monkey in Malay). The common name of this plant 'mistletoe fig' comes from the habit of the plant as epiphytes often grow on larger trees. The scientific name, *deltoidea, triangularis* refers to the shape of the leaves (Starr *et al*., 2003).

According to the Malaysian traditional herbalist (Kamarudin and Latiff, 2002), 7 different varieties of *F. deltoidea* are identified as *motleyana, angustifolia, intermedia, kunstleri, deltoidea, bilobata* and *terengganuensis*.

For the purpose of the analytical and biological study, the variety of *deltoidea* (*F. deltoidea* var. *deltoidea*) has been selected in this resarch (Figure 2.1)

2.1.2 *Ficus deltoidea* Var. *deltoidea*

Distribution: Malaysia (East Johore, South East Pahang), Lingga Archipelagos, Bangka, Sumatra, Borneo (Zunoliza, 2009).

Ecology: generally epiphytic in lowland and mountain forest, up to 1200 m altitude (Kinabalu), also terrestrial on rocks and sea-shores (Zunoliza, 2009).

Morphology: A small shrub smaller fig ripening orange to red. The leaves are lanceolate and penniveined. A strong distinction of this plant is rugose-angular (Zunoliza, 2009).

Figure 2.1: Pictures of *Ficus deltoidea* leaves and fruit

2.1.3 Review of Chemical Constituents of *Ficus deltoidea*

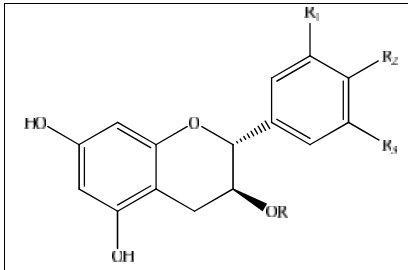
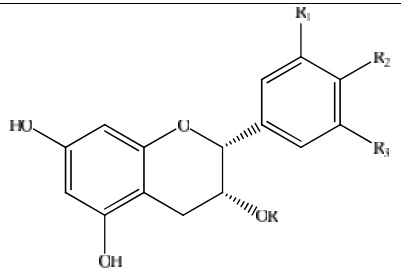
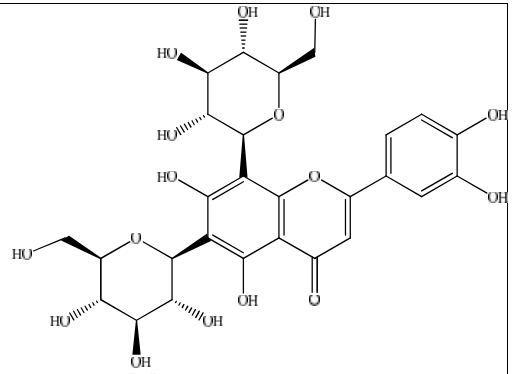
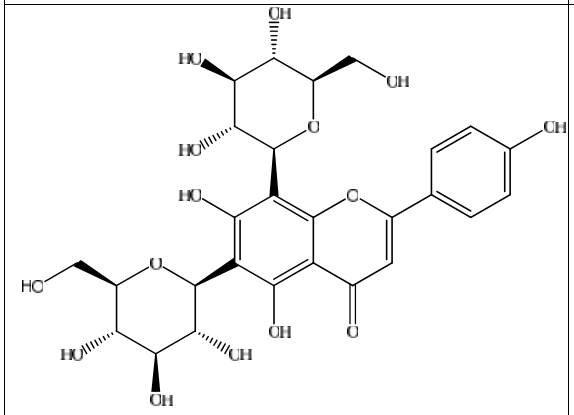
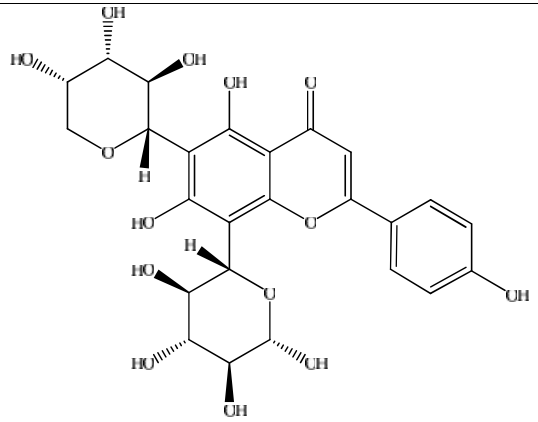
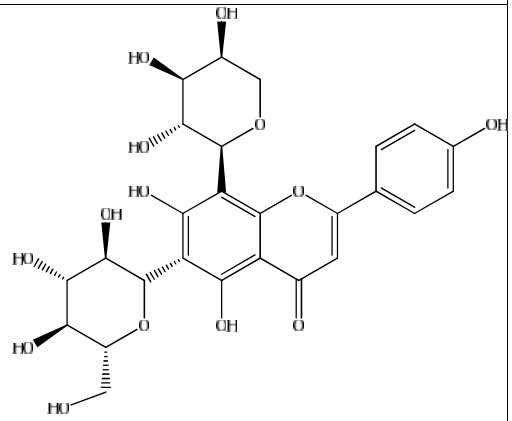
Various chemical constituents including phenolics, triterpenoids and flavonoids have been identified from the aerial part of *F. deltoidea*. Reviews on the identified chemicals of *F. deltoidea* are summarized in Table 2.1.

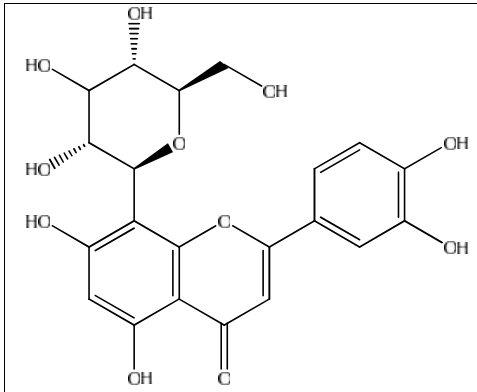
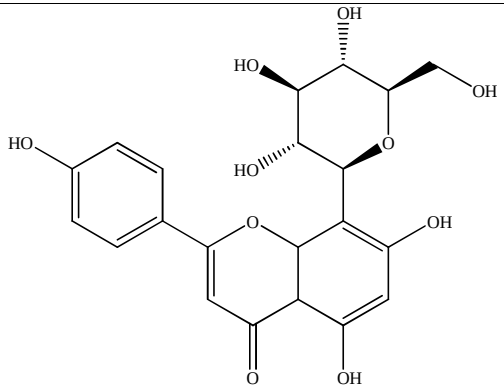
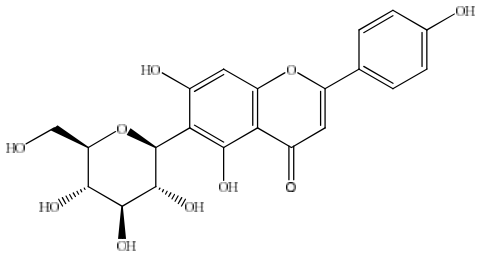
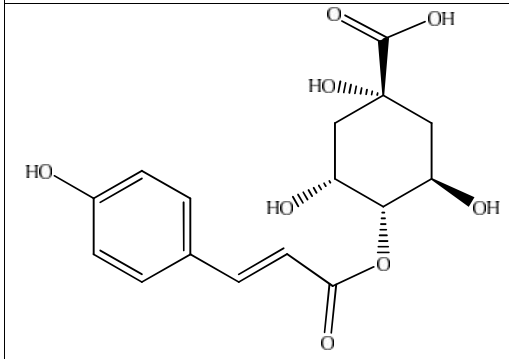
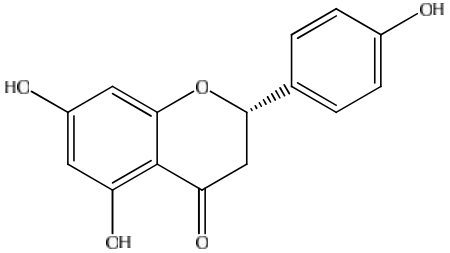
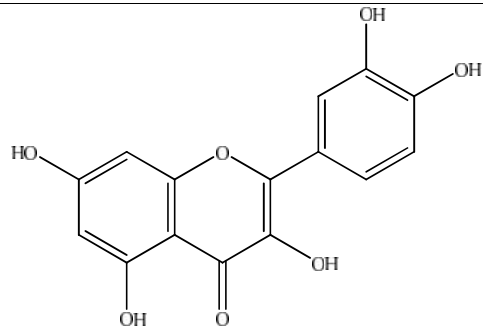
Table 2.1: Chemical constituents of *Ficus deltoidea*

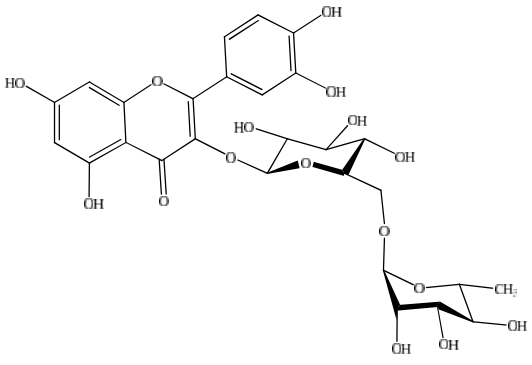
Class of compounds	Chemical constituents	Reference
Flavon-3-ol	Gallocatechin [1], catechin [2], epi gallocatechin [3], epi efzelechin [4], epi catechin [5]	Omar *et al*., 2011
Flavones	Lucenin-2 [6], vicenin-2 [7], isoschaftoside [8], schaftoside [9], orientin [10], vitexin [11], isovitexin [12]	Omar *et al*., 2011; Zunoliza, 2009
Hydroxycinnamates	4-p-coumaroylquinic acid [13]	Omar *et al*., 2011
Flavonoids aglycone (Flavonols)	Naringenin [14], quercetin [15], rutin [16]	Ling *et al*., 2010
Triterpene	β-amyrin cinnamate [17], lupeol [18], β-sitosterol [19], friedelin [20]	Zunoliza, 2009; Suryati *et al*., 2011

Number in brackets indicate the number of the structure

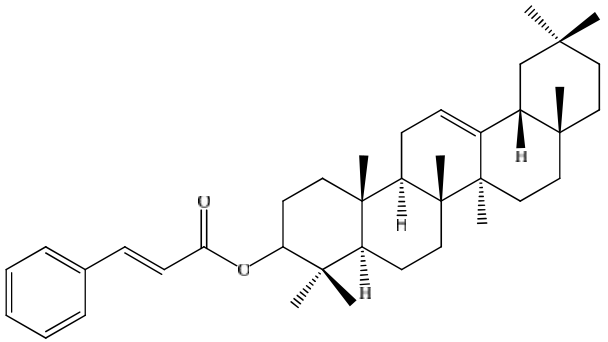
Table 2.2: Chemical structure of *Ficus deltoidea*

 R = H, R_1 = R_2 = R_3 = OH, Gallocatechin [1] R = R_1 = H, R_2 = R_3 = OH, Catechin [2]	 R = H, R_1 = R_2 = R_3 = OH, Epi gallocatechin [3] R = R_1 = R_3 = H, R_2 = OH, Epi efzelechin [4] R = R_1 = H, R_2 = R_3 = OH, Epi catechin [5]	 Lucenin-2 [6]
 Vicenin-2 [7]	 Isoschaftoside [8]	 Schaftoside [9]

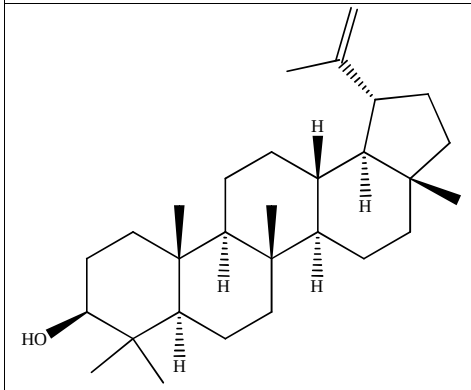
 Orientin [10]	 Vitexin [11]	 Isovitexin [12]
 4-p-coumaroylquinic acid [13]	 Naringenin [14]	 Quercetin [15]



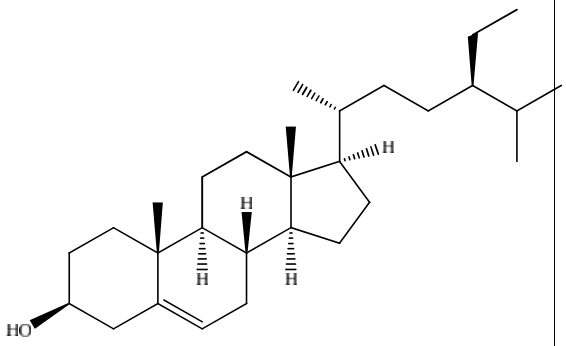
Rutin [16]



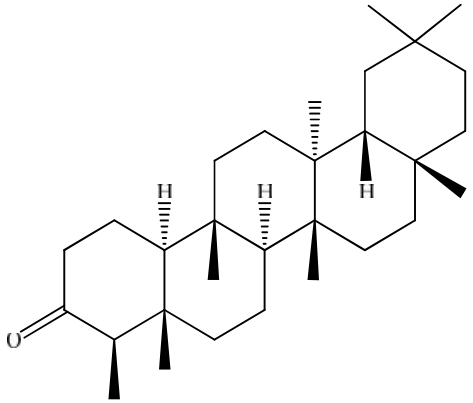
β-amyrin cinnamate [17]



Lupeol [18]



β-sitosterol [19]



Friedelin [20]

2.1.4 Review of Biological and Pharmacological Activities of *Ficus deltoidea*

F. deltoidea is one of the popular plants in Malay traditional medicine its pharmacological properties have not been fully studied. Very less publication indicating scientific findings of this plant have been published. For example *F. deltoidea* is reported to have antioxidant, antihypertension, anti-inflammatory, anti-nociceptive, anti-diabetic, anti-ulcerogenic, anti-melanogenic and wound healing effects. Therefore, research on pharmacological properties of *F. deltoidea* becomes a challenge due to the lack of scientific data about this plant.

Recently Zunoliza (2009) studied the standardized extracts of *F. deltoidea* leaves which were divided into five parts: chemical investigation, chemical profiling, biochemical profiling, stability studies and biological profiling. Five compounds were isolated that included three triterpenoids, β-amyrin cinnamate, β-sitosterol and friedelin and two flavonoids, vitexin and isovitexin. *In vitro* antihypertensive studies using Angiotensin-Converting Enzyme (ACE) assay showed that the extracts have potent ACE inhibitory activity on *in vitro* model. In another study on *F. deltoidea* fruit extract showed that, the plant extract demonstrated inhibitory effects towards ACE activity (Abdullah *et al*., 2008). Both studies suggested that *F. deltoidea* may possess antihypertensive properties.

Antioxidant activity of *F. deltoidea* extracts has been investigated by several studies. The methanolic and aqueous extracts of three varieties of *F. deltoidea* (*terengganuensis, angustifolia* and *deltoidea*) were subjected for antioxidant activity using *in vitro* models such as free radical scavenging activity, reduction power of iron (III), superoxide anion (O_2^-) scavenging xanthine oxidase (XOD), nitric oxide (NO) and lipid peroxidation. In this study, content of total polyphenols, flavonoids,

tannis and their correlation with antioxidant activity were estimated. The results of this study indicated that methanol and aqueous extracts of different varieties of the plant exhibited different radical scavenging activities in different models ($P < 0.05$) and the activity is comparable to quercetin, rutin, butylated hydroxyanisole (BHA), ascorbic acid and allopurinol (Zunoliza *et al*., 2009a).

In another study non-enzymatic and enzymatic antioxidant activity of 13 accessions of aqueous extracts of *F. deltoidea* were investigated. Different accessions of *F. deltoidea* were divided into two groups and generally named as female and male based on the leaf shape and spots. For non-enzymatic antioxidant, several assays were used such as 2,2-diphenyl-1- picrylhydrazil (DPPH) free radical scavenging assay and ferric reducing antioxidant power (FRAP) assay for total antioxidant content. However, enzymatic antioxidant was assayed using ascorbate oxidase, peroxidase, catalase and ascorbate peroxidase. In addition, the content of total polyphenol, flavonoid, phenolic acid and vitamin C in *F. deltoidea* leaves extracts were also analyzed. The results show that extracts from female leaves contain higher content of polyphenols, flavonoids, phenolic acids and vitamin C than those extracts from male leaves. Whereas, in most of the assays female leaf extracts are better than the male leaf extracts. This study suggests that female leaf extract contained more antioxidant compounds. Therefore, they have better antioxidant activity than the male leaves (Hakiman and Maziah, 2009).

The antinociceptive activity of aqueous extract of *F. deltoidea* leaves was evaluated by three models of nociception, namely, acetic acid-induced abdominal writhing, formalin-induced paw licking and hot plate test. The results show that aqueous extract of *F. deltoidea* leaves possess antinociceptive activity as evident in

all the three nociceptive models. This activity is attributed to some pharmacologically active constituents such as flavonoids and tannins which have been discovered from medicinal plants with significant antinociceptive. Thus, it is suggested that *F. deltoidea* can be used in treating conditions associated with painful conditions (Sulaiman *et al*., 2008).

The blood glucose lowering effect of *F. deltoidea* leaf and fruit aqueous extracts were studied using oral glucose tolerance test. The results demonstrated that external glucose load significantly reduced post-treatment at 50 mg/kg dosage of the aqueous extracts of both the leaf and fruit of *F. deltoidea.* From the brine shrimp toxicity test, both extracts were found to be non-toxic (Norhaniza *et al*., 2007).

In another study, the effect of *F. deltoidea* five different extracts (hot and cold aqueous extracts, petroleum ether extract, methanol and ethanol extracts) and three fractions from the methanol extract (acidified-chloroform fraction, bacified-chloroform fraction and n-butanol) on enhanced basal and insulin-stimulated glucose uptake into Chang liver cell line were tested using glucose uptake assay. The results illustrated that, except petroleum ether extract, all *F. deltoidea* extracts and fractions have the ability to enhance either basal or insulin-stimulated glucose uptake into the liver cell line. Ethanol and methanol extracts, and acidified-chloroform and bacified-chloroform fractions showed ability of insulin-mimetic activity, however, ethanol extract possess the highest insulin-mimetic activity. Both methanol extract and n-butanol fraction showed insulin-sensitizing activity while methanol extract possess the highest activity. Therefore, it is found that *F. deltoidea* extracts have the ability of blood glucose lowering effect by the augmentation of glucose uptake into liver cells (Adam *et al*., 2009).

The inhibitory activity of *F. deltoidea* extracts (hot aqueous, ethanol and methanol extracts) on α-glucosidase enzyme in the small intestine using *in vitro* and *in vivo* models was also studied. In *in vitro* study, all the extracts of *F. deltoidea* inhibited rat intestine α-glucosidase while in the enzyme kinetics study, all extracts exhibited a mixed-type inhibition mechanism against the activity of α-glucosidase. In the animal study, all the extracts reduced postprandial hyperglycemia at a dose of 1000 mg/kg body weight in normal and diabetic rats compared to the control group. Methanol extract of *F. deltoidea* showed the highest inhibition of α-glucosidase followed by the ethanol and hot aqueous extracts. This study suggests that the *F. deltoidea* extracts possess antidiabetic property by inhibition of α-glucosidase in the small intestine (Adam *et al*., 2010).

Abdullah *et al*. (2010) evaluated the role of *F. deltoidea* aqueous extract in the enhancement of wound healing in experimental rats and also histology study of healed wounds in rats. In this study, five groups of male *Sprague Dawley* rats were experimentally wounded in the trailing neck area. Group 1 animals as a negative control were treated with sterile deionized water while group 2 rats were treated with thin layer of blank placebo. Group 3 and 4 were topically dressed with thin layer of placebo containing 5% and 10% *F. deltoidea* extracts, respectively. Group 5 wounds were treated by thin layer of intrasite gel as a positive control. The results showed that, placebo containing 5% and 10% *F. deltoidea* extracts significantly accelerate the rate of wound healing compared to sterile deionized water or dressed with blank placebo and the activity is comparable with intrasite gel (positive control).

Anti-inflammatory activity of standardized extracts of leaves of three varieties of *F. deltoidea* (*terengganuensis, angustifolia* and *deltoidea)* was investigated by

using three *in vitro* assays namely, lipoxygenase, hyaluronidase and TPA-induced oedema. For the purpose of standardization, two pharmacologically active markers (vitexin and isovitexin) have been used to standardize methanol and aqueous extracts of different varieties of *F. deltoidea* using high performance liquid chromatography (HPLC) and also percentages of total proteins and polysaccharides have been estimated in all plants extracts. The results indicate that both methanol and aqueous extracts did not show any inhibition in lipoxygenase assay which refers to antiradical properties of plant extracts. However, in hyaluronidase and TPA-induced oedema assays methanol extracts of all three varieties of the plant exhibited moderate inhibitory activity than the aqueous extracts. Thus, this study illusterated that methanol extracts of *F. deltoidea* possess bellow anti-inflammatory properties than the aqueous extracts. This might be due to the presence of active substances endowed with anti-inflammatory activity such as polyphenolic components (Zunoliza *et al*., 2009b).

Anti-ulcerogenic activity of aqueous extract of *F. deltoidea* against ethanol-induced gastric mucosal injury was studied by *in vivo* model. Twenty four adult male Sprague-Dawley rats were divided into 4 groups and were pre-treated: group 1 distilled water as negative control, groups 2 and 3 with 250 and 500 mg/kg *F. deltoidea* extract, respectively (experimental groups) and group 4 with omeprazole as positive control 30 minutes before oral administration with absolute ethanol to generate gastric mucosal injury. After 1 hour, all animals were sacrificed and their stomachs were rapidly removed and ulcer areas and histological sections of gastric walls were determined. The results showed less gastric mucosal lesions produced by ulcerogens after pre-treatment with *F. deltoidea* or omeprazole. Group 3 rats that were pre-treated with 500 mg/kg *F. deltoidea* extract showed more gastric protection

than 250 mg/kg. From the histological studies, it was confirmed that, those rats which were pre-treated with distilled water (negative control), markedly showed extensive and deep gastric mucosal necrotic damage, along with edema and leucocytes infiltration of the submucosal layer. However, better protection of the gastric mucosa by marked reduction in ulcer area and absence of oedema and leukocyte infiltration of submucosa was observed in those rats which were pre-treated with omeprazole or *F. deltoidea* extracts. Therefore, this study showed that, *F. deltoidea* extract by the comparative significant decreases in ulcer areas and inhibition of submucosal edema and leucocytes infiltration of submucosal layer promotes ulcer protection as ascertained (Zahra *et al*., 2009).

Anti-melanogenic efficacy of *F. deltoidea* aqueous extract was investigated by preventing *in vitro* tyrosinase activity and by suppressing tyrosinase gene expression in B16F1 melanoma cells model. Different *in vitro* assays were used in this study namely, MTT assay for measuring cytotoxicity of the extract, melanin assay, mushroom tyrosinase inhibition assay, intracellular tyrosinase activity assay and immunoblot assay. The results of MTT assay showed that highest concentration of the extract dose not affect cell viability as 0.1% (w/v). *F. deltoidea* leaf extract inhibited significantly α-MSH-induced melanin synthesis with dose-dependent manner by treatment. The effect of *F. deltoidea* extract on α-MSH-induced melanin synthesis was comparable with kojic acid, an effective anti-melanogenic agent, used as the positive control in this assay. The extract of *F. deltoidea* inhibited mushroom tyrosinase activity and intracellular tyrosinase activity of B16F1. The inhibition of intracellular tyrosinase activity was analyzed by immunoblot and tyrosinase zymography. It was found to be exerted at the protein expression level. *F. deltoidea* extract also showed the ability of reduction of the expression of microphthalmia-

associated transcription factor (MITF). Therefore, this study finds that *F. deltoidea* leaf extract are able to inhibit tyrosinase enzyme activity directly by down-regulation of the expression of genes involved in the melanogenesis pathways. In conclusion, *F. deltoidea* leaves have strong anti-melanogenic activity and also the potential to be used as novel depigmenting agents for cosmetic (Oh *et al*., 2011).

Table 2.3: Summary of literatures on biological and pharmacological activity of *Ficus deltoidea*

Part of plant	**Type of extract**	**Biological activity**	**Methodology**	**Compounds contribute**	**Reference**
	Methanolic and aqueous extracts		*In vitro* assays: free radical scavenging activity, reduction power of iron (III), superoxide anion (O_2^-), scavenging xanthine oxidase (XOD), nitric oxide (NO) and lipid peroxidation	Polyphenol Flavonoids Tannins	Zunoliza *et al*., 2009a
Leaf	Aqueous extract	Antioxidant	Non enzymatic *in vitro* assays: 2,2-diphenyl-1-picrylhydrazil (DPPH) free radical scavenging assay and ferric reducing antioxidant power (FRAP) assay for total antioxidant content Enzymatic *in vitro* assays: ascorbate oxidase, peroxidase, catalase and ascorbate peroxidise	Polyphenol Phenolic acid Flavonoids	Hakiman and Maziah, 2009
Leaf	Aqueous extract	Anti-inflammatory	*In vitro* assays: lipoxygenase, hyaluronidase and TPA-induced oedema.	Total proteins Total polysaccharides Total poly phenols	Zunoliza *et al*, 2009b
Leaf	Aqueous extract	Antinociceptive	*In vivo* assays: acetic acid-induced abdominal writhing, formalin-induced paw licking and hot plate test	Flavonoids Tannins	Sulaiman *et al*., 2008

Fruit	Aqueous extract	Antidiabetes	*In vivo* assay: oral glucose tolerance	Flavonoids Tannins	Norhaniza *et al*., 2007
	Hot and cold aqueous extracts, petroleum ether, ethanol and methanol extracts		*In vitro* assay: Screened of insulinotrophic properties, using rat pancreatic β-cell lines (BRIN-BD11 cells) and cytotoxocity effect of plant extracts on the cell.	Flavonoids Tannins	Hamid *et al*., 2008
Leaf	Hot and cold aqueous extracts, petroleum ether, ethanol and methanol extracts	Antidiabetes	*In vitro* assay*:* glucose uptake assay *In vivo* assay: α-glucosidase inhibition Assay	Flavonoids Tannins	Adam *et al*., 2009
	Hot aqueous, ethanol and methanol extracts		*In vivo* assay: the effect of extracts on postprandial blood Glucose levels following sucrose loading in normal and diabetic rats	Flavonoids Tannins	Adam *et al*., 2010