PROFILING AND QUANTIFICATION OF *COSMOS CAUDATUS* KUNTH AND *CENTELLA ASIATICA* LINN. AND IN VITRO ANTI CANCER ACTIVITY OF *COSMOS CAUDATUS*

By

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LIST OF ABBREVIATIONS

AA	Asiatic Acid
AAS	Atomic Absorption Spectrometry
ABTS	2,2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
AEAC	Ascorbic Acid Equivalent Antioxidant Capacity
AS	Asiaticoside
As	Arsenic
ATCC	American Type Culture Collection
ATR	Attenuated Total Reflection
BA	Betulinic Acid
CA	*Centella asiatica*
CC	*Cosmos caudatus*
Cd	Cadmium
$CHCl_3$	Chloroform
CO_2	Carbon Dioxide
DAD	Diode Array Detector
DLA	Dalton’s Lymphoma Ascites Tumour Cells
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EA	Ethyl Acetate
EAC	Ehrlich Ascites Tumour Cells

EtOH	Ethanol
FBS	Fetal Bovine Serum
FTIR	Fourier Transform Infrared
GAE	Gallic Acid Equivalent
GC-MS	Gas Chromatography-Mass Spectrometry
HCl	Hydrochloric Acid
HCT	Human Colorectal Carcinoma Cells
Hex	Hexane
Hg	Mercury
H_2O_2	Hydrogen Peroxide
HNO_3	Nitric Acid
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
HUVECs	Human Umbilical Vein Endothelial Cells
IC_{50}	Inhibition Concentration 50
ICH	International Conference on Harmonisation
LOD	Limit of Detection
LOQ	Limit of Quantification
MA	Madecassic Acid
MAPK	Mitogen-activated Protein Kinase
MCF-7	Human Hormone Sensitive and Invasive Breast Cancer Cell Line
MeOH	Methanol
MIC	Minimum Inhibitory Concentration

MLT	Microbial Limit Test
MPCE	Micronucleated Polychromatic Erythrocytes
MPO	Myeloperoxidase
MS	Madecassoside
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
OD	Absorbance
Pb	Plumbum
PBS	Phosphate Buffer Saline
PCA	Principal Component Analysis
PE	Platting Efficiency
RPMI	Roswell Park Memorial Institute
R_2	Regression Coefficient
RSD	Relative Standard Deviation
RSLC	Rapid Separation Liquid Chromatography
R_t	Retention Time
RVSEB	Rappaport Vassiliadis Salmonella Enrichment Broth
SCD	Soybean-Casein Digest Agar
SD	Standard Deviation
SDA	Sabouraud 68 Dextrose Agar
SF	Survival Fraction
TBA	Thiobarbituric Acid Test

T47D	Human Hormone Sensitive Early Stage Breast Cancer Cell Line
UV-Vis	Ultra Violet-Visible
WHO	World Health Organization
XLD	Xylose-Lysine-Desoxycholate Agar

LIST OF UNITS

g	Gram
h	Hour
Kg	Kilogram
L	Liter
M	Molar
mg	Miligram
mg/mL	Miligram per mililiter
min	Minute
mL	Mililiter
mL/min	Mililiter per minute
mm	Milimeter
ng/mL	Nanogram per mililiter
nm	Nanometer
rpm	Revolution per minute
v/v	Volume per volume
v/wt	Volume per weight
wt/wt	Weight per weight
µg/µL	Microgram per microliter
µL	Microliter
µm	Micrometer
µM	Micromolar

LIST OF SYMBOLS

α	Alpha
β	Beta
˚C	Celsius
γ	Gamma
λ_{max}	Lambda max
%	Percent

LIST OF PUBLICATIONS

1. Munira Mohd Amin Sharifuldin, Gheniya Ghafar, Che Norma Ismail, Tang Hui Ying, Pazilah Ibrahim and Zhari Ismail, Microbial limit test (MLT) for selected herbal products in the community pharmacy, International Conference of Natural Product 2010, 10-12 Disember 2010

2. Munira Mohd Amin Sharifuldin, AbdalrahimF. A. Aisha and Zhari Ismail, Total Phenolics, Primary Metabolites and Antioxidant Activity of Centella asiatica Extract, 26th Scientific Conference of Malaysian Society of Pharmacology and physiology , 18-20 May 2012

3. Munira Mohd Amin Sharifuldin, Abdalrahim F.A. Aisha, Zhari Ismail, Quantification of Rutin, Quercitrin and Quercetin in *Cosmos caudatus* Kunth by RP-HPLC, International Conference of Natural Product 2013, 4-6 Mac 2013.

4. Munira Mohd Amin Sharifuldin, Abdalrahim F.A. Aisha, Zhari Ismail, Quantification of madecassoside, asiaticoside, medecassic acid and asiatic acid in *Centella asiatica* by reverse phase HPLC, International Conference of Natural Product 2013, 4-6 Mac 2013.

PEMPROFILAN DAN PENGKUANTITIAN *COSMOS CAUDATUS* KUNTH DAN *CENTELLA ASIATICA* LINN. SERTA AKTIVITI ANTI KANSER *COSMOS CAUDATUS* SECARA IN VITRO

ABSTRAK

Kajian ini bertujuan untuk memastikan kualiti bahan mentah, membangun dan mengesahkan kaedah analitikal bagi pemprofilan sebatian dan pempiawaian ekstrak *C. asiatica* dan *C. caudatus* daripada 3 lokasi berbeza. Tumbuhan-tumbuhan ini diekstrak menggunakan 3 pelarut berbeza dan nilai aktiviti antioksida dikaji. Ekstrak daun *C. caudatus* turut disaring bagi aktiviti anti kanser secara *in vitro*.

Dalam kajian ini, kualiti bahan mentah telah ditentukan berdasarkan beberapa parameter seperti total abu, total abu tidak larut asid, total kelembapan, ujian logam berat dan mikrobial dan nilai ekstrakan. Keputusan bagi ujian-ujian tersebut menunjukkan nilai yang boleh diterima dan berada dibawah limit yang telah ditetapkan.

Pengekstrakan dijalankan menggunakan 96%, 75%, 50%, 25% etanol dan air dan kesemua ekstrak dianalisis secara kualitatif dengan menggunakan spektroskopi Inframerah Terubah Fourier (FTIR) dan spektroskopi Ultralembayung/Tampak (UV/Vis). Spektra yang diperolehi daripada analisis FTIR digunakan pula untuk analisis sebatian prinsipal yang menunjukkan pengkelasan mengikut kumpulan berfungsi dan sebatian kimianya.

Ekstrak turut dianalisis bagi kandungan total metabolit primer dan sekunder. Bagi *C. caudatus*, nilai kandungan saponin, protein, polisakarida dan fenolik tertinggi ialah 46.12%, 57.36%, 7.54% and 4.42% manakala bagi *C. asiatica* pula

ialah 54.60%, 24.58%, 11.98% dan 6.82%. Ujian aktiviti radikal bebas DPPH menunjukkan aktiviti sederhana dalam semua ekstrak.

Analisis kualitatif terhadap ekstrak menggunakan kromatografi lapisan nipis prestasi tinggi (HPTLC) telah dilakukan dengan menggunakan 1 sebatian penanda bagi *C. caudatus* iaitu kuersitrin (R_f: 0.21) dan 4 sebatian penanda bagi *C. asiatica,* iaitu madekasosida (R_f: 0.31), asiatikosida (R_f: 0.39), asid madekasik (R_f: 0.87) dan asid asiatik (R_f: 0.96). Pemilihan sebatian penanda adalah berdasarkan keunikan dan kandungan yang tinggi dalam herba tersebut. Kromatografi cecair prestasi tinggi (HPLC) telah dijalankan untuk mengkuantifikasi 3 sebatian penanda bagi *C. caudatus* dan 4 sebatian penanda bagi *C. asiatica*. Peratusan amaun rutin, kuersitrin dan kuersetin yang terkandung dalam *C. caudatus* adalah 0.13 - 0.94%, 1.51 - 13.78% dan 0.18-0.92%. Peratusan amaun madekasosida, asiatikosida, asid madekasik dan asid asiatik yang terkandung dalam ekstrak *C. asiatica* adalah dalam lingkungan 0.10 - 9.86%, 0.14 - 7.74%, 0.41 - 3.32% dan 0.15 - 1.44%.

Bagi aktiviti anti kanser, fraksi-fraksi daripada *C. caudatus* dinilai dari segi sifat toksik, apoptosis dan sifat anti tumor menggunakan sel karsinoma kolorektal manusia (HCT 116). Pecahan F2, F7 dan F8 menunjukkan potensi sifat toksik bergantung kepada dos dengan nilai IC_{50} masing-masing ialah 15.53 ± 0.4, 32.72 ± 0.3 dan 34.16 ± 1.4 μg/mL. Penelitian ke atas potensi membran mitokondria, struktur kromatin dan morfologi nuklear sel, menunjukkan sel berada dalam keadaan apoptotik setelah dirawat menggunakan fraksi-fraksi tersebut. Ujian terhadap sifat migrasi, ketelapan dan pembentukan koloni menunjukkan fraksi-fraksi ini menghalang ketiga-tiga bentuk pergerakan sel yang diperlukan bagi percambahan sel kanser secara metastasis.

Kesimpulannya, pembangunan kaedah HPLC baru dapat membantu dalam menentukan kualiti sesuatu bahan mentah dan produk bagi proses pemiawaian dan juga kerja-kerja rutin. Berdasarkan keputusan kajian, *C. caudatus* boleh dipertimbangkan sebagai salah satu sumber bagi agen anti kanser kerana ia menghalang laluan karsinogenesis.

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ABSTRACT

This study aims to check the quality of raw materials, develop and validate analytical methods for chemical profiling and standardization of *C. asiatica* and *C. caudatus* extracts from 3 different locations. The plants were extracted with 3 different solvents and were studied for antioxidant activity. *C. caudatus* leaves extracts were also screened for *in vitro* anti-cancer properties.

In this study, the quality of the raw materials have been determined based on several parameters such as total ash, total insoluble ash, total moisture content, heavy metal test, microbial limit test and extractive values. The results showed acceptable values compared to the reference limits.

Extraction was done using 96%, 75%, 50% and 25% ethanol and water and the extracts were analyzed qualitatively using Fourier Transform Infrared (FTIR) and Ultraviolet/visible (UV/Vis) spectroscopy. The spectra collected from FTIR analyses were subjected to principal component analysis which showed that the extracts were clustered based on the same functional group and chemical constituents.

The extracts were also analysed for the primary and secondary metabolites content. For *C. caudatus*, the highest total glycosaponins, total protein, polysaccharides and phenolics content was 46.12%, 57.36%, 7.54% and 4.42%, respectively, while the highest total glycosaponins, total proteins, polysaccharides and phenolics content for *C. asiatica* was 54.60%, 24.58%, 11.98% and 6.82%, respectively. The DPPH scavenging activity showed moderate activity in all extracts.

Qualitative analysis of the extracts by high performance thin layer chromatography (HPTLC) was done using 1 marker compound in *C.* caudatus which was quercitrin (R_f: 0.21) and 4 marker compounds in *C. asiatica,* namely madecassoside (R_f: 0.31), asiaticoside (R_f: 0.39), madecassic acid (R_f: 0.87) and asiatic acid (R_f: 0.96). Marker compounds were chosen based on the uniqueness and amount in that herb. High performance liquid chromatography (HPLC) was done to quantify 3 marker compounds in *C. caudatus* and 4 marker compounds in *C. asiatica*. The percentage of rutin, quercitrin and quercetin in *C. caudatus* was 0.13 - 0.94%, 1.51 - 13.78% and 0.18 - 0.92%, respectively. The percentage of madecassoside, asiaticoside, madecassic acid and asiatic acid in *C. asiatica* extracts was in the range 0.10 - 9.86%, 0.14 - 7.74%, 0.41 - 3.32% and 0.15 - 1.44%, respectively.

For anti cancer activity, the fractions obtained from *C. caudatus* extract were evaluated for cytotoxicity, apoptosis and antitumorgenicity on human colorectal carcinoma cell HCT 116. The F2, F7 and F8 fractions showed potent dose dependent cytotoxicity with IC_{50} value of 15.53 ± 0.4, 32.72 ± 0.3 and 34.16 ± 1.4 μg/mL, respectively. From the observation on mitochondrial membrane potential, chromatin structure and nuclear morphology, it showed apoptotic on the treatment cells. Assay on cell migration, cell invasion and clonogenicity showed that the fractions inhibit these three pathways that are required for metastasis and cancer cell proliferation.

In conclusion, the new development of HPLC method can help in determining the quality of the raw materials and product for standardization process as well as routine works. *C. caudatus* can be considered as a source for anti cancer agent as it perturbs the carcinogenesis pathway.

CHAPTER ONE

INTRODUCTION

1.1 Significance of Medicinal Plants

Since long time ago, humans have been using herbs and spices as vegetables and treatment for many diseases. They were consumed for thousands of years as traditional herbal medicine due to their health benefits. In Malaysia, the market of herbal product is experiencing a tremendous increase, and this phenomenon shows that more people are interested in using herbal products to treat diseases or as daily supplements. Although the research and production of modern scientific drugs are growing rapidly, many people still prefer herbal products as alternative medicines. Malaysia spends nearly 1.2 billion to import herbal products every year. According to the World Health Organization (WHO), more than 3.5 billion consumers in developing countries still rely on herbal plants to treat a variety of diseases and health problems (Balick and Cox, 1997). In United States of America for example, billions of dollars has been spent to buy capsules, tablets, teas and tonic herbs for medicinal purposes. In other countries such as Canada, Europe and Germany the percentage of people using herbal medicines is increasing and herbal medicine has become well established as part of health care system (Jantan, 2006).

Herbaceous plants are not only important to the population of the world to treat a variety of diseases, but it is also used as a source of drugs for modern

medicine such as aspirin, codeine and digoxin. Nowadays, there are nearly 116 types of compounds derived from herbal plants used as prescription drugs (Li, 2009).

In Malaysia, there are more than 120 species of traditional vegetables or herbs known as ‘salad’ came from various families and usually their leaves, shoots and rhizomes are eaten fresh or cooked. It is very popular due to its appealing taste and smell and also its nutritional benefits. Based on several studies, these vegetables have been shown to contain high amount of carbohydrates, proteins, minerals and vitamins, and also have been proved to have some medicinal values such as blood cleansing, reduce high blood pressure, fever, and glucose level and accelerate wound healing. There are some studies that have been conducted which prove that these vegetables also play an important role in reducing the risk of cancer and aging process (Abas *et al.*, 2006).

In Malay traditional medicine, Chinese traditional medicine, Ayurvedic medicine, naturopathy and homeopathy, herbal products used are produced from crude plant preparations. Several studies conducted have shown that crude drug contains a mixture of phytochemicals such as alkaloids, terpenoids, glycosides, tannins, flavonoid, carbohydrates, proteins, lipids, and nucleic acid (Jantan, 2006).

1.2 Quality Control of Herbal Medicine

Herbal products have been used for long time and their advantages and potentials were proven in various studies and reports. However, there are still reports of side effects and failures of these herbal medicines that lead to health problems. The presence of heavy metals and microbes in the herbal products which exceeds the limit indicates poor quality of the products. There are also number of herbal products that are sold without a clear explanation regarding their contents, dosage and usage.

To ensure the quality of these products is maintained within the guidelines and limits set, a quality control process should be established.

The first step in quality assurance is to ensure the product composition using pharmacognostic authenticated methods such as scientific names and knowledge about plants and parts of plants commonly used. To control the quality of raw materials, an assessment of the physicochemical properties such as color, odor, microscopic examination, loss on drying, moisture and ash content values on the herbal materials is very important. Microbial and heavy metals limit test should also be done to ensure the quality of raw materials and to avoid toxicity. Presence of waste fertilizers and pesticides should be monitored using methods that have been set (Hussain *et al.*, 2009). Among others, thin-layer chromatography, gas chromatography, high-performance liquid chromatography, mass spectrometry, infrared-spectrometry, ultraviolet-visible spectrometry, either used alone or in combination, can be successfully used for standardization purpose and to control the quality of both the raw material and the finished herbal drugs (Calixto, 2000).

Scientific assessment of the safety and effectiveness of herbal products is very important both in terms of medical and economic aspects. Contamination and adulteration may cause toxicity and serious adverse events of herbal product administratered afterwards. Toxicity of herbal products may also be due to inherent amout of plant constituents and ingredients, manufacturing malpractice and contamination. The contaminants may include microorganisms, pesticides and heavy metals. This justifies the importance of assessing the safety of herbal products (Mosihuzzaman and Choudhary, 2008) .

Quality and medicinal value of herbs can be affected by several factors such as the use of fresh plants, light exposure, temperature, nutrients, water availability,

collection time and method, drying, packaging, storage, age, part of the plant collected and other factors. Extraction method and solvent, contamination with microorganisms, heavy metals, pesticides and fertilizers are also the variables that have a big effect on the quality, safety and efficacy of herbal drugs. These factors explain the variations in the composition of herbal product. Thus, proper standardization and quality control of raw material and the herbal preparations themselves should be strictly carried out (Calixto, 2000).

Example of standardized herbal preparations that commonly used to treat various diseases is phytotherapeutic agents or phytomedicines. This type of phytotherapeutic agents usually consists of complex mixtures of one or more plants. In Germany, herbal drugs were used in the treatment of common cold (66%), flu (38%), digestive and intestinal diseases (25%), headache (25%), insomnia (25%), stomach ulcer (34%), nervousness (21%), circulatory disorders (15%), bronchitis (15%), skin diseases (15%), and fatigue and exhaustion (12%) (Calixto, 2000).

Phytotherapeutic agents are usually marketed as standardized preparations in the form of liquid, solid (powdered extract), or viscous preparations that were prepared by various methods of extraction such as maceration, percolation or distillation for volatile oils. Solvents such as ethanol, water, or mixtures of ethanol and water are commonly used for the preparation of liquid extract and the solvents were evaporated to produce the extracts in powder form. In order to improve their therapeutic efficacy, some phytotherapeutic agents are greatly concentrated to improve the effect (Calixto, 2000).

In this study, two types of local medicinal herbs have been selected, namely *Centella asiatica* (CA) locally known as '*pegaga*' and *Cosmos caudatus* (CC) locally known as '*ulam raja*'. *Centella asiatica* Linn., (Umbeliferaceae) is a popular

medicinal plant and it is commonly served as a vegetable salad or *ulam* in Malaysian language. It is a green, slender, creeping plant with a root at the nodes and it has been used since long time ago in the Ayurvedic medicine as a memory enhancer and to treat wounds and diarrhea (Ali, 2008).

C. caudatus is a traditional medicinal plant from Compositae family. It is known in Malaysia as *Ulam Raja* which means the king's salad. The fresh leaves are consumed for their taste and health benefits such as antioxidant activity, to improve blood circulation and to promote the formation of healthy bones (Abas *et al.*, 2003).

1.3 Justification of the Research

Currently *C. asiatica* and *C. caudatus*, have the potential for different pharmacological properties. Various methods have been developed to study the chemical composition found in these plants. High Performance Liquid Chromatography (HPLC) and high performance thin layer Chromatography (HPTLC) methos have been developed to detect and quantify the presence of the active chemical constituents in the plant. However, these methods still need to be improved so that the results are obtained more quickly and accurately. The number of cancer patients around the world is increasing day by day. Many studies are now actively committed to finding the best remedy to cure this disease. One alternative method for treating cancer is by using herbal plants. Some plants that have a high content of antioxidant activity or active chemical that acts as an antioxidant has potential as an anticancer agents. However, information studies in this area have not been convincing and plants found to have anticancer potential is still limited. Referring to National Pharmaceutical Control Bureau (NPCB) website, there are over 80 *C. asiatica* registered products are available in market nowadays (NPCB, 2002-

2013). Although a number of products manufactured from these herbs are available in the market and many people consume it directly as vegetable, there is still lack of information in terms of chemical components, pharmacological properties such as anticancer and other bioactivities, quality and safety of the plants. Until now, the fact of the traditional use of herbal plants that have been used since long time ago has not been scientifically validated and comprehensively studied by researchers.

1.4 Objectives of the Study

The objectives of this study are as the following:

1) To analyze the quality of *Cosmos caudatus* and *Centella asiatica* raw material from 3 different sources of raw materials.
2) To investigate the chemical profile of various extracts prepared by different extraction solvents from 3 different sources of raw materials.
3) To develop and validate analytical methods for phytochemical analysis of *Centella asiatica* and *Cosmos caudatus* extracts using suitable marker compounds.
4) To study the *in vitro* anti-cancer properties of *Cosmos caudatus* leaf fraction.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Cosmos caudatus*

"*Cosmos caudatus* is a member of the Asteraceae family. It is an erect annual herb that can reach up to 2 m high. The stems are slender, terete, and glabrous to sparsely pubescent. The leaves measures about 20 cm long, 2 - 3 pinnatisect with each segment being lanceolate to 6 mm broad, acute, aristate, margins entire and ciliate. The peduncles are slender, and elongate. The flower heads 1 - 3 in an open cluster. The heads radiate, outer involucral bracts are 8, linear-subulate to lanceolate, acute measures 8 - 11 mm long. The ray florets are mostly pink measuring 10 - 15 mm long, the limb entire or 2 - 3 denticulate at the apex. The corollas are yellow measuring 5 - 6 mm long. The achenes are mostly black measuring 1 - 3 mm long, fusiform, compressed, slightly curved, the upper 1/3 produced into a brown, ascending-strigose beak, pappus awns 2, slender, diverging, retrorsely barbed measuring 2.5 - 4 mm long" (Globinmed, 2003)

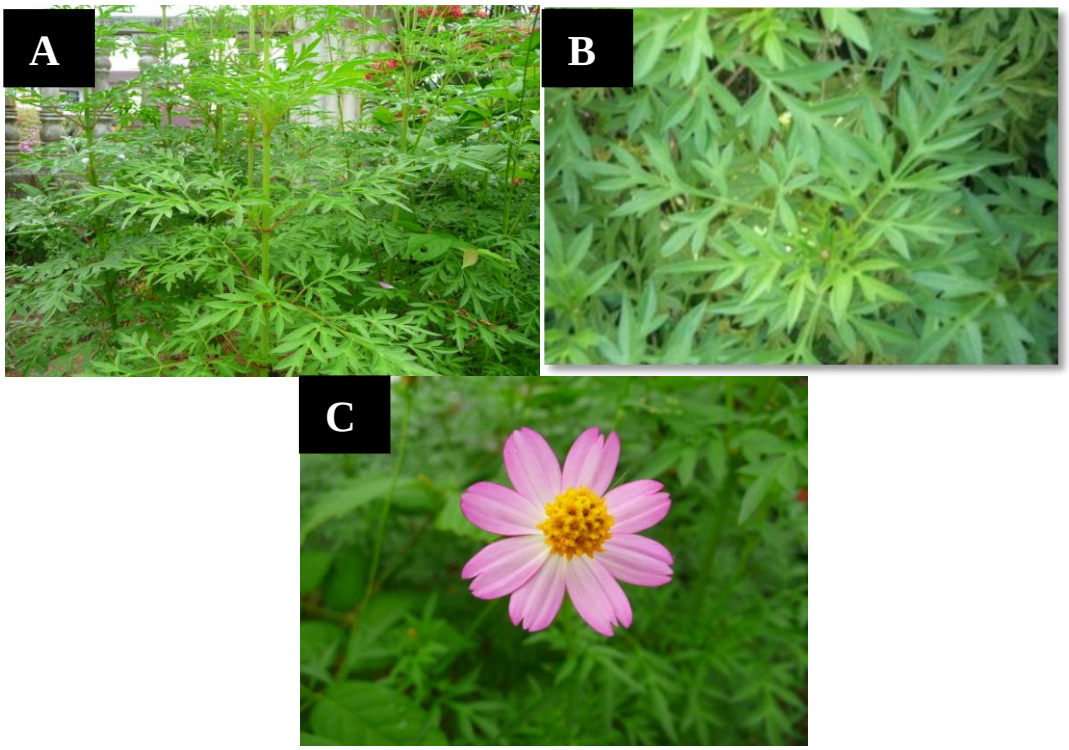


Figure 2.1 Picture of *Cosmos caudatus*. A) whole plant, B) leaves of *Cosmos caudatus* and C) flower of *Cosmos caudatus*

2.1.1 Taxonomy

Kingdom	: Plantae
Subkingdom	: Viridaeplantae
Infrakingdom	: Streptophyta
Division	: Tracheophyta
Subdivision	: Spermatophytina
Infradivision	: Angiospermae
Class	: Magnoliopsida
Superorder	: Asteranae
Order	: Asterales
Family	: Asteraceae
Genus	: Cosmos
Species	: *Cosmos caudatus* Kunth

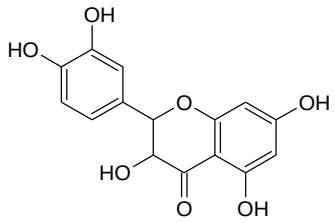
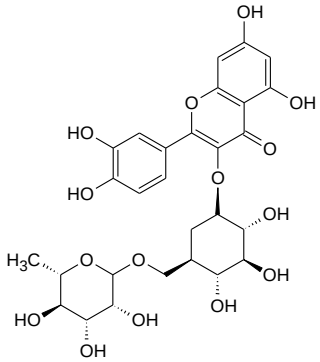
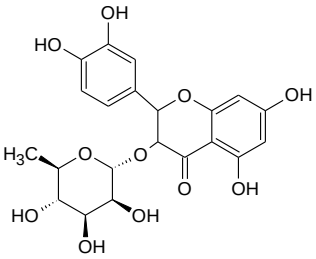
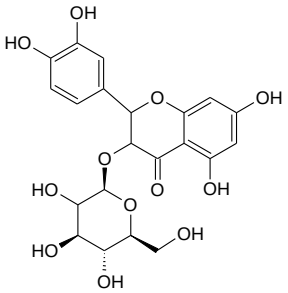
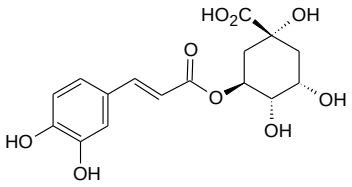
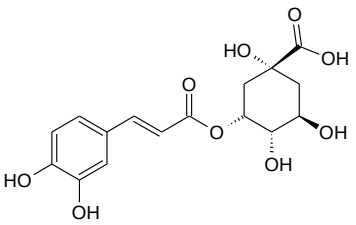
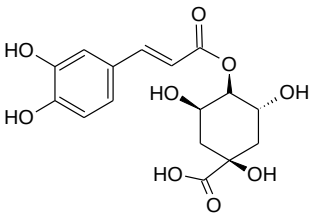
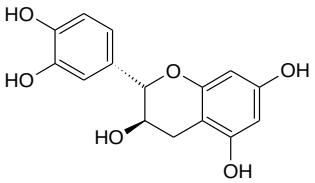
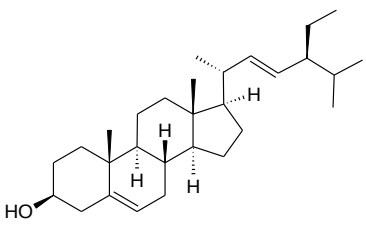
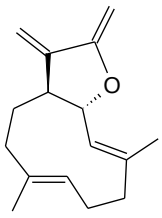
(*Integrated Toxonomy Information System ITIS*)

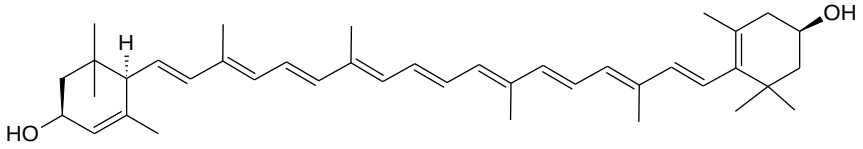
2.1.2 Review of Chemical Constituents of *Cosmos caudatus*

Previous studies showed many compounds have been isolated and identified from *C. caudatus* extract and 4 of the compounds were quercetin 3-O-β-arabinofuranoside, quercetin 3-O-α-rhamnoside, quercetin 3-O-β- glucoside, and quercetin. All 4 compounds isolated from *C. caudatus* showed strong antioxidant activity (Abas, 2005). The major antioxidants in *C. caudatus* were attributed to a number of proanthocyanidins that existed as dimers through hexamers, quercetin glycosides, chlorogenic, neochlorogenic, cryptochlorogenic acid and (+)catechin (Shui *et al.*, 2005). The chloroform extract of the leaves of *C. caudatus* afforded costunolide, stigmasterol, lutein and 4,4-bipyridine (Ragasa *et al.*, 1997).

Six compounds have been isolated from *C. caudatus* roots and the structures were predicted to be one hydroxyeugenol and 5 coniferyl alcohol derivatives. Structures of the isolated compounds were established by referring to the spectral data and the compounds were identified as Z-coniferyl alcohol- 3'-acetyl-4-isobutyrate and l',2'-dihydroxy-coniferyl alcohol-3'-isobutyryl-4-isobutyrate. The other compounds were identified to be, l'-acetoxy-eugenol-4-isobutyrate, l',2'-epoxy-Z-coniferyl alcohol-3'-(2-methylbutyryl)-4-isobutyrate, l',2'-epoxy-Z-coniferyl alcohol-3' acetyl-4-isobutyrate and l',2'-epoxy-Z-coniferyl alcohol-3'-isobutyryl- 4-isobutyrate (Fuzzati *et al.*, 1995).

Table 2.1 Chemical structure for *Cosmos caudatus* compounds

 quercetin	 rutin
 quercitrin	 isoquercitrin
 chlorogenic acid	 neochlorogenic acid
 cryptochlorogenic acid	 (+)catechin
 stigmasterol	 custunolide



lutein

2.1.3 Review of Biological and Pharmacological Activities of *Cosmos caudatus*

Cosmos caudatus showed high antioxidant activity (>70%) in 3 bioassay pathways which are lipid peroxidation, superoxide and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity. This shows that their great antioxidant potential and capacity to fight free radicals and prevent oxidative damage to body tissues and cells (Vimala *et al.*, 2005). In thiobarbituric acid test (TBA) analysis, *C. caudatus* methanolic extract showed the highest antioxidant effect compared to other plants (Huda-Faujan *et al.*, 2009). Phytochemical analysis of the methanolic extract of *C. caudatus* showed that 4 compounds (quercetin 3-O-β-arabinofuranoside, quercetin 3-O-α-rhamnoside, quercetin 3-O-β-glucoside and quercetin) isolated showed strong antioxidant activity (Abas, 2005). From another previous study, flavonoid content in mg/100 g fresh weight (fw) of *C. caudatus* was 52.19 and it showed the greatest total phenols among the vegetables analyzed, with 1.52 mg GAE/100 g fw. *C. caudatus* also showed the highest antioxidant activity as measured by ferric cyanide reducing power, DPPH and ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) scavenging, and inhibition of linoleic acid oxidation (Andarwulan *et al.*, 2010).

C. caudatus was found to have extremely high antioxidant capacity of about 2400 mg ascorbic acid equivalent antioxidant capacity (AEAC) per 100 g of fresh sample. More than 20 antioxidants were identified and the major antioxidants in *C. caudatus* are proanthocyanidins that existed as dimers through hexamers, quercetin

glycosides, chlorogenic, neo-chlorogenic, crypto-chlorogenic acid and (+)-catechin. High content of antioxidants in *C. caudatus* may be helpful in certain activities such as ability to reduce oxidative stress (Shui *et al.*, 2005).

Previous study reported the antimicrobial activity of *C. caudatus* leaf extracts and from the preliminary antimicrobial screening, it showed inhibition by the *n*-hexane, diethyl ether, and ethanol extracts against 5 microbial strains comprise of 2 Gram positive bacteria: *Bacillus subtilis*, *Staphylococcus aureus*, 2 Gram negative bacteria: *Escherichia coli, Pseudomonas aeruginosa* and 1 fungi: *Candida albicans* by the disc diffusion method. Minimun inhibitory concentration (MIC) values ranged from 6.25 - 25 mg/mL for the tested crude extracts, and this proved *C. caudatus* Kunth could be a potential source of new antimicrobial agents especially to treat infections caused by the tested microbial strains and proved the traditional uses of this herb (Rasdi *et al.*, 2010).

Previous study also showed that *C. caudatus* methanolic extract has cytotoxic effect on breast cancer cell line (T47D) using methylthiazolyldiphenyl-tetrazolium bromide (MTT) method. The result showed the dose dependant activity and the IC_{50} of the extract was 344.915 µg/mL. The cells were identified suffering apoptosis after a double staining test. It has been known that *C. caudatus* methanolic extract contain some aglicone flavonoids and quercetin glycoside that have been reported to possess anticancer property (Pebriana *et al.*, 2008).

In a study, costunolide, stigmasterol, lutein and 4,4'-bipyridine from *C. caudatus* chloroform extract were tested for antimutagenicity potential and antimicrobial activity. Results of the study indicated that at a dosage of 8.0 mg/kg body weight, costunolide reduced the number of micronucleated polychromatic erythrocytes (MPCE) induced by mitocycin C by 85 % by the micronucleus test. For

stigmasterol and lutein, they indicated a 79 and 81 % reduction in MPCE. On the other hand, 4,4'-bipyridine did not exhibit antimutagenic activity. Antimicrobial tests showed complete inhibitory activity against *Staphyloccus aureus* and *Saccharomyces cereviseae*, partial inhibitory activity against *Bacillus subtilis*, slight inhibitory activity against *Candida albicans* and negative inhibitory activity against *Escherichia coli* and *Pseudomonas aeruginosa* at concentrations of 100 µg/mL and 1 µg/mL for costunolide while for stigmasterol and 4,4'-bipyridine, they indicated slight inhibitory activity against *C. albicans* and *S. cereviseae* and negative inhibitory activity against *S. aureus, E. coli*, *B. subtilis* and *P. aeruginosa*. In the earlier anti microbial study, lutein showed high activity in antimicrobial test (Ragasa *et al.*, 1997).

2.2 *Centella asiatica*

Centella asiatica (L.) Urban or known as 'pegaga' belongs to the Apiaceae (Umbelliferae) family and it contains about 20 different species. *Hydrocotyle asiatica* L. is the other name that is most commonly found and usually used as its synonym in scientific work or research papers. Different country use different common name to describe *Centella asiatica* (Table 2.1).

C. asiatica found mostly in Southeast Asia, Sri Lanka, in parts of China, in the western South Sea Islands, Madagascar, South Africa, in the southeast of the U.S.A., Mexico, Venezuela and Columbia, as also in the eastern regions of South America. This herb grows at altitudes between 0 and 2500 metres above sea level in moist and dense shade area. Usually, for cultivated *C. asiatica*, it can be harvested 6 months after planting, and at any time throughout the year (Brinkhaus *et al.*, 2000).

Table 2.2 List of other common names for *Centella asiatica* (Brinkhaus *et al.*, 2000)

Country	**Name**
Chinese	Luei Gong Gen,Tungchian
English	Indian Pennywort
French	Hydrocotyle asiatique
German	Asiatischer Wassernabel
Indonesian	Kaki kuda, Pegagan, Antanan gede, Gagan-gagan, Gang-gagan, Kerok batok, Panegowan, Rendeng, Calingan rarnbar, Kos tekosan, Pagaga, Tungke-tungke, Papaiduh, Pepiduh, Piduh, Puhe beta, Kaki kuta, Tete karo, Tete, Kadho
Italian	Idrocotile
Japanese	Tsubo-kusa
Spanish	Blasteostimulina (asiaricoside)

"*C. asiatica* is a slender creeping herb with long-stalked, green reniform leaves with rounded apices and a smooth texture with palmate netted veins. The petiole is relatively long, up to 20 cm. Its flowers are sessile, white or reddish and hermaphrodite. Each flower contains 2 styles and 5 stamens which is mericarp in nature. The rootstock consists of rhizome which is growing vertically down while the stolon grows horizontal and interconnecting one plant to another" (Zhang, 2009).

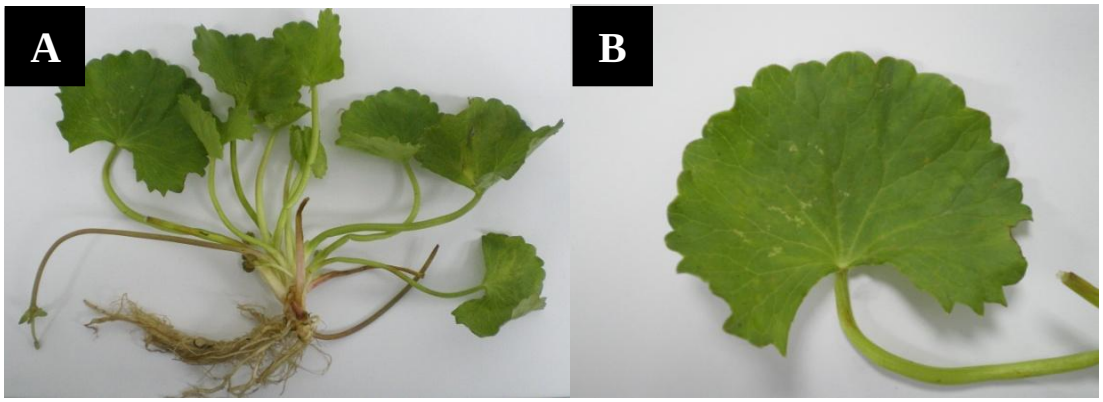


Figure 2.2 Picture of *Centella asiatica.* A) the whole plant of *Centella asiatica* and B) the leaf of *Centella asiatica*

2.2.1 Taxonomy

Kingdom	: Plantae
Subkingdom	: Viridaeplantae
Infrakingdom	: Streptophyta
Division	: Tracheophyta
Subdivision	: Spermatophytina
Infradivision	: Angiospermae
Class	: Magnoliopsida
Superorder	: Asteranae
Order	: Apiales
Family	: Apiaceae
Genus	: Centella L.
Species	: *Centella asiatica* (L.) Urban

(*Integrated Taxonomy Information System ITIS*)

2.2.2 Review of Chemical Constituents of *Centella asiatica*

Tabel 2.3 shows the list of chemical constituents of *Centella asiatica* and its classes.

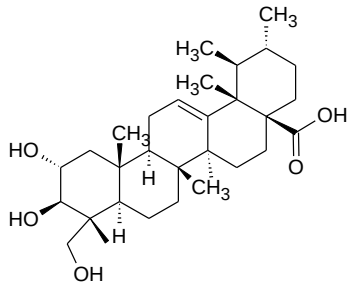
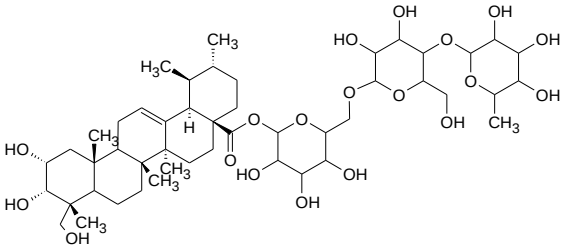
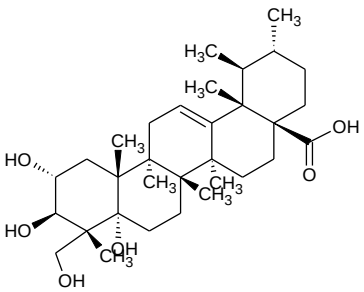
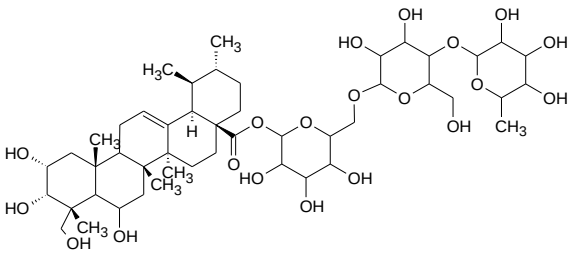
Table 2.3 Chemical constituents of *Centella asiatica* (Chong and Aziz, 2011)

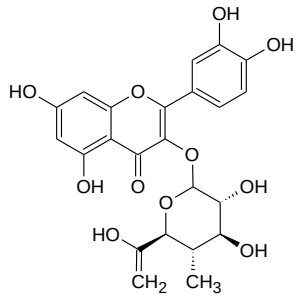
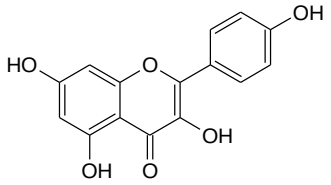
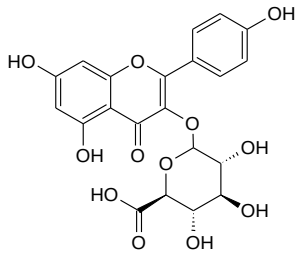
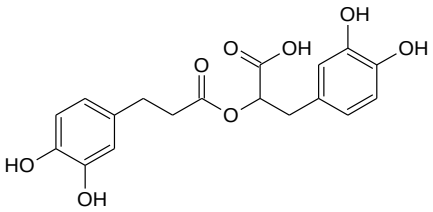
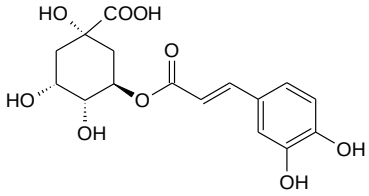
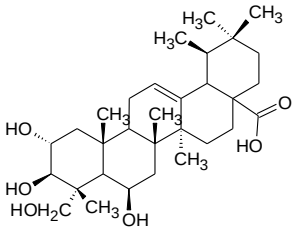
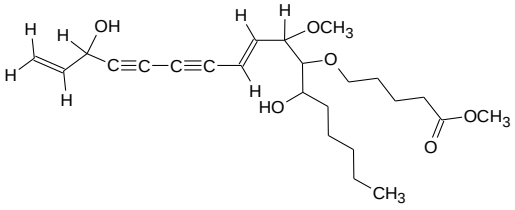
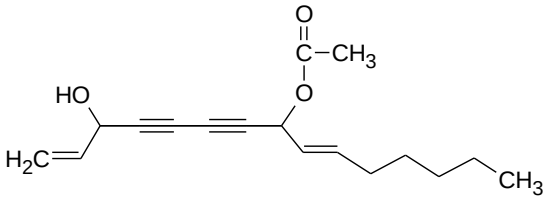
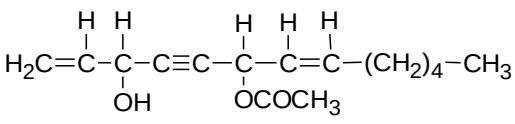
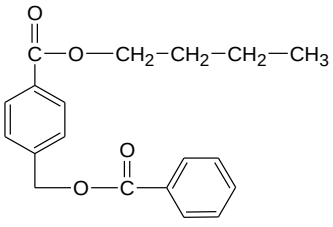
Classes of chemical constituents		**Chemical constituents**
Monoterpenes	Acyclic monoterpenes	3-nonen-2-one
	Monocyclic monoterpenes	Linalool, myrcene, γ-terpinene, terpinolene, limonene, α-terpinene, α-phellandrene, ρ-cymene, terpinen-4-ol, pulegone, menthone, methyl carvacrol, methyl thymol,
	Bicyclic monoterpenes	α-thujene, α-pinene, β-pinene, camphene, bornyl acetate, chrysanthenyl acetate
Sesquiterpenes	Acyclic sesquiterpenes	Trans-β-farnesene, decan-1-ol
	Monocyclic sesquiterpenes	Germacrene a, germacrene b, germacrene d, β-elemene, γ-elemene, γ-curcumene, bicyclogermacrene, bicycloelemene, humulene epoxide, α-humulene
	Bicyclic sesquiterpenes	Epibicyclosesquiphellandrene, α-cadinene, δ-cadinene, caryophyllene oxide, β-caryophyllene, β-acoradiene
	Tricyclic sesquiterpenes	Spathulenol, allo-aromadendrene, viridiflorol, epiglobulol, mintsulfide, α-copaene
Diterpenes	Acyclic diterpenes	Neophytadiene
Triterpenes	Ursane-type pentacyclic triterpenes	Asiatic acid, madecassic acid, brahmic acid, 6β-hydroxyasiatic acid, 2α, 3α-dihydroxyurs-12-en-28-oic acid, 2α, 3β, 23-trihydroxyurs-20-en-28-oic acid, 2α,

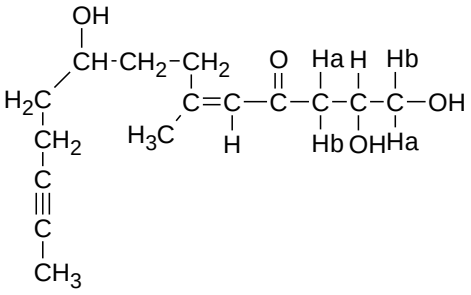
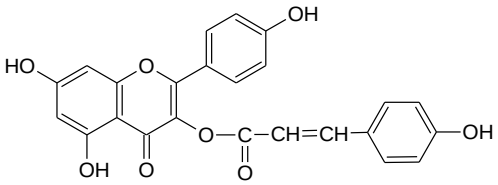
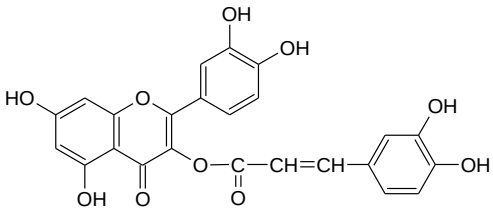
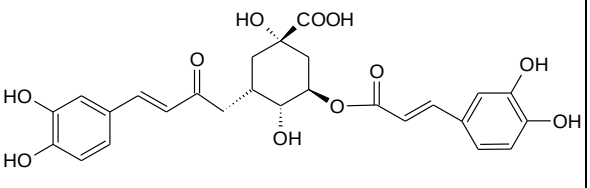
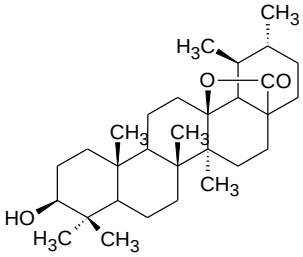
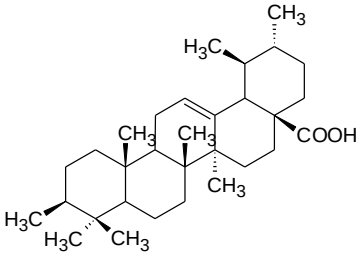
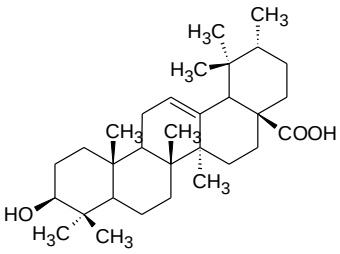
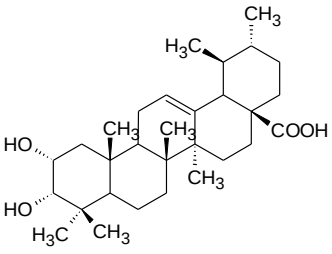
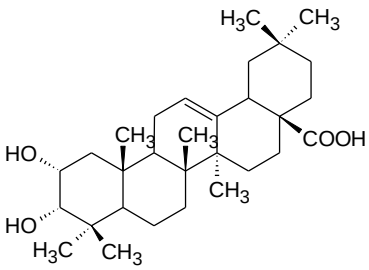
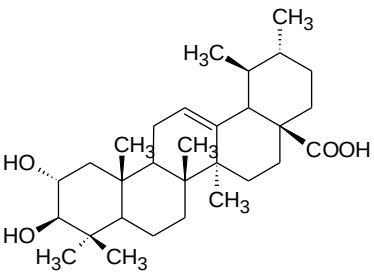
		3β, 20, 23-tetrahydroxyurs-28-oic acid, pomolic acid, corosolic acid, ursolic acid
	Ursane-type pentacyclic triterpenes saponins	Asiaticoside a, madecassoside, brahminoside, asiaticoside c, asiaticoside d, asiaticoside e, asiaticoside f, asiaticoside, 2α, 3β, 23-trihydroxyurs-20-en-28-oic-acid o- α-l-rhamnopyranosyl-(1→4) -o-β-d-glucopyranosyl-(1→6)-o-β-d-glucopyranosyl ester, centellasaponin b, centellasaponin c, scheffuroside b, 3-o-[α-l-arabinopyranosyl] 2α, 3β, 6β, 23-α tetrahydroxyurs-12-ene-28-oic acid, 23-o-acetylmadecassoside
	Oleanane-type pentacyclic triterpenes	3-epimaslinic acid, terminolic acid
	Oleanane-type pentacyclic triterpenes saponins	Asiaticoside b, centellasaponin d, scheffoleoside a, 23-o-acetylasiaticoside b
	Steroid type triterpenes	Stigmasterol, sitosterol, campesterol, sitosterol 3-o-β-glucoside, stigmasterol 3-o-β-glucoside
Tetraterpenes		β-carotene
Phenols	Flavonoids	Kaempferol, kaempferol-3-o-β-d-glucuronide, castilliferol, quercetin, quercetin-3-o-β -d-glucuronide, castillicetin
	Phenylpropanoids	Rosmarinic acid, chlorogenic acid, 3,4-di-o-caffeoyl quinic acid, 1,5-di-o-caffeoyl quinic acid, 3,5-di-o-caffeoyl quinic acid, 4,5-di-o-caffeoyl quinic acid, isochlorogenic acid
	Tannin	Tannin, phlobatannin
Carbohydrate	Monosaccharide	Glucose, mesoinositol
	Oligosaccharide	Centellose
	Polysaccharide	Pectin, arabinogalactan

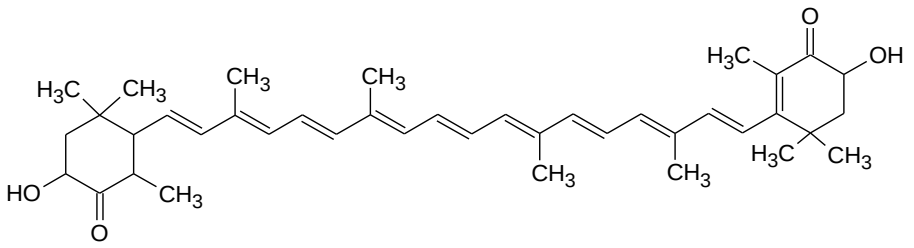
Vitamin	Ascorbic acid, nicotinic acid, β-carotene
Mineral	Calcium, phosphorus, iron, potassium, calcium, magnesium, manganese, zinc, sodium, copper
Amino acid	Alanine, arginine, aspartic acid, glutamic acid, leucine, iso-leucine, valine, methionine, lysine, histidine, tyrosine, phenylalanine, threonine, glycine, serine, threonine, proline, cystine
Polyacetylene	8-acetoxycentellynol, cadiyenol, dotriacont-8-en-1-oic acid, 11-oxoheneicosanyl, cyclohexane
Others	Asiaticin, centellicin, centellin

Table 2.4 Chemical structure of *Centella asiatica* compounds

 asiatic acid	 asiaticoside
 madecassic acid	 Madecassoside

 quercetin-3-O-glucuronide	 kaempferol
 kaempferol-3-O-glucuronide	 rosmarinic acid
 chlorogenic acid	 terminolic acid
 cadiyenol	 8-acetoxycellynol
 centellin	 asiaticin

 centellicin	 castiliferol
 castilicetin	 isochlorogenic acid
 ursolic acid lactone	 ursolic acid
 pomolic acid	 2α,3α-dihydroxyurs-12-en-28-oic acid
 3-epimaslinic acid	 corosolic acid



β –carotene

2.2.3 Traditional uses of *Centella asiatica*

C. asiatica is traditionally used to treat burns, cough, dermatitis, diarrhea, dysmenorrhea, hepatitis, syphilis, jaundice, anemia, epilepsy, bronchitis, cholera, constipation, asthma, measles, smallpox, rheumatism, epistaxis, hypertension, leucorrhoea, nephritis, urethritis, toothache, fever, leprosy and wounds. It is also used as a tonic and an analgesic. Other than that, this herb also used in the therapy of albinism, anemia, cellulite, dizziness, dysentery, dysmenorrhea, dysuria, haematemesis, hemorrhoids, neuralgia, and varices; and as an antipyretic and anti inflammatory (Zhang, 2009).

2.2.4 Review of Biological and Pharmacological Activities of *Centella asiatica*

A study on anti-tumor effect of methanol extract from the whole plant of *C. asiatica* and its partially purified acetone fractions (AF) using Ehrlich ascites tumour cells (EAC) and Dalton's lymphoma ascites tumour cells (DLA) showed IC_{50} of methanolic extract was found to be 62 µg/mL for EAC and 75 µg/ml for DLA, and that of AF was 17 µg/mL for EAC and 22 µg/mL for DLA for the short term in vitro cytotoxicity (Babu *et al.*, 1995).

Another study showed that methanolic extract of *C. asiatica* induced apoptosis in human hormone sensitive and invasive breast cancer cell line (MCF-7)

as indicated by nuclear condensation, increased annexin staining, loss of mitochondrial membrane potential and induction of deoxyribonucleic acid (DNA) breaks identified by TdT-mediated dUTP-biotin nick end labelling (TUNEL) reactivity. This extract and asiatic acid inhibited the proliferation of MCF-7, in a concentration dependent manner and it showed that LD_{50} (median lethal dose) value of this extract was found to be 66 mg for MCF-7. The highest concentration of the extract (82 mg) inhibited MCF-7 cell growth almost equivalent to growth inhibition obtained by 10 mM tamoxifen which is a known antiestrogen drug currently used in breast cancer patients, while 10 µM asiatic acid induced ~95 % cell death in 48 h. This showed that methanolic extract possess only moderate cytotoxicity compared to the higher cytotoxicity of asiatic acid (Babykutty *et al.*, 2008).

Madecassoside contained in *C. asiatica* extract has significant wound-healing activity and is one of the major reasons for the use of *C. asiatica* in the successful treatment of burn injury (Liu *et al.*, 2008). The extract of *C. asiatica* increased the wound breaking strength and it showed the effect of attenuating the known effects of dexamethasone healing that can overcome the wound-healing suppressing action in a rat model (Shetty *et al.*, 2006). The drug "Titrated Extract from *C. asiatica*" (TECA), used for its stimulating properties on the healing of wounds, is a mixture of 3 terpenes including asiatic acid, madecassic acid and asiaticoside. Asiatic acid was found to be the only component responsible for collagen synthesis stimulation and asiaticoside exerted a preferential stimulation of collagen synthesis (Maquart *et al.*, 1990; Maquart *et al.*, 1999; Shukla *et al.*, 1999).

The healing effects of *C. asiatica* water extract and asiaticoside, an active constituent of *C. asiatica* was suggested as anti-gastric ulcers drugs. Previous study showed that this extract and asiaticoside can accelerate the healing of gastric kissing

ulcers (Cheng *et al.*, 2004). They were found to promote angiogenesis, facilitate epithelial proliferation, and suppress Myeloperoxidase (MPO) activity during ulcer healing stage. Results were observed after 0.25 g/kg *C. asiatica* water extract or 10 mg/kg asiaticoside administration, and it proved that asiaticoside was the most active ingredient that can enhance ulcer healing (Cheng *et al.*, 2004). From another study, oral administration of *C. asiatica* water extract (0.05 g/kg, 0.25 g/kg and 0.50 g/kg) before ethanol administration significantly inhibited gastric lesions formation by strengthening the mucosal barrier (58% to 82% reduction) and decreased mucosal MPO activity (Cheng and Koo, 2000).

Madecassoside could protect human umbilical vein endothelial cells (HUVECs) from oxidative injury, which was probably achieved by inhibiting cell apoptosis via protection of mitochondria membranes and down regulation of the activation of caspase-3 and p38 mitogen-activated protein kinase (MAPK). Madecassoside (10, 30, 100 μmol/L) could reverse morphological changes, elevate cell viability, increase glutathione levels, and decrease lactate dehydrogenase and malondialdehyde levels caused by hydrogen peroxide (H_2O_2) in a dose dependent manner. It attenuated apoptosis, preventing the activation of caspase-3 and the loss of mitochondrial membrane potential, as well as the phosphorylation of p38 MAPK in HUVECs (Bian *et al.*, 2012).

One of the previous studies proved that *C. asiatica* water extract that contains asiaticoside, had a promising antibacterial effect against *Staphylococcus aureus* compared to hexane and ethanol extracts (Taemchuay *et al.*, 2008). Total triterpenoid fraction of *C. asiatica* (TTFCA) is effective in venous insufficiency, reducing ankle edema, foot swelling, and capillary filtration rate and by improving microcirculatory parameter. It displayed a significant activity in venous hypertensive microangiopathy

and its effects are dose-dependent (Cesarone *et al.*, 1994; Cataldi *et al.*, 2001; Incandela *et al.*, 2001a).

The methanol and ethyl acetate extracts from the aerial part of *C. asiatica* as well as the pure asiaticoside, can impart anxiolytic activity (Wijeweera *et al.*, 2006). Previous study demonstrated anxiolytic effect of a standardized extract of *C. asiatica* containing triterpenoids not less than 80% (CAe) in both acutely and chronically stressed animals. These effects could be mainly accounted by madecassoside and asiaticoside, suggesting a possible use of CAe for the treatment of both acute and chronic anxiety in the pathological state (Wanasuntronwong *et al.*, 2012).

The total triterpenes from *C. asiatica* had shown antidepressant activity (Chen *et al.*, 2003). In the tail suspension and forced swimming test of male mice, asiaticoside significantly decreased immobility time and these results suggested that asiaticoside may have antidepressant-like action (Liang *et al.*, 2008).

From another earlier study, *C. asiatica* showed high antioxidant activity especially in ethanol extract from different parts (roots, petioles and leaves) of *C. asiatica*. Higher antioxidant activity was shown in the roots of *C. asiatica* compared to leaves or petioles in all types of solvent used (Hamid *et al.*, 2002). In other study, the antioxidant levels and enzymes activity were found to increase significantly in both the liver and kidney after oral treatment with crude methanolic extract of *C. asiatica* on lymphoma-bearing mice (Jayashree *et al.*, 2003). *C. asiatica* also showed high antioxidative activity in leaves and root extracts (Hamid *et al.*, 2002; Pittella *et al.*, 2009; Jayashree *et al.*, 2003; Zainol *et al.*, 2003).

Previous study showed the radioprotective activity in *C. asiatica* extract and this histologic finding indicates that tetrandrine and madecassol are able to reduce acute radiation reactions by their anti-inflammatory activity (Chen *et al.*, 1999;