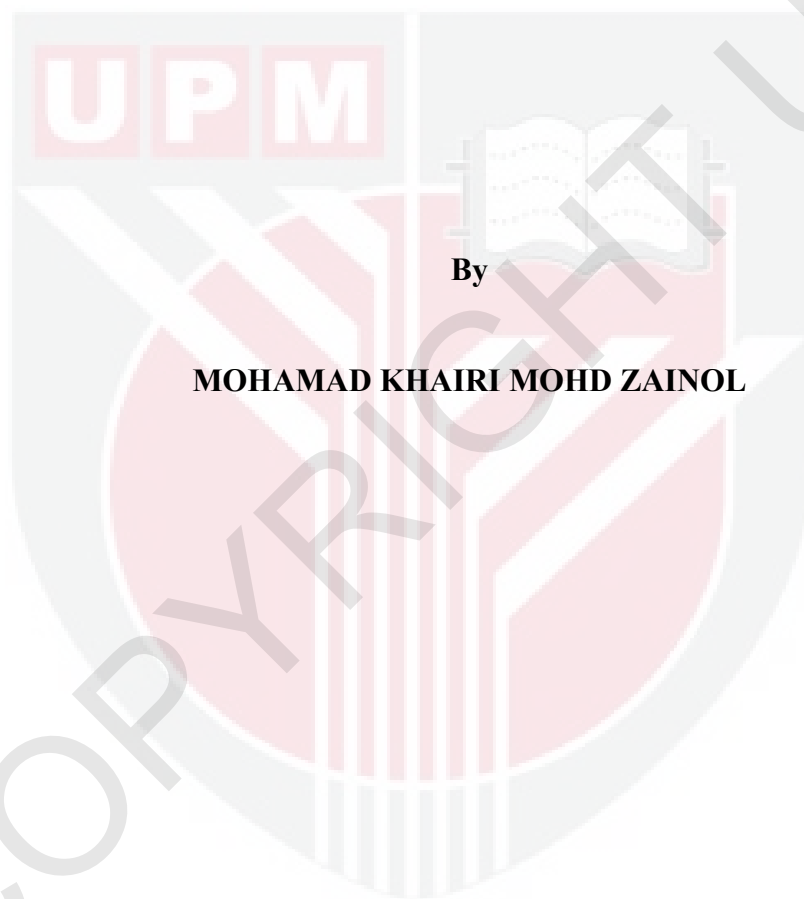


**DETERMINATION OF FLAVONOIDS IN *Centella asiatica* (L.) URBAN AND
THEIR UTILIZATION IN HERBAL NOODLE**



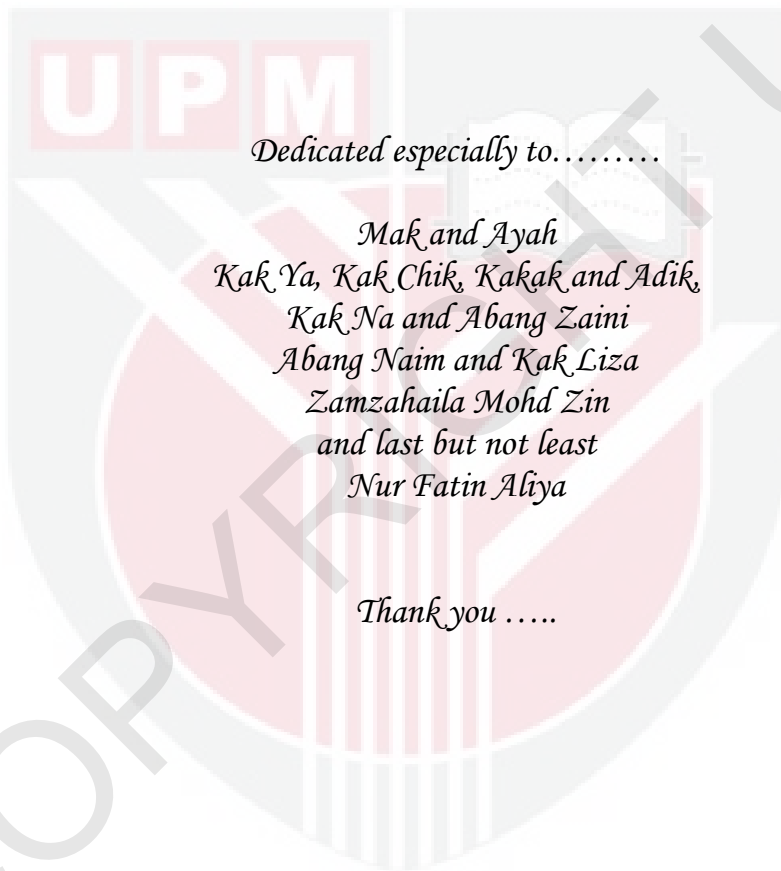
By

MOHAMAD KHAIRI MOHD ZAINOL

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfilment of the Requirements for the Degree of Master of Science**

August 2004

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Dedicated especially to.....

*Mak and Ayah
Kak Ya, Kak Chik, Kakak and Adik,
Kak Na and Abang Zaini
Abang Naim and Kak Liza
Zamzahaila Mohd Zin
and last but not least
Nur Fatin Aliya*

Thank you

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

DETERMINATION OF FLAVONOIDS IN *Centella asiatica* (L.) URBAN AND THEIR UTILIZATION IN HERBAL NOODLE

By

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Chairman : Associate Professor Azizah Abdul Hamid, PhD

Faculty : Faculty of Food Science and Technology

'Pegaga' or *Centella asiatica* has been medically recognized to be effective in the treatment of skin diseases and many other illnesses. Due to the presence of abundant amount of active compounds, *C. asiatica* is potentially a good source to alleviate or prevent the development of chronic diseases, cancer and others. The objective of this study was to develop and optimize a functional food namely *C. asiatica* herbal noodle containing flavonoids from *C. asiatica*. The study included determination and quantification of flavonoids (bioactive compounds) and selection of the best formulation using RSM to develop the product containing *C. asiatica*. The first stage of the study was conducted to evaluate the highest antioxidative activity and total phenolic compounds in different parts namely leaf, root and petiole among four different accessions of *C. asiatica* namely Ca 10, CA 05 Ca 08 and CA 11 with the objective to determine the best accession that would then be used in the developed products. Methanol was used as an extraction solvent and antioxidative assay was biochemically done using ferric thiocyanate method (FTC) and thiobarbituric acid test (TBA) while total phenolic compounds (TPC) was determined using the Folin-

Ciocalteu phenol method. Results from FTC and TBA test showed that no significant ($p < 0.05$) difference was exhibited in antioxidative activity between leaf extract of *C. asiatica* especially CA 05 as compared to that of α -tocopherol and BHT while petiole extracts showed somewhat negligible activity. On the other hand, the antioxidative activities measured correlated well with total phenolic compounds (TPC) for leaf extract of CA 05. Therefore leaf of CA 05 was used for further analysis to determine the compound responsible for the high antioxidative activities. The results also suggested that more than one compound attributed to antioxidative activity of different parts of *C. asiatica* and flavonoids are one of phenolic compounds that were known to be potent antioxidant that might be responsible for the antioxidative activities in *C. asiatica*. Further study was done to determine the stability of flavonoids (catechin, quercetin and rutin) during drying and to identify the optimum drying method that could sustain the most flavonoids in *C. asiatica* which were then incorporated into food product. Reverse-phase high-pressure liquid chromatography (RP-HPLC) with a Symmetry C₁₈ column (Waters, USA) and a water/methanol mobile phase was used to determine individual flavonoids in leaf *C. asiatica* CA 05. The results showed that vacuum oven drying method was the optimum treatment among the different drying techniques based on factors of cost and yield of flavonoids sustained. The next area of study was focused on incorporating *C. asiatica* into the product, analyzed for bioactive compound present in it and tested for consumer acceptance. Response surface methodology (RSM) using Central Composite Design with quadratic model was used in the product formulation, development and optimization. Three independent variables were chosen (*C. asiatica* extract, salt and sodium hydroxide or 'air abu') in the development and optimization of the product. Finally, the products were analyzed

for the presence of flavonoids and the sensory evaluation (dependent variables). *C. asiatica* herbal noodles containing pegaga extract (formulation of: 10% *C. asiatica* extract, 5g salt and 5g 'air abu') was the most optimum product based on the dependent variables collected. This research would then be able to serve as reference for new products in the food industry and wider the selection of consumption of natural products.



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

**PENGenalPASTIAN BAHAN FLAVONOID DARI *Centella asiatica* (L.)
URBAN DAN PENGGUNAANNYA DALAM MEE HERBA**

Oleh

MOHAMAD KHAIRI MOHD ZAINOL

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Fakulti : Sains and Teknologi Makanan

'Pegaga' atau *Centella asiatica* telah dikenali sebagai tumbuhan yang efektif untuk mengubati penyakit kulit, hipertensi dan lain-lain. Kajian ini dijalankan untuk menghasilkan makanan berfungsi yang mengandungi flavonoid daripada *C. asiatica*. Kajian ini mencangkupi penentuan dan mengenalpastian kuantiti flavonoid di dalam *C. asiatica* serta pemilihan formulasi terbaik untuk menghasilkan makanan berfungsi tersebut. Peringkat awal kajian telah melibatkan penentuan tahap aktiviti antioksidan dan kandungan bahan fenol dalam daun, akar dan batang pegaga daripada empat jenis pegaga yang berbeza iaitu CA 01, CA 05, CA 08 dan CA 11, untuk mengenalpasti bahagian dan jenis pegaga terbaik yang akan digunakan dalam penghasilan produk herba iaitu mee herba pegaga. Analisis antioksidan dijalankan dengan menggunakan kaedah tiocianat ferik (FTC) dan ujian asid tiobarbiturik (TBA). Aktiviti antioksidan daripada ekstrak-ekstrak ini kemudiannya akan dibandingkan dengan antioksidan piawai α -tokoferol dan toluena hidroksida butilat (BHT). Manakala kandungan bahan fenol (TPC) dapat ditentukan dengan menggunakan kaedah Folin-Ciocalteu fenol. Keputusan daripada kaedah FTC and

TBA menunjukkan tiada perbezaan yang signifikan ($p < 0.05$) ditunjukkan dalam aktiviti antioksidan antara ekstrak daun pegaga terutamanya CA 05 berbanding α -tokoferol dan BHT. Selain itu, aktiviti antioksidan yang telah diukur juga menunjukkan korelasi yang baik dengan kandungan bahan fenol (TPC) terutamanya untuk bahagian daun CA 05. Keputusan ini mencadangkan daun CA 05 boleh digunakan sebagai sumber bahan aktif dalam penghasilan makanan berfungsi. Selain itu, didapati bahan fenol juga mungkin bertanggungjawab dalam aktiviti antioksidan yang tinggi dalam pegaga terutamanya didalam daun CA 05. Kajian ini mencadangkan bahawa kemungkinan lebih daripada satu bahan aktif yang bertanggungjawab dalam aktiviti antioksidan didalam bahagian-bahagian berbeza pegaga manakala flavonoid, bahan fenolik yang diketahui mempunyai tahap aktiviti antioksidan yang tinggi, berkemungkinan merupakan bahan yang paling memainkan peranan dalam aktiviti antioksidan *C. asiatica*. Kajian seterusnya melibatkan tahap kestabilan flavonoid (katekin, kuersetin dan rutin) di dalam pegaga terhadap pengeringan dan mengenalpasti teknik pengeringan yang dapat meminimalkan kehilangan flavonoid, yang seterusnya akan dimasukkan kedalam produk. Kromatografi cecair tekanan tinggi fasa terbalik (RP-HPLC) dengan kolum Symmetry C₁₈ (Waters, USA) dan fasa gerak air/metanol telah digunakan untuk mengenalpasti kehadiran flavonoid didalam daun *C. asiatica* CA 05. Pengeringan dengan bervakum dikenalpasti sebagai teknik terbaik untuk pengeringan daun pegaga dalam usaha untuk mengurangkan kehilangan flavonoid berdasarkan kepada faktor kos dan banyaknya hasil. Kajian seterusnya melibatkan penghasilan makanan berfungsi (mee herba) yang ditambah bahan aktif daripada *C. asiatica* dan seterusnya menganalisa kandungan biomarkernya dan juga tahap penerimaan pengguna. Kaedah metodologi respon permukaan (RSM) menggunakan teknik *Central Composite*

Design (CCD) dengan persamaan kuadrat digunakan dalam formulasi, penghasilan dan pengoptimuman produk. Tiga pembolehubah tetap telah dipilih iaitu ekstrak *C. asiatica*, garam and natrium hidroksida atau 'air abu') dalam penghasilan mee herba pegaga tersebut. Seterusnya, produk yang telah disediakan dianalisa untuk mengenalpasti kandungan bahan aktif, dan kajian ujirasa terhadap produk-produk yang dihasilkan telah dijalankan untuk mengukur kandungan nutrien dan tahap penerimaan produk. Berdasarkan ciri-ciri fizikal dan sensori mee yang dianalisa, mee yang menggunakan formulasi (10% ekstrak pegaga, 5g garam dan 5g air abu) menunjukkan hasil yang optima berdasarkan kepada data-data daripada ujikaji (analisis biomarker dan tahap penerimaan pengguna). Kajian ini seterusnya boleh dijadikan sebagai rujukan dalam usaha menghasilkan produk-produk baru dalam industri makanan terutamanya produk herba.

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I certify that an Examination Committee met on 28th August 2004 to conduct the final examination of Graduate Student on her Master of Science thesis entitled “Determination of flavonoids in *Centella asiatica* (L.) Urban and their utilization in herbal noodle” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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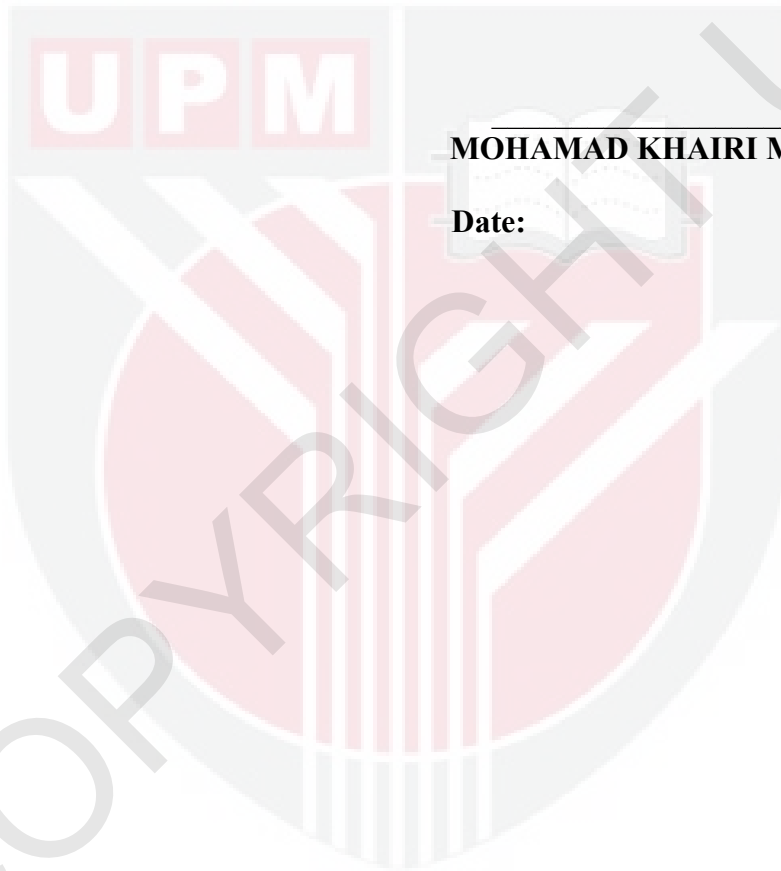
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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any degree at UPM or other institutions.



MOHAMAD KHAIRI MOHD ZAINOL

Date:

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LIST OF ABBREVIATIONS

AOA	Antioxidative activities
AE	Antiradical efficiency
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CCD	Central composite design
C. I. E.	Commision Internationale de L'Eclairage
DPPH	2,2-diphenyl-1-picrylhydrazyl
FTC	Ferric thiocyanate
h	Hour
HCl	Hydrochloric acid
HPLC	Reverse phase high pressure liquid chromatography
L	Liter
LDL	Low density lipoprotein
mg	Milligram
ml	Milliliter
nm	Nanometer
mm	Millimeter
µg	Microgram
µm	Micrometer
mM	MilliMolar
MAD	Malonaldehyde
MeOH	Methanol
Min	Minutes
N	Normality
Na DEDTC	Sodium diethyldithiocarbamic
PG	Propyl gallate
RP-HPLC	Reverse phase high pressure liquid chromatography
RSM	Response surface methodology
rpm	Revolution per minute
SAS	Statistical Analysis System
TFA	Trifluoroacetic acid
TBA	Thiobarbituric acid
UV	Ultraviolet
UV/Vis	Ultraviolet / visible
v/v	Volume per volume.
w/v	Weight per volume

CHAPTER 1

GENERAL INTRODUCTION

For thousand of years, plants have been used in maintaining and improving the quality of human life. The World Health Organization (WHO) estimated that 80 % of earth's inhabitants rely on traditional medicine for their primary health care needs, and the use of these therapies involves plant extracts or their biochemically active components (Vimala and Adenan, 1999).

Malaysian rainforest offers a rich diversity of species of herbal and medicinal plants, which are largely unexplored. The total area of the rainforest is estimated to be 19.12 million hectares, which covers more than 58% of the country's land area. This area contains more than 20,000 plant species, including 2,000 that have been reported to possess medicinal values (Jaganath and Ng, 1999). Therefore, the potential for discovering useful and valuable products for food, health, cosmetics, flavour and fragrance, industrial biochemical's, herbal remedies and pharmaceuticals are tremendous. One of the discoveries of medicinal plants today is 'pegaga' or scientifically known as *Centella asiatica* (L.) Urban. This herb was recommended by the World Health Organization (WHO) (2004) as one of the most important medicinal plant species to be conserved and cultivated.

C. asiatica or 'pegaga' is a slender, creeping plant and rooting at the nodes, grows wildly in both tropical and sub-tropical countries. They are known as one of the local herbs that is claimed to possess various physiological effects. Reports from different

places revealed that *C. asiatica* has been used for various ailments, including wound healing, memory improvement, treating mental fatigue (Goh *et al.*, 1995), bronchitis, asthma, dysentery, leucorrhoea, kidney trouble, urethritis (Jaganath and Ng, 1999), antiallergic, anticancer, curing leukorrhea and toxic fever (Kan, 1986). It is also commonly used as porridge to combat nutritional deficiencies among pre-school children in Sri Lanka (Cox *et al.*, 1993). In the Chinese Pharmacopoeia, *C. asiatica* is used as antipyretic, diuretic, and antidote for the treatment of heatstroke, diarrhoea, eczema and snake's bite (Jaganath and Ng, 1999).

The decoction of leaves was used to treat leprosy and rheumatism, while infusion of the toasted leaves or juices extracted from the leaves, together with food, is used to relieve minor dysentery in children (Goh *et al.*, 1995). The poultice leaves are used to treat sores, or pound leaves into a paste to apply it to the body for fever. Juices from the roots are used to clean ulcerous wounds (Goh *et al.*, 1995).

Nowadays, synthetic antioxidants have restricted use in food industries as regarding to various studies that shown them to be carcinogenic and mutagenic agents (Madhavi *et al.*, 1995). This finding is especially true for butylated hydroxyl anisole (BHA) and butylated hydroxy toluene (BHT). Thus, with increasing consciousness of consumers with regards to safety of food additives and the higher manufacturing cost and lower efficiency of natural antioxidant, a need for identifying alternative natural and safer sources of food antioxidant is created (Wanasundara and Shahidi, 1998). Therefore, search for natural antioxidant, especially of plant origin, has greatly increased in recent years (Lörliger, 1993). Antioxidative compounds obtained from natural sources such as grains, oilseeds, beans, leaf waxes, bark, roots, spices,

fruits and vegetables have been widely investigated (Chen *et al.*, 1996). According to Pratt (1992), most of these bioactive compounds can be easily found in wood, bark, stem, leaf, fruit, root, flower and seed of many higher plants. All these studies also supported by experimental results that suggest the plants contain high antioxidant pigments such as carotenoids, lycopenes and flavonoids, which are produced naturally by the plants in order to protect against oxidative damages. (Wanasundara and Shahidi, 1998).

Recent researches have suggested that diets rich in phenolic compounds are always associated with longer life expectancy (Hertog and Hollman, 1996). It is an established fact that phenolic compounds, such as flavonoids, anthraquinones, anthocyanidins and xanthenes possess remarkable antioxidative activities. Epidemiological studies had shown that high consumption of fruits and vegetables containing flavonoids lower the mortality rate from chronic diseases (Hertog *et al.*, 1992). They also reported that flavonoids from fruits and vegetables would give some cardioprotective effects in the Zutphen Elderly study. Flavonoids are ubiquitously found in all edible plants and reported to be potent antioxidants, antitumor, antibacterial, anticoagulant, antiplatelete, antiinflammation and vasodilating properties. Among the common flavonoids studied for their antioxidative activities are known as catechins, flavonols (quercetin, myricetin and kaempferol), apigenin and luteolin.

Growing concern about the side effects and limitations of modern scientific medicines have lead to a renewed curiosity about 'natural' products. Consumers are now interested in 'natural' products, as part of an increased focus on health, fitness,

and well being. They perceived natural ingredients as 'good' and 'pure' and botanical medicines as more 'natural' and better for the patients and more friendly to the environment than synthetic drugs. Previous report by Ministry of Agriculture Malaysia in 1999, the total sales of the natural segment are estimated at 10 % of the US\$ 65 billion personal care and cosmetic market estimates between 8-10 % per year compared with the synthetic ingredient-based segment of 3-5 %. Therefore, it is interesting and worthwhile to investigate and identify a safer, 'natural' product from tropical edible higher plants.

Functional foods or nutraceuticals can be defined as any food that contains substances that can be considered as the whole food that provides medical or health benefits, including prevention and treatment of diseases (Goldberg, 1994). They may range from isolated nutrients, dietary supplements and diets to 'genetically' engineered 'designer' food, herbal products and processed products such as cereals, soup and beverages (Pszczola, 1992). The food may contain physiologically active ingredients including fibres, polysaccharides, fatty acids, peptides, proteins, antioxidants, probiotics, vitamins, minerals, phytochemicals, antimicrobials and others. There are two types of functional foods namely fermented foods and foods containing disease-preventing phytochemicals (Pszczola, 1992).

The major proponents of herbal products view herbs as medicine in a way that is fundamentally different from herbalists. Herbal products can ensure that sufficient amounts of the herb's constituents or the biomarkers are present to deliver an efficacious product. Another reason favoring the use of herbal products is that they assure positive identification that the active is what it is claimed to be. Natural or

herbal products do have advantages yet it needs consistently strong evidence with guaranteed constituents in it such as the biomarkers (Peters *et al.*, 1998).

When it is necessary to establish the positive identification of compounds, high-pressure liquid chromatography (HPLC) is normally used. These readings establish a fingerprint of the chemicals contained in the plant or extract. To establish the identity of a plant, matching biochemical fingerprints are all that is needed (Crozier *et al.*, 1997). However, it is possible to use the same process to only establish the presence of the marker compound of standardized products. Therefore, the objectives of this research were to:

- To evaluate antioxidative activities of root, leaf and petiole of four different accession of *C. asiatica* (CA 01, CA 05 CA 08 and CA 11) and identify the compound responsible.
- To identify the drying technique that can sustain the optimum amount of compounds that are responsible to the antioxidative activities of *C. asiatica*
- To develop and optimize a herbal product namely *C. asiatica* herbal noodle base on RSM formulation that will contain significant amount of the biomarkers and also accepted by consumers.

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