STUDIES OF WATER-SOLUBLE EXTRACT OF *LABISIA PUMILA* VAR. *ALATA* OF SELECTED GEOGRAPHIC ORIGINS

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ABSTRACT

Labisia pumila (Myrsinaceae) or “kacip Fatimah”, is traditionally used in Malay traditional medicine in the form of decoction as postpartum tonic. Today, it is sold as functional food and beverage in response to consumer interest in its potential health benefits. However, nutrient data on this plant, for example, remains scarce. The aim of this research project was to determine the properties and attributes of water-soluble extracts of *Labisia pumila* var. *alata* to reveal its potential health benefits as functional food and beverage. The nutrient composition, total phenolic content, total flavanoid content and antioxidant activities of LP of various sources, *viz.*, Malaysian and Indonesian origins, were studied to determine the source with specific properties and attributes. PLS and PCA were then used to classify and to understand the correlation between total solid, total acidity, and solubility in cold and hot water, proximate properties, minerals, heavy metals, total phenolic content, and antioxidant activities of *Labisia pumila* var. *alata* leaves (LP) due to geographic origin. Based on results obtained, there were no significant difference in yield, total solid, total acidity, and solubility in cold and hot water of lyophilized LP extract due to geographic origin ($p>0.05$). The extract from Halimun-Salak Mountain, Bogor (LPB) was found to have the highest pH content. LPT was found to have the highest gallic acid value (1.86%), total phenolic content (140.49 mg GAE/g lyophilized plant material), antioxidant activity $_{(FRAP)}$ (17.57 mg GAE/g lyophilized plant material), $DPPH_{(EC50)}$ (78.79 µg/mL), protein (9.87±0.18 %), moisture (10.11±0.06 %), mineral Na (20.20 mg/100 g), K (1128 mg/100 g), Mg (476.1 mg/100g), and Zn (2.32 mg/100 g). The highest values of Ca and Fe were for LP from Pahang (LPR) at 265.8 mg/100g Ca and 41.61 mg/100g Fe, respectively. There was a significant difference ($p<0.05$) between lyophilized extracts of various geographic origins and proximate content, mineral and heavy metals composition, and antioxidant activities. The PCA models showed data correlated with of *Labisia pumila* var. *alata* of various geographic origins and proximate composition, mineral and heavy metals composition, and antioxidant activity. The first two factors (F1 and F2) accounted for 61.25% and 20.48%. The factors loading of F1 had a positive correlation with antioxidant activities (FRAP and DPPH), total phenolic content, moisture, and crude fiber. The strong positive loadings of F2 were total flavonoid content and lipid content. The cluster centroid in cluster analysis (CA) resulted in four groups of *Labisia pumila* var. *alata* samples based on geographic origins.

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ABSTRAK

Labisia pumila (Myrsinaceae) atau "kacip Fatimah", digunakan secara meluas di dalam perubatan tradisional Melayu dalam bentuk rebusan sebagai tonik selepas bersalin. Ketika ini, *Labisia pumila* var. *alata* dikomersilkan sebagai makanan dan minuman berfungsi memandangkan ianya mempunyai potensi dan memberi manfaat terhadap kesihatan. Bagaimanapun, sehingga hari ini data nutrisi bagi tumbuhan ini masih belum lengkap. Tujuan projek penyelidikan ini adalah untuk mengkaji ciri-ciri dan sifat-sifat ekstrak larut air dari *Labisia pumila* var. *alata* dalam merungkai manfaat kesihatan dan potensinya sebagai makanan dan minuman berfungsi. Komposisi nutrisi, jumlah kandungan fenolik, jumlah kandungan flavanoid dan aktiviti antioksidan daripada daun-daun *Labisia pumila* var. *alata* (LP) daripada kawasan-kawasan tertentu di dua negara berbeza iaitu Malaysia dan Indonesia telah dikaji. *Part Least Square* (PLS) dan *principal component analysis* (PCA) digunakan untuk mengelaskan dan memahami kaitan antara jumlah pepejal, jumlah keasidan, dan kelarutan dalam air sejuk dan panas, analisa proksimat, mineral, logam berat, jumlah kandungan fenolik, dan aktiviti antioksidan daripada LP di kedudukan geografi berbeza. Berdasarkan hasil analisis yang diperolehi, tidak terdapat perbezaan yang signifikan dalam hasil jumlah pepejal, jumlah keasidan, dan kelarutan dalam air sejuk dan panas dari ekstrak lyophilized LP berdasarkan lokasi geografi berbeza ($p>0.05$). LP dari Gunung Halimun-Salak, Bogor (LPB) didapati mempunyai kandungan pH tertinggi. Manakala LP dari Gunung Tilu, Bogor (LPT) didapati mempunyai kandungan tertinggi dalam hal jumlah gallic asid (1.86%), jumlah kandungan fenolik (140.49 mg GAE/g lyophilized LP), aktiviti antioksidan$_{(FRAP)}$ (17.57 mg GAE/g lyophilized LP), DPPH$_{(EC50)}$ (78.79 µg/mL), protein (9.87±0.18%), kandungan air (10.11 ± 0.06%), mineral Na (20.20 mg/100 g), K (1128 mg/100 g), Mg (476.1 mg/100g), dan Zn (2.32 mg/100 g). Nilai tertinggi Ca dan Fe adalah daripada LP dari Pahang (LPR) dengan masing-masing memberi nilai Ca (265.8 mg/100g) dan Fe (41.61 mg/100g). Namun, terdapat perbezaan yang signifikan ($p <0.05$) antara ekstrak LP lyophilized daripada pelbagai kedudukan geografi berbeza dengan kandungan proximate, mineral, logam berat, dan aktiviti antioksidan. Model PCA menunjukkan terdapat kaitan antara *Labisia pumila* var.*alata* dari pelbagai kedudukan geografi dengan kandungan proximate, komposisi mineral dan logam berat, dan aktiviti antioxidant. Faktor pertama dan kedua (F1 dan F2) masing-masing menunjukkan nilai 61.25% dan 20.48% dimana F1 mewakili aktiviti antioksidan FRAP, DPPH, TPC, kandungan air, kandungan gallic asid, dan serat kasar; manakala F2 mewakili TFC dan kandungan lipid. Analisis kelompok (CA), sentroid, mengelaskan LP menjadi empat kumpulan sampel berdasarkan asal-usul geografi berbeza.

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LIST OF ABBREVIATIONS

AS	Analytical sensitivity
AAS	Atomic absorption spectrometer
BHA	Butylated hidroxyanisole
CA	Cluster analysis
CE	Catechin equivalent
CGA	Chlorogenic acid
CV	Coefficient variation
DAD	Diode array detector
DPPH	2,2 –diphenyl-1-pycrylhydrazyl
EC_{50}	Effective concentration to 50% inhibition
FAO	Food and Agriculture Organization
FDA	Food Drug Administration
FRAP	Ferric reducing antioxidant potential
GA	Gallic acid
GAE	Gallic acid equivalent
GWE	Gingseng water extract
HPLC	High performances liquid chromatography
ICPMS	Inductively coupled plasma-mass spectrometry
LC	Liquid chromatography
LP	*Labisia pumila* var. *alata*
LOD	Limit of detection
LOQ	Limit of quantization
PCA	Principal component analysis
PLS	Part least square
RDA	Recommended dietary allowances
RNI	Recommended nutrient intake
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RFS	Free radical scavengers

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TFC	Total flavonoid content
TPC	Total phenolic content
TPTZ	Tris (2-pyridyl)-1,3,5-triazine
UV-vis	Ultra violet – visible
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

According to the World Health Organization, traditional medicine is the sum total of knowledge of skills and practices based on the theories, beliefs and experiences indigenous to different culture that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses. It has been estimated that in some Asian and African countries, 80% of the population depend on traditional medicine for primary health care while in many developed countries, 70% to 80% of the population has used some form of alternative or complementary medicine. Herbal treatments are the most popular form of traditional medicine. The international marketplace derived annual revenues in Western Europe of US$ 5 billion in 2003-2004, US$ 14 billion in China in 2005 and US$ 160 million in Brazil 2007 (WHO, 2008).

The current high rate of demand and methods of harvesting medicinal plants, particularly from the wild, for the herbal market has lowered natural population sizes for many species of plants. Rapid deforestation and other anthropogenic activities in the Malay Archipelago have rapidly shrunk the populations of indigenous medicinal plants as well. Thus, research on medicinal plants is a high priority agenda in many parts of the world today.

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Labisia pumila (Myrsinaceae), popularly known as “Kacip Fatimah” in Malay, has been used by many generations of Malay women to induce and facilitate childbirth as well as a post-partum medicine (Burkill, 1935). The plants are usually boiled and water soluble extract or decoction taken as a drink. Interest has recently been shown in the herbal preparation to determine its mode of action and potential pharmacological application. In the mean time commercial preparations as canned tonic drinks have been marketed without knowledge of the mode action, potential toxicity and side effects. Because it is given to women during post-partum, the possibility of it being a phytoestrogen was considered most likely. An earlier *in-vitro* study using human endometrial adenocarcinoma cell of the Ishikawa-Var I showed that the ethanolic extract of the roots of *L. pumila* var. *alata* exhibited a weak but specific estrogenic effect on the cells, resulting in enhanced secretion of alkaline phosphatase (Jamal, 1999). A study by Husniza *et al.* (2000) showed the water extracts of *L. pumila* to be able of displacing estradiol binding to antibodies raised against estradiol, suggesting the presence of estrogen-like compounds in the extract.

The search for antioxidant from natural sources has received much attention. In previous studies, antioxidant activities are found more in *Labisia pumila* var. *alata* than in the other variety (Norhaiza *et al*., 2009). *Labisia pumila* var. *alata* could prevented the changes in bone biochemical markers in rats (Shuid *et al.*, 2010) and as an anti-photo aging cosmetic ingredient (Hyun-kyung *et al*, 2010). Plant phenolic compounds can reduce the deleterious effects of reactive oxygen species (ROS) on a number of biological and pathological processes (Sawa *et al.*, 1999). The antioxidant activity of the aqueous *Labisia pumila* var. *alata* extracts have been reported as providing significant protection to human dermal fibroblasts, from cell damage caused by UV irradiation (Choi *et al*., 2010), most likely due to the presence of flavonoids (Norhaiza *et al*., 2009).

1.2 PROBLEM STATEMENT

There is now an increasing demand for functional food and beverage; companies are touting the presence of antioxidants in their products in response to consumer interest in the potential health benefits of antioxidants and minerals in the diet.

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According to Stone (1988), there exist three varieties of *Labisia pumila* in Malaysia, namely, *Labisia pumila* var. *alata, L. pumila* var. *pumila and L. pumila* var. *lanceolata.* Each variety commands a different use so it is important to ensure that the right variety is used in each case. The leaves of *Labisia pumila* var. *alata* have become a popular ingredient of the functional foods and beverages market. Thus, it is pertinent that the reproducibility of the extraction process to produce *L. pumila* var. *alata* leaves water soluble extract and reliable chemical profiling methods are achieved in order to ensure consistency and safety of this important ingredient.

An increasing demand for functional food and beverage from these plant in Malaysia, have forced companies to import raw material to meet market needs. Based on a study on the distribution of *Labisia pumila* var. *alata* in Indonesia, this plant has been found to flourish there (Sunarno, 2005). *Labisia pumila* has been found growing in Halimun-Salak Mountain in Bogor, on Java Island (Setiawan, 2005); Jambi, Riau and Aceh on Sumatera Island (Rahayu *et al.*, 2007). Variations of chemical profiles and nutritional composition may arise due to external factors such as geographic, climatic, altitude and soil type (Chew *et al.*, 2011; Hougthon and Raman, 1998).

At present, there is no literature report regarding the properties and attributes, namely; nutrient composition, total phenolic content, total flavanoid content and antioxidant activities of *L. pumila* var. *alata* of various geographical origins. As sources of raw materials of functional foods and beverages market may vary, it is also important to evaluate and document properties and attributes of LP of various sources.

1.3 RESEARCH AIM

Labisia pumila (Myrsinaceae) or “kacip Fatimah”, traditionally used in Malay traditional medicine in the form of decoction as postpartum tonic, has found popular and global use as functional food and beverage. Consumers in this market sector expect to derive specific health benefits from consumption of *Labisia pumila* food or beverage. The aim of this research project was to determine the properties and attributes of water-soluble extracts of *Labisia pumila* var. *alata* and their sources, to reveal the plant’s

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potential health benefits as functional food and beverage as such data on the plant remains scarce.

1.4 RESEARCH OBJECTIVES

This study was undertaken to investigate various parameters of the water-soluble extracts of the leaves of *Labisia pumila* var. *alata* of different geographic origins. It embarks on the following objectives:

1. To carry out reproducible extraction process to produce and chemically profile *L. pumila* var. *alata* water-soluble extracts of leaves by High Performance Liquid Chromatography (HPLC),
2. To quantitate the nutrient composition (yield, pH, total solid, acidity, solubility in cold and hot water, proximate, mineral and heavy metal contents) of water-soluble extracts,
3. To quantitate the total phenolic content and total flavonoid content of water-soluble extracts,
4. To evaluate the antioxidant activities of water-soluble extracts,
5. To correlate the above parameters with *L. pumila* var. *alata* leaves samples of various geographical origins.

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CHAPTER 2

LITERATURE REVIEW

2.1 FUNCTIONAL FOOD AND ITS MARKET

The advent of research and development has led to increased awareness in the importance of health, thereby, changing public opinion on food. The changes demand that food products are not only nutritious but are also safe. This type of food is known in the Japanese market as “Foods for Specified Health Use” (FOSHU). Functional foods comprise: (i) conventional foods containing naturally occurring bioactive substances, (ii) foods enriched with bioactive substances (e.g., antioxidants), and (iii) synthesized food ingredients introduced to traditional foods (e.g., antioxidant). Probiotics and prebiotics, soluble fiber, omega-3 – polyunsaturated fatty acids, conjugated linoleic acid, plant antioxidants, vitamins and minerals, certain proteins, peptides and amino acids, as well as phospholipids constitute functional components of such food (Grajek *et al*., 2005).

Functional foods are foods that provide health benefits beyond basic nutrition due to certain physiologically active components, which may or may not have been manipulated or modified to enhance their bioactivity. Functional foods are not pills or capsules but are consumed as part of normal everyday diet. The difference between functional food, food supplement, medicinal food and nutraceutical are shown in Table 2.1.

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Table 2.1: Definition of functional food, food supplement, medicinal food and nutraceutical

Type	Definition	Form	Examples
Functional Food*	Defined as a food similar in appearance to a conventional food (beverage, food matrix), consumed as part of the usual diet which contains biologically active components with demonstrated physiological benefits and offers the potential of reducing the risk of chronic disease beyond basic nutritional functions. Biologically active components should be dietary (nutrient or not) compound present in unmodified whole food or added to a food vehicle	beverages, food	*Labisia pumila* beverage, biscuit high calcium, omega-3 enriched eggs.
Food Supplement**	Defined as vitamins, mineral, herbs, or other botanical, amino acids, and other substances intended to supplement the diet by increasing the total dietary intake, or as any concentrate, metabolite, constituent, extract or combination of these ingredients	pills, tablet, capsule, powder, soft gel, and liquid	Supplement, Vitamin C.
Medicinal food**	Defined as foods formulated to be consumed or administered entirely under the supervision of a physician and are intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements based on recognized scientific principles are established by medical evaluation	liquid, semi liquid, etc	Glucose (infusion), electrolyte.
Nutraceutical***	Defined as food or food products that provide health and medical benefits, including the prevention and treatment of disease or a product isolated or purified from foods, and generally sold in medicinal forms not usually associated with food and demonstrated to have a physiological benefit or provide protection against chronic disease.	powder, liquid, etc	Curcumin, lycopene.

Sources: * FAO (2004); ** Ross (2011); ***Kalra (2003)

The functional food industry is growing steadily worldwide. Innovative products are being launched continuously and competition is fierce. The market is growing at an annual rate of 8-14%, but the exact size of markets is difficult to measure. Depending on the source of data and definition of this category of products, the global market could range between US$7 to US$167 billion (Jamal, 2006). The Malaysian market for herbal products has been estimated to be worth RM 4.5 billion growing at a rate of 15-20%. However, 90% of the raw material used are imported (Jamal, 2006).

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Functional food and beverage companies are touting the presence of antioxidants in their products in response to consumer interest in the potential health benefits of antioxidants in the diet. The most important and the most frequently used functional food components are derived via extraction of antioxidants from plant materials. Today's increasing demand for functional foods and beverages are not in tandem with available information on nutritional data of these foods and beverages.

2.2 *LABISIA PUMILA*

2.2.1 Botanical overview

Labisia pumila (vernacular name: kacip Fatimah) is classified under the family of Myrsinaceae, of sub herbaceous plants. It exists in three known varieties, namely, *L. pumila* var. *alata*, *L. pumila* var. *pumila* and *L. pumila* var. *lanceolata* (Stone, 1988). Sunarno (2005) revision of the genus *Labisia* had expanded it from three species into six species. The three (3) additional species are *L. longistyla*, *L. malouiana* and *L. obtusifolia*.

Labisia pumila's root is tough and woody with long primary roots but few secondary roots. *Petioles* ranged 5–12 cm long, wing 3–5 mm wide at each side. The tip of the leaf is pointed with a base that is tapered or rather broad – rounded. The leaf has a slight odor and taste. The whole leaf is about 5-35cm long and 2-8cm wide finely toothed with numerous veins. It is of a dark green color on adaxial and lighter green on the axial. Flowers on the shrub are very small, generally white or pink, in spike like panicle of small clusters. They range from 6-30 cm long with sepals, petals and stamens. The petals wrap around and enclose the stamens. *Anthers* 0.8 mm, o*vary* broadly ovoid, placenta broadly ellipsoid. The fruits are about 5cm in diameter and are either bright red or purple (Sunarno, 2005). The botanical classification is outlined in Figure 2.1.

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Kingdom: Plantae
Subkingdom: Tracheobionta
Super Division: Spermatophyta
Division: Magnoliophyta
Class: Magnoliopsida
Sub Class: Dilleniidae
Ordo: Primulales
Family: Myrsinaceae
Genus: Labisia
Species: *Labisia pumila*

Figure 2.1: Botanical classification of *Labisia pumila*

2.2.2 Distribution and growth habit of *Labisia pumila* (Myrsinaceae)

Labisia pumila is found mainly in the lowland and hill forests of peninsular Malaysia at an altitude between 300 and 700m (Burkill, 1935). Sunarno (2005) reported the distribution of *Labisia pumila* in Myanmar, Thailand, Malaysia, Sumatera, Peninsular Malaysia, Java, Borneo, Philippines, and Lesser Sunda Islands. *Labisia pumila* has been found growing in Halimun-Salak Mountain in Bogor, on Java Island (Setiawan, 2005) and in Jambi, Riau and Aceh, on Sumatera Island (Rahayu *et al.*, 2007). Setiawan (2005), reported that the physical parameter affecting the growth of *Labisia pumila* on Halimun Mountain are altitude and slope of hill.

2.2.3 Phytochemical and pharmacological studies

Labisia pumila has generally been used as traditional medicine. Anti-oxidant activities have been found higher in *Labisia pumila* var. *alata* as compared to *L. pumila* var. *pumila* (Norhaiza *et al.*, 2009). According to Jaafar *et al*. (2007), the phenolic content in leaves was highest compared to stem or root in *L. pumila* red and green variety. According to Ali and Khan (2009), methanolic extract of *Labisia pumila* contained phenolic compounds, glycerogalactolipid, cerebrosides, α-tocopherol, sterols and lipids. Yusoff and Wan Mohamud (2006 and 2011) reported the presence of gallic acid, a well known antioxidant, in water soluble extracts of *Labisia pumila* var. *alata*.

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Pharmacology studies have shown that the petroleum ether extract of *Labisia pumila* var. *alata* had toxic effects on kidneys and livers of rats (Effendy *et al*., 2006). An aqueous extract of *Labisia pumila* var. *alata* could decrease corticosterone levels with no effect on the immune status of pregnant rats (Pandey *et al.*, 2008) and could prevent the changes in bone biochemical markers in rats (Shuid *et al.*, 2010). Choi *et al.* (2010), suggested that *Labisia pumila* var. *alata* extracts have tremendous potential as an anti-photoaging cosmetic ingredient. The aqueous extract of *Labisia pumila* var. *alata* has been found to inhibit estradiol binding to antibodies raised against estradiol, suggesting the presence of estrogen-like compounds in the extract (Husniza *et al.*, 2000). According to Singh *et al.*, (2009) a dose of 50 mg/kg of an aqueous extract of *Labisia pumila* var. *alata* corresponded to no-adverse-effect-level (NOAEL), whereas higher doses were associated with some toxicity concerns. While there have been many reports on the phytochemical and pharmacology of *Labisia pumila* var. *alata*, nutritional data on aqueous extracts and their correlation to geographical origin remain obscure.

2.2.4 Use of *Labisia pumila* in traditional medicine

Labisia pumila is traditionally consumed by women as an herbal medicine to treat menstrual irregularities, painful menstruations and to help contracting the birth channel after delivery and alleviate sickness to the bones (Zakaria and Mohd, 1994). Other benefits of *Labisia pumila* consumption include alleviating fatigue, menopausal symptoms, promotion of emotional well being, prolonging energy, relieve of constipation, as anti-dysmenorrheal and anti-flatulence.

2.2.5 Biological variation

Biological variation is an important point to consider when extracts are fractioned and tested. According to Hougthon and Raman (1998), one of the considerations is the variation in amount and identity of the chemical constituents found in the materials to be examined. The authors also suggested variation in the chemical profile of source material will probably cause a difference in the activity of extract when tested biologically.

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Variation may occur due to the existence of distinct phenotypes of particular species. While the amount of the constituents may vary, their relative proportions are expected to remain about the same. Second, variation may arise due to external factors such as climate, altitude and soil type, so that the same chemo type will produce different profiles according to its environment. When multiple collections are made, the TLC chemical profiles of individual samples may be compared against the profile of the original material (Hougthon and Raman, 1998). According to Wu *et al.* (2009), flavonoids, *viz*., hyperin, quercitrin, and quercetin, varied remarkably in the plants of *Houttuynia cordata* from different provinces in China, and variation in quercitrin were significantly correlated to the biological characteristics of the plant but not correlated to the geographic region where the plant grows.

2.3 HIGH PERFORMANCES LIQUID CHROMATOGRAPHY (HPLC) IN CHEMICAL PROFILING

2.3.1 High Performances Liquid Chromatography (HPLC)

HPLC is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture. HPLC instruments consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector (Fig. 2.2).

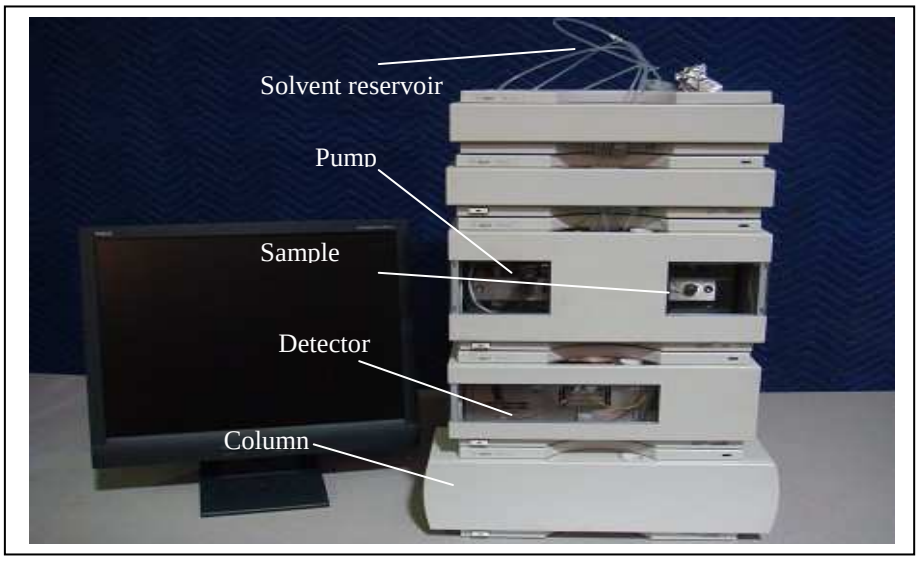

Figure 2.2: Basic instrumental components of HPLC. (Agilent, 2006)

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Compounds are separated by injecting a sample mixture onto the column. The different components in the mixture will pass through the column at different rates due to differences in their portioning behavior between the mobile liquid phase and the stationary phase. Figure 2.3 shows the schematics of the main processes in an HPLC.

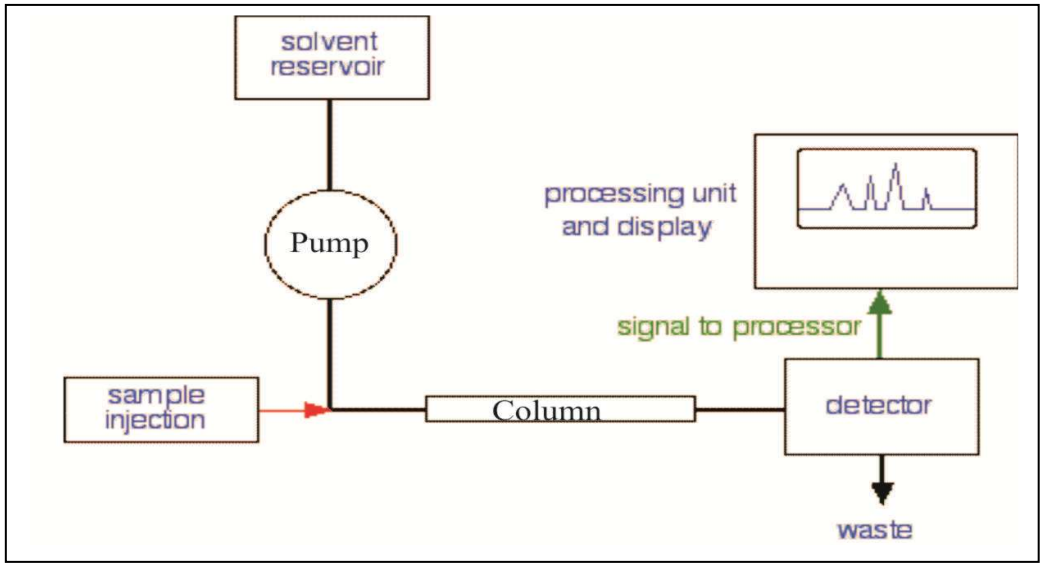

Figure 2.3: Schematic of an HPLC.

There was many method of water extraction for HPLC analysis. Hu *et al.* (2011) prepared water extract of ginseng (WEG) by added ginseng root (100 g) into 600 mL of distilled water and extraction was performed by heating at 95 °C, it was then filtered and lyophilized. The resulting powder (yield, 40 g) was dissolved in double distilled water and sequentially passed through 0.22 μm filters for sterilization, and then diluted in RPMI 1640 medium before use at final concentration of 0.001, 0.01, 0.1 or 0.2 mg raw herb/mL.

In material leaves, Tang Tung *et al.* (2009) prepared water soluble extract of *Acacia confusa* Merr leaves by boiling double-distilled water of leaves and allowed to infuse for 4 hours. The extract was decanted, filtered under vacuum, concentrated in a rotary evaporator, and then lyophilized. The resulting crude extract (7.3 g) was fractionated successively with EtOAc, *n*-butanol (BuOH), and water to yield soluble fractions of EtOAc (0.9 g), BuOH (2.0 g), and H_2O (4.1 g). Meanwhile, Sakanaka *et al.* (2005) prepared water soluble extracts of tea by adding 50 g of

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