ANTIOXIDANT ACTIVITY OF DIFFERENT FRACTIONS FROM *TINOSPORA CRISPA* STEMS EXTRACTS

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ABSTRACT

"Patawali (Malay) or *Tinospora crispa* is a plant that has many traditional uses for skin treatment. *Tinospora crispa* is a woody and glabrous plant in the family Menispermaceae. In cosmetics, *Tinospora crispa* can be used externally for its strong antioxidant activity and free radical scavenging properties. Therefore, the objective of the study was to fractionated all the five fractions from the *Tinospora crispa* stems extract, to test antioxidant activity of the extracted fractions using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay and finally to evaluate total content of phenolics in the fractions extracted from Tinospora crispa stems. The methodology of the study involves defatting of the plant material, separation and extraction of the moderately polar extract (organic extract) along with the aqueous extract. The fractions are then tested using the DPPH assay for free radical scavenging property to prove its antioxidant activity. The antioxidant activity of the plant fractions are compared with positive control ascorbic acid and butylated hydroxytoulene (BHT). On the other hand, total content of phenolics in fractions extract from the stem was determined using Folin-Ciocalteu method. Moreover, thin layer chromatography method was used to identify phenolics in fraction F3 and fraction F4 while alkaloids were also identified in fraction F4. The research carried out proved that the fractions extracted had significant antioxidant activity especially in fraction F3 and F4, 88.40 ± 0.37 % and 64.91 ± 0.58 % of inhibition repectively which was realatively comparable with the positive control ascorbic acid and butylated hydroxytoluene (BHT). Besides, the total phenolic content in F3 was reported the highest followed by F4, 73.54 ± 2.22 μg GAE per mg of sample and 62.10 ± 1.42 μg GAE per mg of sample respectively. As an overall conclusion, the research clearly supports that the *Tinospora crispa* stems extract have significant antioxidant activity due to the presence of phenolics in the fractions that can be further studied. The research too emphasizes the extract from the plant stems to be a good source of herbal medication that can be produced in industries to compliment the society need for curing diseases such as cancer and rapid aging.

ABSTRAK

"Patawali atau *Tinospora crispa* merupakan tumbuhan yang mempunyai banyak kegunaan tradisional untuk rawatan kulit. *Tinospora crispa* berkayu dan glabrous dalam keluarga Menispermaceae. Dalam kosmetik, *Tinospora crispa* boleh digunakan secara luaran untuk aktiviti antioksidatifnya yang kuat kerana sifatnya yang boleh memerangkap radikal bebas. Oleh itu, objektif kajian adalah untuk mengasingkan dan mengumpul kesemua lima pecahan ekstrak daripada tumbuhan ini. Kemudian untuk menguji aktiviti antioksidatif pecahan yang diekstrak menggunakan "2,2-diphenyl-1-picrylhydrazyl" (DPPH) dan akhirnya untuk menilai kandungan jumlah fenolik yang diekstrak daripada batang *Tinospora crispa* di dalam setiap ekstrak. Metodologi kajian ini melibatkan pengasingan wax daripada batang tumbuhan dan pengeluaran ekstrak sederhana organik bersama-sama dengan ekstrak akueus. Pecahan kemudiannya diuji dengan menggunakan DPPH membuktikan aktiviti antioksidatifnya serta membandingkannya dengan asid askorbik kawalan positif dan "butylated hydroxytoulene" (BHT). Sebaliknya, kandungan jumlah phenolics di dalam pecahan ekstrak daripada batang ditentukan dengan menggunakan kaedah "Folin-Ciocalteu". Selain itu, kaedah kromatografi digunakan untuk mengenal pasti phenolics dalam pecahan F3 dan F4 manakala alkaloid yang juga dikenal pasti dalam F4 pecahan. Penyelidikan yang dijalankan membuktikan bahawa pecahan yang diekstrak mempunyai aktiviti antioksidan yang ketara terutama dalam pecahan F3 dan F4, 88.40 ± 0.37 % dan 64.91 ± 0.58 % perencatan masing-masing yang setanding dengan kawalan positif asid askorbik dan "butylated hydroxytoluene" (BHT). Selain itu, jumlah kandungan fenolik dalam F3 dilaporkan tertinggi diikuti oleh F4 sebanyak 73.54 ± 2.22 μg GAE/ mg sampel dan 62.10 ± 1.42 μg GAE/ mg sampel masing-masing. Kesimpulannya, penyelidikan ini dengan jelas menyokong bahawa ekstrak batang *Tinospora crispa* mempunyai aktiviti antioksidan yang ketara disebabkan oleh kandungan fenolik di dalamnya. Penyelidikan ini juga menekankan ekstrak daripada batang tumbuhan ini boleh menjadi sumber yang baik untul membuat ubat-ubatan herba di dalam indutri yang akan selaras dengan keperluan masyarakat bagi mengubati penyakit-penyakit seperti kanser dan penuaan yang cepat.

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LIST OF SYMBOLS

λ	Wavelength
%	Percentage
$\pm$	Uncertainty
>	Greater than
cm	Centimeter
h	Hour
µL	Microliter
g	Gram
Kg	Kilogram
L	Liter
min	Minutes
mg	Milligram
mL	Milliliter
µg	Microgram
nm	Nanometer
R_f	Retention factor

LIST OF ABBREVATIONS

BHT	Butylated hydroxytoluene
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DMSO	Dimethyl Sulfoxide
EtOH	Ethanol
MeOH	Methanol
TLC	Thin Layer Chromatography
UV-Vis	Ultraviolet-Visible

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Tinospora crispa is a woody and glabrous plant in the family of Menispermaceae. It is found in primary rainforests or mixed deciduous forests throughout a large part of Asia and Africa including all parts of Thailand, Malaysia and Indonesia (Pathak et al., 1995).

Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and repercussion injury of many tissues, a central nervous system injury, gastritis and cancer (Pourmorad et al., 2006). Due to environmental pollutants, radiation, chemicals, toxins, deep fries and spicy foods as well as physical stress, free radicals cause depletion of the immune system antioxidants, the change in gene expression and induce abnormal proteins. The oxidation process is one of the most important routs for producing free radicals in food, drugs, and even living systems (Dillard and German, 2000 and Turkoglu et al., 2007).

Antioxidant means "against oxidation". An antioxidant is any substance that retards or prevents deterioration, damage or destruction by oxidation. In definition of the term by Halliwell and Gutteridge (1999) stated that an antioxidant is 'any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate'. On the other hand, a free radical is a compound with one or more unpaired electrons in its outer orbital. Such

unpaired electron(s) make these species very unstable and therefore quite reactive with other molecules due to the presence of unpaired electron(s) and they try to pair their electron(s) and generate a more stable compound. Antioxidants can be classified taking into account their mechanism of action, although there are other possible classifications. Bearing in mind the mechanism of action, they can be divided into primary antioxidants, synergistic and secondary antioxidants (Rajalakhmi et al., 1996). Some substances considered as antioxidants are ascorbate, tocopherols, some enzymes, carotenoids and bioactive plant phenols. The health benefits of fruits and vegetables are largely due to the antioxidant vitamin content supported by a large number of phytochemicals, some with greater antioxidant properties. Sources of tocopherols, carotenoids and ascorbic acid are well recognized antioxidants.

Antioxidants are important species which possess the ability of protecting organisms from damage caused by free radical-induced oxidative stress. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators (Canadanovic-Brunet et al., 2005). A number of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been extensively added to foodstuffs, although their use has begun to be questioned because of their toxicity (Ito et al., 1985), so there is considerable interest in preventive medicine and in the food industry in the development of natural antioxidants obtained from botanical sources, especially herbal plants (Djilas et al., 2003). Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value. Moreover, the increasing use of plant extracts in the food, cosmetic and pharmaceutical industries suggests that, in order to find active compounds, a systematic study of medicinal plants is very important (Nostro et al., 2000).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical which has an unpaired valence electron at one atom of nitrogen bridge (Eklund et al., 2005). DPPH is one of the free radicals widely used for testing preliminary radical scavenging activity of a compound or plant extract. It is stable and it contains an odd electron in its structure

that is usually utilized for the detection of the radical scavenging activity in chemical analysis.

1.2 PROBLEM STATEMENT

Antioxidant in general is important for the anti-ageing study. Besides, T*inospora Crispa* is reported to contain different phenolic compounds with probable free radical scavenging property (Amom et al., 2009). The antioxidant activities may vary depending on the structural factors, such as number of phenolic hydroxyl or methoxyl group, flavone hydroxyl, keto groups, free carboxylic groups and other structural features. Thus, this study is simply based on further study of on *Tinospora Crispa* fractions extracted to know their variation in antioxidant properties and their total content of phenolics. This will be done because the role of free radicals in many disease conditions has been well established (Pathak et al., 1995; Quisumbing et al., 1978 and Rahman et al., 1999). Several biochemical reactions in our human body can damage crucial bio-molecules due to presence of free radical and increase the rate of ageing in human while cause severe health risks. Hence, this study is done to extract different fractions from *Tinospora crispa* stems and then to determine the antioxidant activity of the different fractions extracted with other positive controls such as ascorbic acid and butylated hydroxytoluene using DPPH Assay. Finally, the total content for phenolics in the respective fractions extracted from T*inospora crispa* stems is determined.

1.3 OBJECTIVES

i. To extract different fractions from *Tinospora crispa* stems extracts.
ii. To determine antioxidant activity of the different fractions extracted from the *Tinospora crispa stems* using DPPH Assay.
iii. To determine total content of phenolics in the respective fractions extracted from *Tinospora crispa* stems.

1.4 SCOPE OF RESEARCH

As a way to accomplish the objective of this study, the scope of this research focuses on extraction of different fractions from *Tinospora crispa* stems and the comparison of the antioxidant activity of the different fractions extracted with other established antioxidants such as ascorbic acid and butylated hydroxytoluene (BHT) using DPPH Assay. Besides, it also focuses on determination of the total content of phenolics in the respective fractions extracted from *Tinospora crispa* stems. Finally it involves identification of the phenolics and alkaloids present in some selected fractions of extract.

1.4.1 SIGNIFICANCE OF THE STUDY

The extracted fractions from *Tinospora crispa* stems are believed to have anti-oxidative properties which can help in anti-ageing of cells. Ageing of cells can cause severe health risks to humans. Therefore, through this test of free radical scavenger using 2,2-diphenyl-1-picrylhydrazyl DPPH of the fractions extracted from *Tinospora Crispa*, it is trusted to help reduce ageing of cells that may eventually decrease health risks. If this study proves that the fractions extracted from *Tinospora Crispa* has anti-oxidative properties, it may be of great use to the medicinal industry and help treat various deceases such as cancer, atherosclerosis, senility, aging, and many more. Thus, the fractions are further studied to determine the responsible antioxidants which can take part in maintenance of good health as do vitamins, minerals and enzymes. Antioxidants can be added to foodstuffs to prevent free radical damage as they scavenge the free radical (Botsoglou et al., 2002 and Shetty, 1997). Besides, the ready availability of the plant source widely distributed in Asia is a major factor that influences the plant selection in this study. Other than that, the plant source is also cheap and is viable for industrial applications.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

In this chapter, the detailed facts on the plant material *Tinospora crispa*, antioxidants, the various type of antioxidants, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent functionality were discussed.

2.2 *TINOSPORA CRISPA* PLANT MATERIAL

Tinospora crispa as in Figure 2.1 is an indigenous climber plant that commonly grows wild in Asean countries including Malaysia. Known by various local names like 'akar patawali' and 'akar seruntum' (Noor and Ashcroft, 1989). Most species of the genus *Tinospora* are among the most widely employed medicinal plants throughout a large part of Asia and Africa (Pathak et al., 1995). In general folklore, the stem decoction is considered antipyretic, useful as an antimalarial and a wash for skin ulcers. Traditionally an infusion is used to treat fever due to malaria and also in cases of jaundice and for use against intestinal worms. The antimalarial effect was confirmed in a study (Rahman et al., 1999).

An infusion of the stem is drunk as a vermifuge while a decoction of the stem is used for washing sore eyes and syphilitic sores. Its stem has been used by traditional folklore for various therapeutic purposes such as treatment for diabetes, hypertension, stimulation of appetite and protection from mosquito bites. Based on research done it

was also used as hypoglycemic drug an antipyretic agent in both man and domestic animals (Kongsaktrakoon et al., 1994; Noor et al., 1989 and Pathak et al., 1995). This bitter tasting plant locally known as Makabuhai in Philippines is used for the treatment of stomach troubles,. ulcers and fevers, as a tonic and a febrifuge for malaria and smallpox, as a vulnerary for itches and wounds, and many other purposes (Quisumbing, 1978).

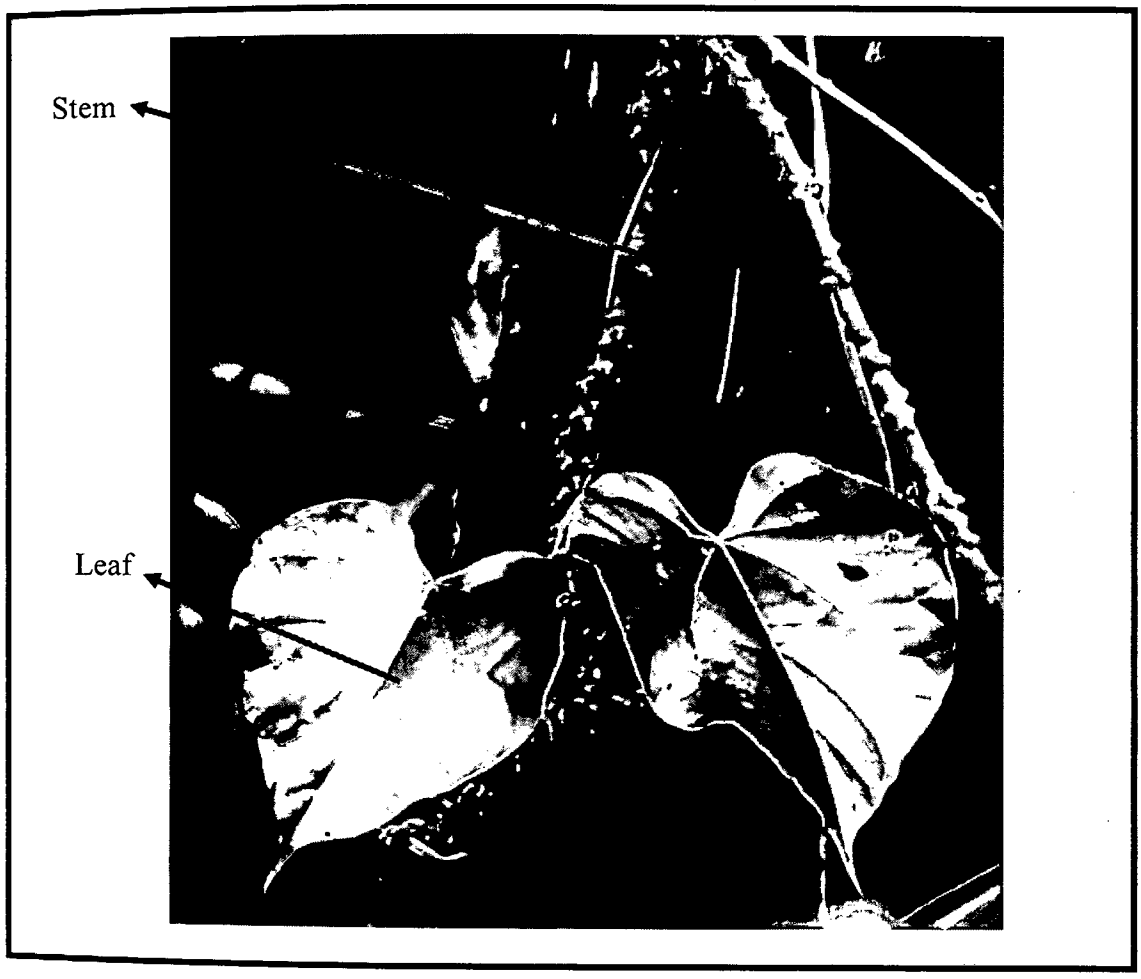


Figure 2.1: *Tinospora crispa* plant material

Source: Noor et al. (1989)

2.3 ANTIOXIDANTS

Antioxidant compounds play an important role as a health-protecting factor. Antioxidants are substance that significantly prevents or delays the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Halliwell and Gutteridge, 1990). Oxygen-centered free radicals and other reactive oxygen species (ROS) have been associated with the beginning of many diseases and degenerative processes in ageing (Halliwell, 1994). Since oxidative stress is common in chronic degenerative diseases, dietary antioxidants in plant sources may provide a beneficial effect. Antioxidants are known to protect cells against the damaging effects of reactive oxygen species (ROS) such as singlet oxygen, superoxide and hydroxyl radicals, among others (Halvorsen et al., 2002 and Wu et al., 2004).

Almost all organisms are well protected against free radical damage by oxidative enzymes such as superoxide dismutase and catalase or chemical compounds such as α-tocopherol, vitamin C (ascorbic acid), carotenoids, polyphenol compounds and glutathione (Betancur-Ancona et al., 2004). However, these systems are frequently insufficient to totally prevent the damage, especially under the conditions of severe oxidative stress, resulting in diseases and accelerated ageing (Ames et al., 1993). The balance between antioxidation and oxidation is believed to be a critical concept for maintaining a healthy biological system. Hyper physiological burden of free radicals causes imbalance in homeostatic phenomena (mechanisms) between oxidants and antioxidants in the body. This imbalance leads to oxidative stress involved in aging and various human diseases like atherosclerosis, stroke, diabetes, cancer and neurodegenerative diseases such as Alzheimer's disease and Parkinsonism (Halliwell, 1994).

2.4 TYPES OF ANTIOXIDANTS.

2.4.1 Phenolics

Phenolic compounds are secondary metabolites which are synthesized in plants. They possess biological properties such as antioxidant, anti-apoptotic, anti-aging, anti-carcinogenic, anti-inflammatory, anti-atherosclerotic, cardiovascular protection, improvement of the endothelial function, as well as inhibition of angiogenesis and cell proliferation activity. Most of these biological actions have been attributed to their intrinsic reducing capabilities (Han et al., 2007). Phenolic compounds are one of the most important, numerous and ubiquitous groups of compounds in the vegetable kingdom, being synthesised by plants during normal development and in response to different situations such as stress and ultraviolet radiation (Naczk and Shahidi, 2004). These substances are obtained from phenylpropanoid metabolism and there are more than 8000 different known structures (Herrero et al., 2005). However, these large ranges of structures possess a common structural feature: an aromatic ring with one or more hydroxy substituents. The way to classify these components it is not clearly established; a possible classification can be based on the number of constitutive carbon atoms in conjunction with the structure of the basic phenolic skeleton, including for instance, simple phenols, phenolic acids, coumarins, flavonoids and stilbenes (Antolovich et al., 2000).

Plant polyphenols are aromatic hydroxylated compounds, commonly found in vegetables, fruits and many food sources that form a significant portion of our diet, and which are among the most potent and therapeutically useful bioactive substances. Phenolic derivatives represent the largest group known as 'secondary plant products' synthesized by higher plants, probably as a result of antioxidative strategies adapted in evolution by respirative organisms starting from precursors of cyanobacteria. Many of these phenolic compounds are essential to plant life, e.g., by providing defense against microbial attacks and by making food unpalatable to herbivorous predators (Bennick, 2002).

Although a precise chemical definition may be given for plant phenolics, it would inevitably include other structurally similar compounds such as the terpenoid sex hormones. Therefore, an operational definition of metabolic origin is preferable, and thus the plant phenols being regarded as those substances derived from the shikimate pathway and phenylpropanoid metabolism, following the phosphoenolpyruvate → phenylalanine → cinnamate → 4-coumarate course, leading to chalcone, flavanone, dihydroflavonol, and anthocyanin as in Figure 2.2 (Robards and Antolovich, 1997).

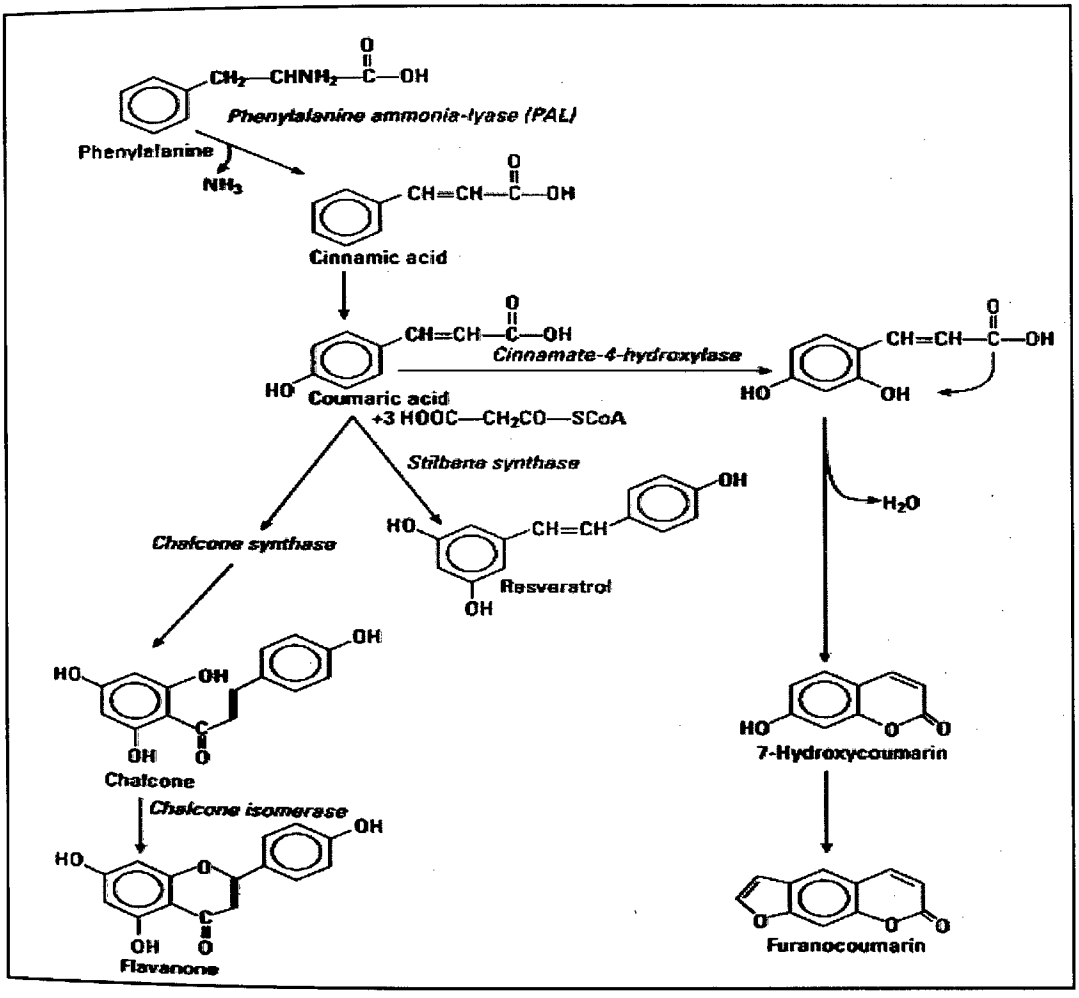


Figure 2.2: Schematic biochemical pathway of the synthesis of flavanone, resveratrol, and furanocoumarin.

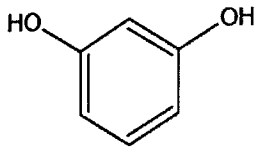
Source: Robards and Antolovich (1997)

Significant antioxidant, antitumor, antiviral and antibiotic activities are frequently reported for plant phenols. They have often been identified as active principles of numerous folk herbal medicines. In recent years, the regular intake of fruits and vegetables has been highly recommended, because the plant phenols and polyphenols they contain are thought to play important roles in long term health and reduction in the risk of chronic and degenerative diseases. Recognition of the benefits brought by these natural products to human health has encouraged the inclusion in everyday diets of some typical plant-derived food and beverages, among the most preferred examples being olive and vegetable oils, citrus and other fruit juices, chocolate, tea, coffee and wine.

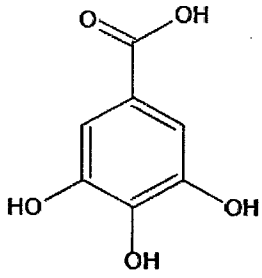
Over eight thousand naturally occurring phenolic compounds are known (Balasundram et al., 2006). These substances contain at least one aromatic ring with one or more attached –OH groups, in addition to other substituents (Bennick, 2002), and can be divided into 15 major structural classes (Harborne and Simmonds, 1964). Major classes of plant phenolics with 'the type of carbon skeleton, class name (example)' format include: C6, simple phenols (resorcinol); C6-C1, phenolic acids (*p*-hydroxybenzoic acid); C6-C2, acetophenones and phenylacetic acids; C6-C3, hydroxycinnamic acids (caffeic acid); C6-C4, hydroxyanthraquinones (physcion); C6-C2-C6, stilbenes (resveratrol); C6-C3-C6, flavonoids (quercetin); $(C6\text{-}C3)_2$, lignans (matairesinol); $(C6\text{-}C3\text{-}C6)_2$, biflavonoids (agathisflavone); $(C6\text{-}C3)_n$, lignins; $(C6\text{-}C3\text{-}C6)_n$, and condensed tannins (procyanidin) (Balasundram et al., 2006).

Tannins are considered to be polyphenolic metabolites of plants with a molecular weight larger than 500 and with the ability to precipitate gelatin and other proteins from solution (Mehansho et al., 1987) and to give typical phenol reactions such as forming a blue colour with $FeCl_3$ (Khanbabaee and Van Ree, 2001). Tannins may be subdivided into hydrolyzable and condensed tannins; the former are esters of gallic acid (gallo- and ellagi-tannins) while the latter are polyhydroxyflavan-3-ols, also known as proanthocyanidins (Porter et al., 1989). (Bennick, 2002) defines hydrolyzable tannins consisting of a polyhydric alcohol, such as glucose, to which gallic acid or its dimer

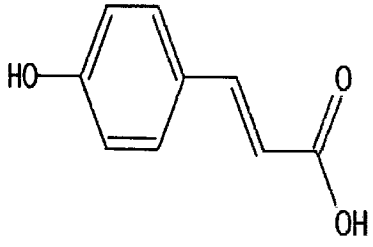
hexahydrodiphenic acid is linked in ester linkages, whereas the condensed tannins contain the monomeric unit of a flavan-3-ol such as catechin or epicatechin that is linked through C-C bonds. Basic plant phenolic structures with examples are shown in Figure 2.3.



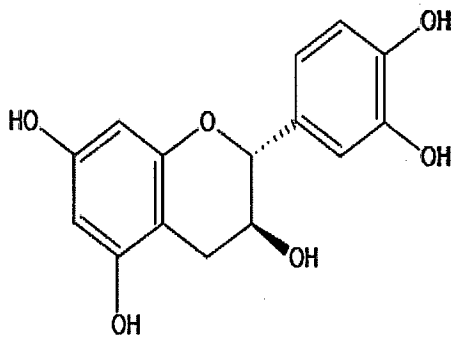
Simple phenols (e.g., resorcinol)



Hydroxybenzoic acids (e.g., gallic acid)



Hydroxycinnamic acids (e.g., p-Coumaric acid)



Flavanols (flavan-3-ols) (e.g., (+)-catechin)

Figure 2.3: Basic plant phenolic structures with examples

Source: Bennick (2002)