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

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Report submitted in partial fulfillment of the requirements for the award of the degree of Bachelor of Applied Science (Honor) Industrial Chemistry

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SUPERVISOR'S DECLARATION

I hereby declare that we have checked this project report and in our opinion this project is satisfactory in terms of scope and quality for the award of the degree of Bachelor of Applied Science (Honor) in Industrial Chemistry.

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STUDENT'S DECLARATION

I hereby declare that the work in this report is my own except for quotations and summaries which have been duly acknowledged. The report has not been accepted for any degree and is not concurrently submitted for award of other degree.

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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
±	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).

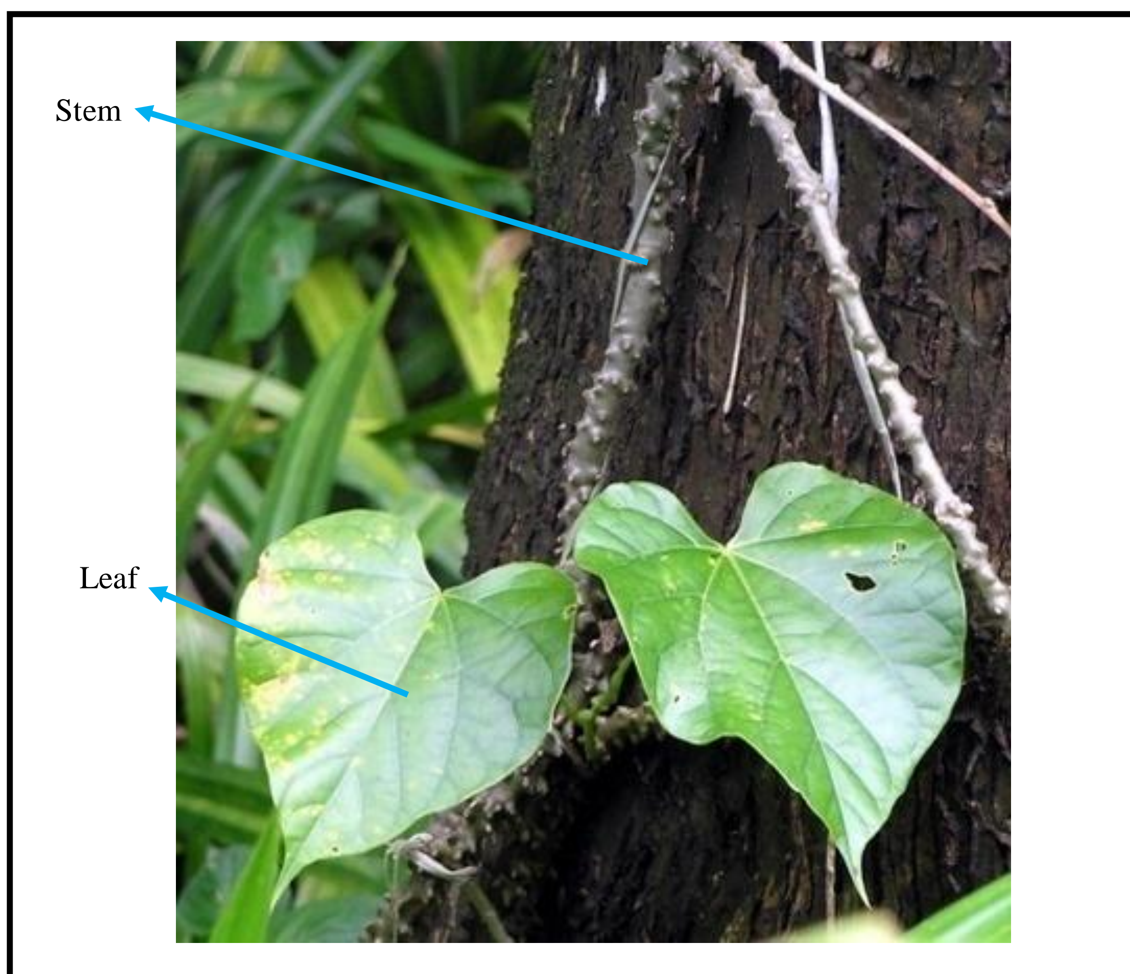


Figure 2.1: *Tinospora crispa* plant material

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).

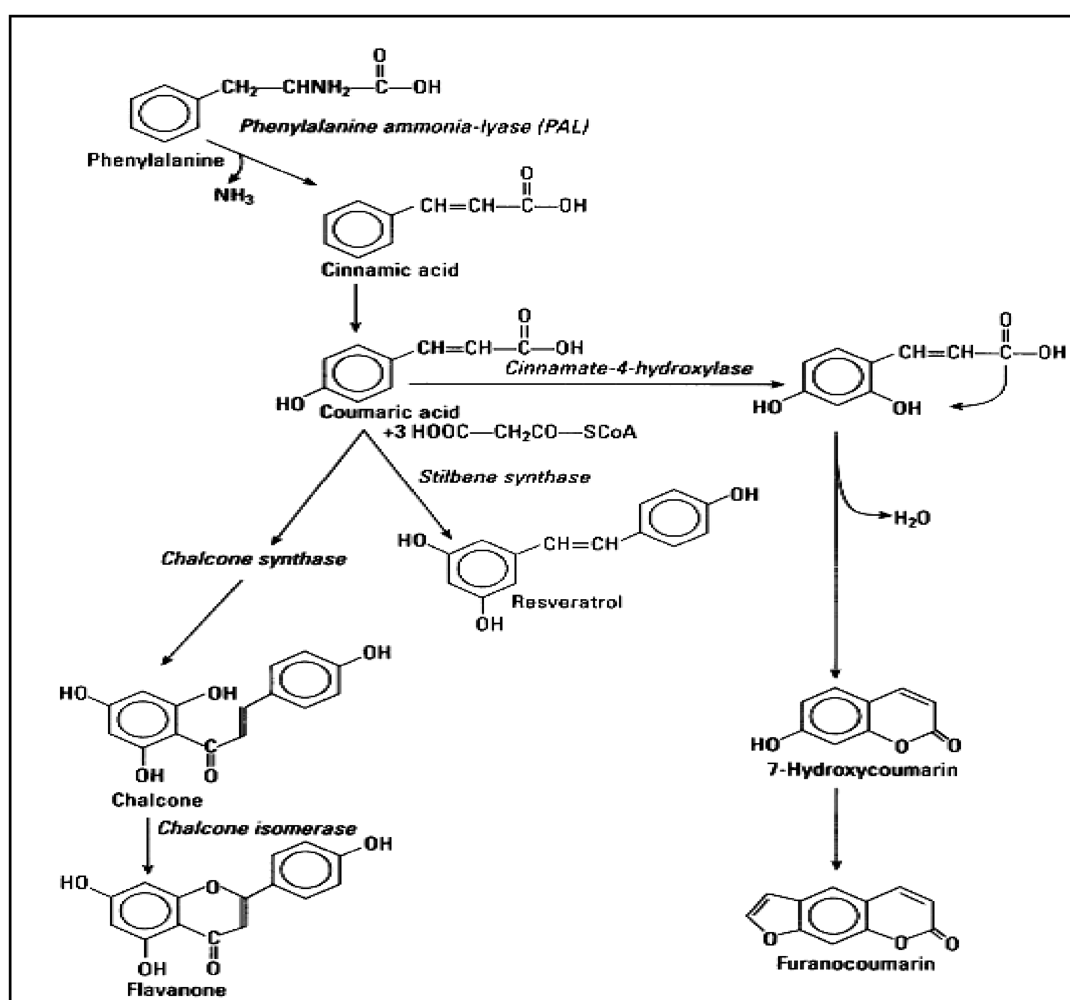


Figure 2.2: Schematic biochemical pathway of the synthesis of flavanone, resveratrol, and furanocoumarin.

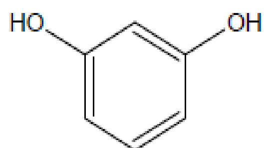
Source: Robards and Antolovich (1997)

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.

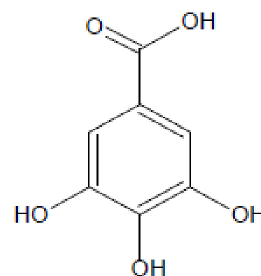
Over eight thousand naturally occurring phenolic compounds are known (Balasundram et al., 2006). These substances contain at least one aromatic ring with one or more attached –OH groups, in addition to other substituents (Bennick, 2002), and can be divided into 15 major structural classes (Harborne and 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

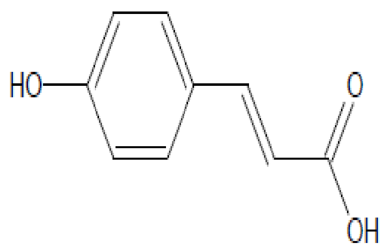
hexahydrodiphenic acid is linked in ester linkages, whereas the condensed tannins contain the monomeric unit of a flavan-3-ol such as catechin or epicatechin that is linked through C-C bonds. Basic plant phenolic structures with examples are shown in Figure 2.3.



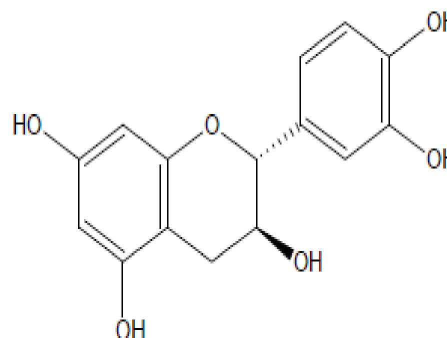
Simple phenols (e.g., resorcinol)



Hydroxybenzoic acids (e.g., gallic acid)



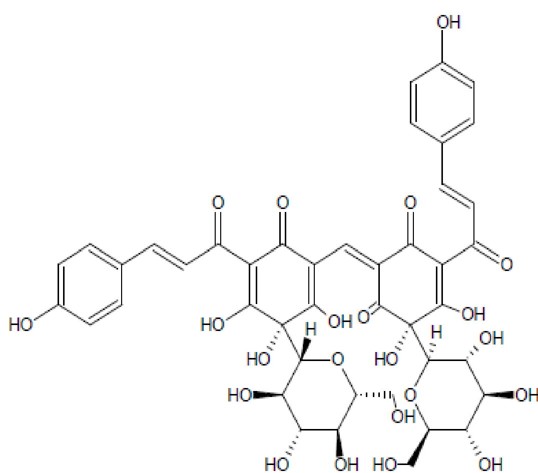
Hydroxycinnamic acids (e.g., p-Coumaric acid)



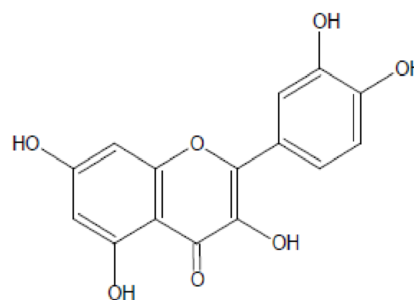
Flavanols (flavan-3-ols) (e.g., (+)-catechin)

Figure 2.3: Basic plant phenolic structures with examples

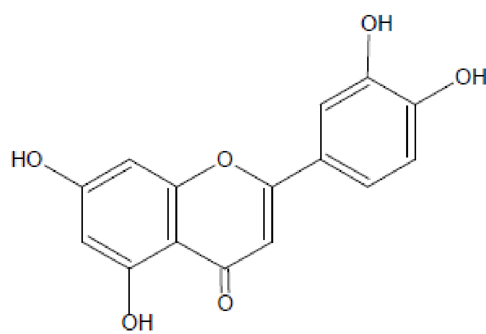
Source: Bennick (2002)



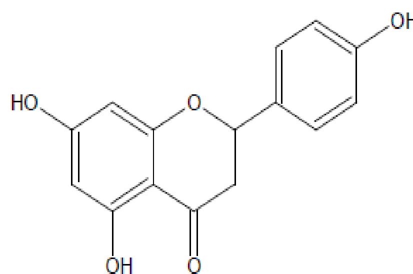
Chalcones (e.g., carthamine)



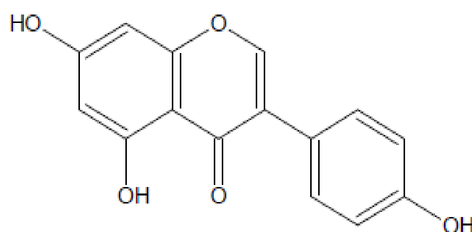
Flavonols (e.g., quercetin)



Flavones (e.g., luteolin)

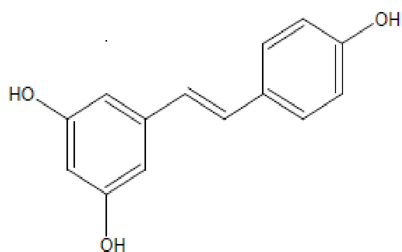


Flavanones (e.g., naringenin)

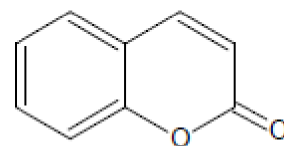


Isoflavones (e.g., genistein)

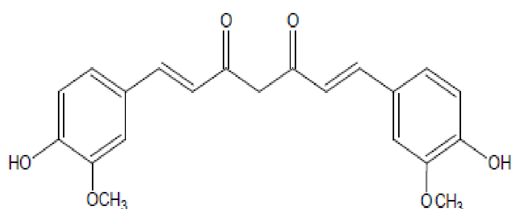
Figure 2.3: Continued



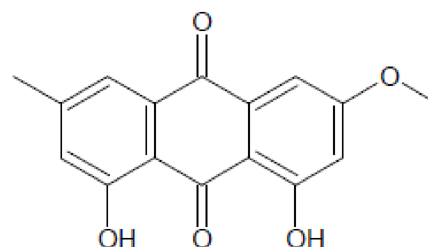
Stilbenes (e.g, resveratrol)



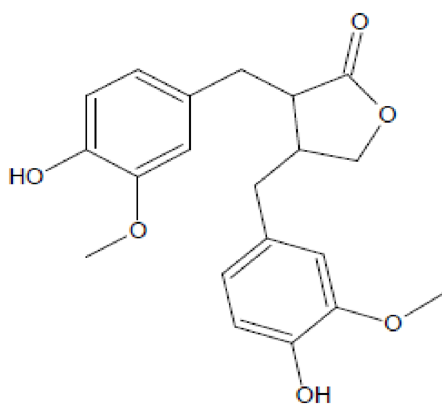
Coumarin (1,2-benzopyrone)



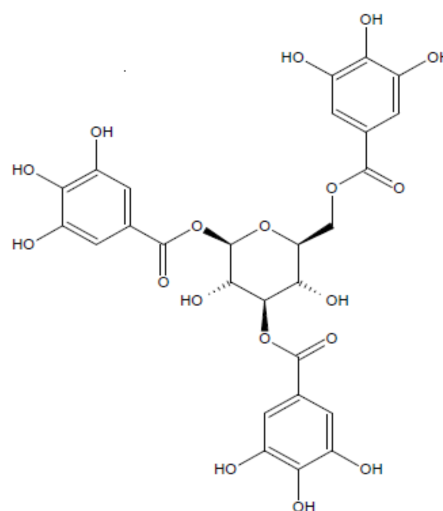
Curcuminoids (e.g., cucumin)



Hydroxyanthraquinones (e.g., Physcion)



Lignans (e.g., matairesinol)



Tannins (e.g., Corilagin: monomeric ellagitannin)

Figure 2.3: Continued

The anti-oxidative activity of phenolic compounds in plants is mainly due to their redox properties and chemical structure, which can play an important role in neutralizing free radicals, chelating transitional metals and quenching singlet and triplet oxygen, by delocalization or decomposing peroxides (Zheng and Wang, 2001, Pizzale et al., 2002). These properties are linked to beneficial health functionality of phenolic antioxidants due to their inhibitory effects against development of many oxidative-stress related diseases such as (Cardiovascular disease) CVD, cancer and diabetes (Huang et al., 1992). Plant phenolics serve as antioxidants by virtue of the hydrogen-donating properties of their phenolic hydroxyl groups (Lindsay and Astley, 2002), as well as by donating electrons to stop free radical chain reactions (Bajpai et al., 2005). Phenolic antioxidants are products of secondary metabolites in plants and are good sources of natural antioxidants in human diets. Due to the carcinogenic potential of synthetic forms natural phenolic antioxidants are also being targeted as alternatives to minimize or retard oxidative deterioration in food and to improve the health-related functional value of the food (Botsoglou et al., 2002 and Shetty, 1997).

Aromatic plants such as herbs and spices are especially rich in their phenolic content, and have been widely used to extend the shelf life of foods (Botsoglou et al., 2002, and Adam et al., 1998) and in traditional medicine as treatment for many diseases (Huang et al., 1992, and Shetty, 1997). Phenolic compounds have attracted considerable attention in the past few years due to their many potential health benefits. They have been demonstrated to render antibacterial, antiviral, anticarcinogenic, anti-inflammatory and vasodilatory actions (Breinholt et al., 1999; Duthie et al., 2000 as well as Shahidi and Naczk, 1995, 2004).

2.4.2 Ascorbic acid

Ascorbic acid as in Figure 2.4 is also referred to as ascorbate or vitamin C, is a required nutrient for humans. Ascorbate is synthesized by plants and most mammals but not by human. Lack of dietary ascorbate results in the clinical syndrome scurvy, which is fatal if untreated, is reported by (Chatterjee et al., 1975 and Nishikimi et al., 1994).

Ascorbate is reversibly oxidized with the loss of one electron to form the free radical, semidehydroascorbic acid, which is further oxidized to dehydroascorbic acid. Ascorbate is often called an outstanding antioxidant. In chemical terms this is simply a reflection of its redox properties as a reducing agent. In physiologic terms this means that ascorbate provides electrons for enzymes, for chemical compounds that are oxidants, or for other electron acceptors. In addition to its redox potential, other properties of ascorbate make it an excellent electron donor in biological systems. First, its intermediate free radical is relatively nonreactive, especially with oxygen (Buettner, 1993).

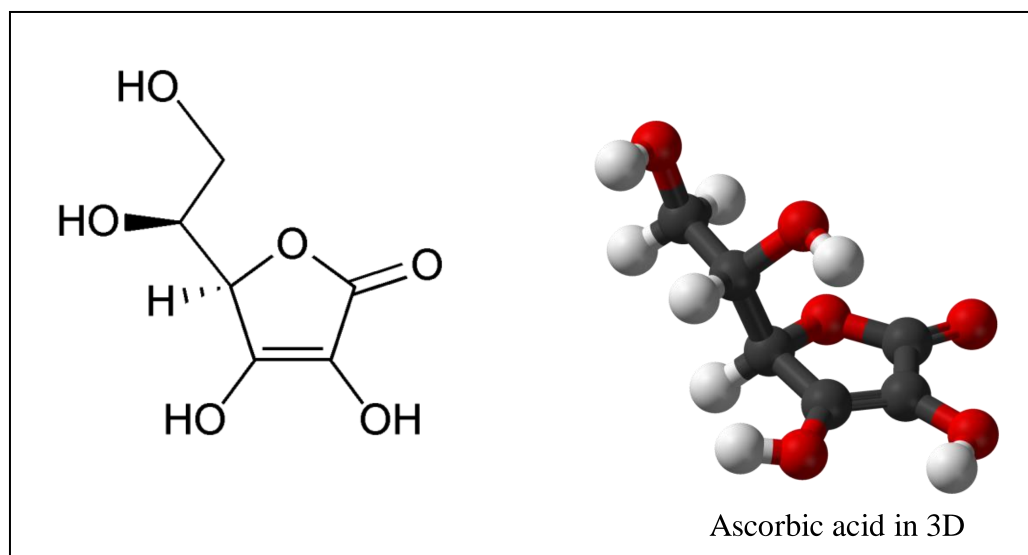


Figure 2.4: Ascorbic acid

Source: Nishikimi et al. (1994)

2.4.3 Butylated Hydroxytoluene (BHT)

Butylated hydroxytoluene (BHT), also known as butylhydroxytoluene, is a lipophilic (fat-soluble) organic compound that is primarily used as an antioxidant food additive (E number E321) as well as an antioxidant additive in cosmetics, pharmaceuticals, jet fuels, rubber, petroleum products, electrical transformer oil and embalming fluid. Butylated hydroxytoluene (BHT), chemically 2, 6-di-tert-butyl-p-cresol (DBPC), is a white crystalline solid with a faint characteristic odor. It is insoluble in water and in propylene glycol, but is freely soluble in alcohol. It is obtained by alkylation of p-cresol with isobutene or by monobutylation of m, p-cresol mixtures. BHT is used as a chemical antioxidant for food, cosmetics, and pharmaceuticals much like butylated hydroxyanisole (BHA) (Safer and Al-Nughamish, 1999).

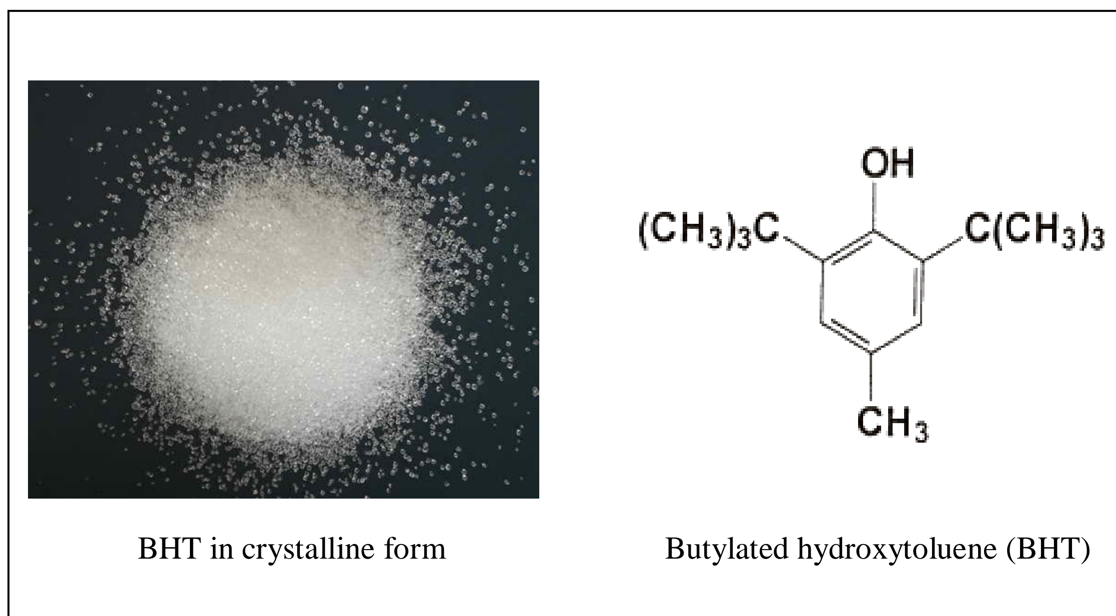


Figure 2.5: Butylated hydroxytoluene (BHT)

Source: Safer and Al-Nughamish (1999)

2.5 2,2-DIPHENYL-1-PICRYLHYDRAZYL (DPPH)

2,2-diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical (Blois, 1952) containing an odd electron in its structure. DPPH has been used to test the antioxidant ability of flavones in vitro (Cotelle et al., 1996). Any substance that can donate a hydrogen atom (antioxidant) to the solution of DPPH can reduce the stable free radical and change the color of solution from violet to pale yellow. Non-reacted radical form of DPPH absorbs in the visible range, and spectroscopic method is based on the measurements of color intensity at 517 nm. In 1922, (Goldschmidt and Renn, 1922) discovered the violet-coloured free stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), which now is used as erythrocyte sedimentation rate (ESR) standard (Chen et al., 1961) and as colorimetric reagent (Hazell and Russel, 1958) and for redox processes. Because DPPH can be kept indefinitely with little decomposition and because it neither dimerizes nor reacts with oxygen (Poirier et al., 1952) it proved to be quite useful in a variety of investigations, such as polymerization inhibition or radical chemistry (Fargere et al., 1995), the determination of antioxidant properties of amines, phenols or natural compounds (vitamins, plant extracts, medicinal drugs) and for inhibiting homolytic reactions. DPPH is intensely violet like $KMnO_4$ and its reduced counterpart 2,2-diphenyl-1-picrylhydrazine (DPPH-H) is orange-yellow (Glavind and Halmer, 1967).

The stable free radical DPPH is well known as a good hydrogen abstractor yielding DPPH-H as by product. This is a redox type process and was first mentioned by Goldschmidt and Renn (1922) in DPPH oxidation of hydroquinone to benzoquinone. More generally, the process was proved to be a homolytical breaking by the DPPH of an H-X bond (X = C, O, N, S, Cl, Br) like in hydrocarbons, alcohols, phenols, thiols, amines, enols, hydroxylamines, N-alkoxynitroanilines, hydracids (Covaci et al., 2001).

The DPPH radical can participate in homolytic additions with other radicals species R., yielding in many instances R-DPPH-H-substituted derivatives. It was also shown that DPPH-H anion (DPPH−) can be oxidized to the DPPH radical. Some aspects of DPPH chemistry, such as its instability in alkaline media, the improvement of

hydrogen abstractions from hydracids in the presence of salts $M^+ X^-$, and the reaction between DPPH and the N-methoxy-2,4,6-trinitroaniline anion yielding quantitatively the anion DPPH− and the aminyl radical of N-methoxy-2,4,6-trinitroaniline, suggested the electron-abstractor character of DPPH from some anionic species A− (Covaci et al., 2001). Active oxygen species such as hydrogen peroxide, hydroxyl radical, and superoxide anion radical, are readily generated in many cells by metabolic processes such as respiration, ischemia/reperfusion, and oxidation of fatty acids, and they are highly toxic to cells by damaging such components as DNA, lipids, and enzymes.

Cells can be injured, and even killed under the most serious conditions, when the content of active oxygen species exceeds the cellular antioxidant capacity. DPPH was used as indicator for measuring the antioxidant capacity in human plasma (Yukowa and Nakajima, 1999). DPPH was used as scavenger for many other radicals, due to the easiness in following of this process, the violet colour of DPPH faints into the yellow colour of its reduced congener (DPPH-H), with a high λ-shift in the visible spectra (from 520 nm to 330 nm) .The basis of this method was introduced by (Blois, 1952) more than 50 years ago. The Figure 2.6 is a picture of DPPH radical.

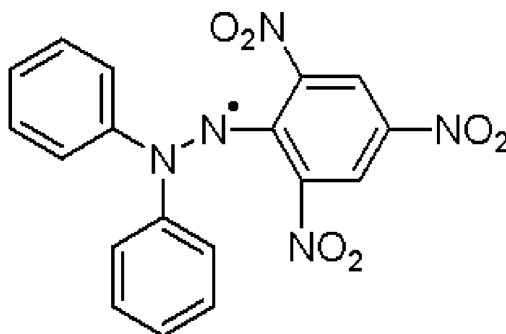


Figure 2.6: 2,2- Diphenyl-1- picrylhydrazyl (DPPH)

Source: Yukowa and Nakajima (1999)

CHAPTER 3

MATERIALS AND METHODS

3.1 INTRODUCTION

This chapter discusses about the comprehensive study of the materials, apparatus, instruments and the methodology of research carried. The methodology of the research is sub divided into five parts which include extraction of the fractions from *Tinospora crispa* stems and antioxidant activity test using 2,2-diphenyl-1-picrylhydrazyl, determination of total phenolic content in the fractions extracted, identification of alkaloid compounds in fraction 4 and identification of phenolic compounds in fraction 3.

3.2 MATERIALS

Stems of *Tinospora crispa* was obtained from district in Bentong, Pahang. The plant was authenticated by Professor Dr. Halijah Ibrahim (Botanist from University Malaya). The solvents hexane, chloroform, methanol, ethanol and Dragendorff's reagent used were from Merck, Germany. 2,2-diphenyl-1-picrylhydrazyl (DPPH) used was from Sigma-Aldrich, Germany. Ascorbic acid, butylated hydroxytoluene (BHT), sodium bicarbonate, Folin - Ciocalteu reagent, gallic acid, sulfuric acid, vanilin and dimethyl sulfoxide (DMSO) used was from R&M Chemicals, United Kingdom. Distilled water and deionized water used were from laboratory resources.

3.3 APPARATUS

Beakers, evaporator flask, separation funnel, conical flasks, petri dishes, glass vials, measuring cylinder, dropper, empty container bottles 500 ml, retort stand, micro plates (Greiner Flat transparent 96W), micro pipettes and tips, tea bag filter, sieve, funnels, glass capillaries, TLC developing chamber and TLC aluminium plates.

3.3 INSTRUMENTS

Rotavapor RII Büchi Switzerland, Ultrasonic bath, Microplate Reader Infinite M200 Pro Tecan, Grinder and Thin Layer Chromatography (TLC) Visualizer.

3.5 METHODOLOGY

3.5.1 Extraction of *Tinospora crispa* stem extracts

Initially, grinded *Tinospora crispa* stems of 1 kg was defatted with 2 L of hexane solvent. The defatted material was shaken for 30 min and the solution was filtered out using sieve. Next, hexane solvent was added to the filtered residue and was shaken again, repeating for 2 cycles. The extract obtained is fraction 1. The filtered residue of stems obtained was soaked with 4:1 ratio of methanol to water solution. This mixture of solution was left to settle for a day.

The solution cake formed was sonicated in the ultrasonic bath for about 30 min and filtered out. The filtered residue was added with methanol and water solution (4:1), shaken for 30 min and filtered. This step was repeated for 3 cycles. The filtrates were collected and evaporated by the rotary evaporator until one third (1/3) of the extract is left in the evaporator flask.

The concentrated extract was acidified with Sulfuric acid 50 % at pH 2. Chloroform was added to the acidified extract to the ratio of 2:1 and was shaken for 30mins. This step was repeated for three cycles. The aqueous and organic layer of the *Tinospora crispa* extract was separated using the separation funnel. The organic layer was collected and evaporated using rotary evaporator until one third (1/3) of extract was left. The concentrated organic extract was obtained as fraction 3. Next, the aqueous layer was basified to pH-10 with 25 % ammonia solution under the fume hood and was partitioned with solvent chloroform: methanol (3:1). The aqueous extract was partitioned in ratio of 1:2 with the solvent using a separation funnel. The organic layer was separated from the separation funnel and rotary evaporated to 1/3 of its original volume to obtain fraction 4. Finally, the aqueous layer was removed from the separation funnel as fraction 5. The solvent fractionation method used to extract stems extracts are based on the method by Harbone et al., 1988. All fractions collected were dried individually on a petri dish. Flow chart of extraction of fraction 1,2,3,4 and 5 from *Tinospora Crispa* stems is summarized in Figure 3.1.

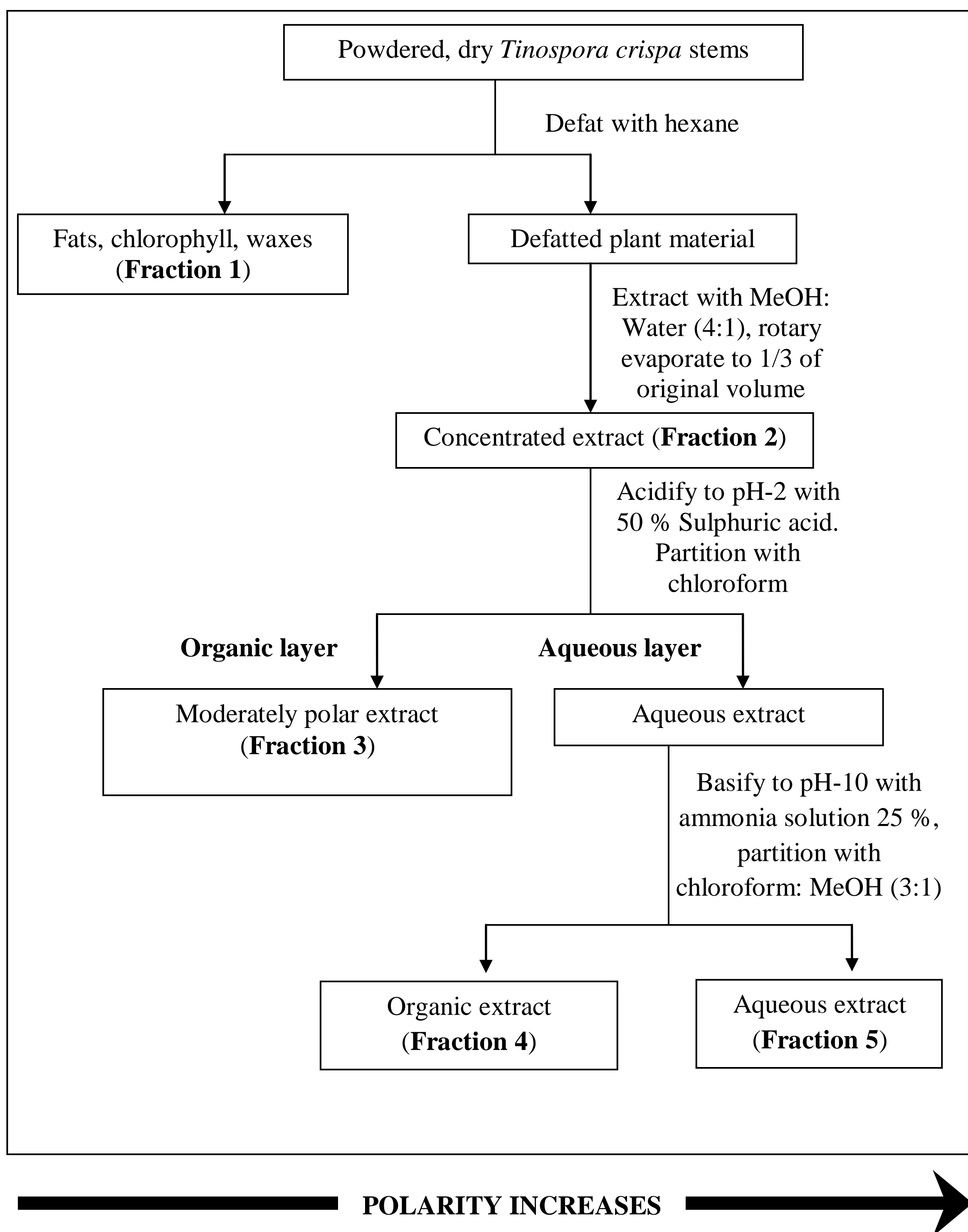


Figure 3.1: Flow chart of extraction of fraction 1,2,3,4 and 5 from *Tinospora Crispa* stems.

3.5.2 2,2-Diphenyl-1-picrylhydrazyl DPPH assay

The scavenging activity of DPPH free radicals was determined according to the method recommended by Lu and Yeap (2000) with minor modification. Initially, 0.45 mM of DPPH was prepared by adding 1.8 mg of DPPH into 10 mL of absolute ethanol. 100 µL of the 0.45 mM DPPH will be added into 50 µL of the samples, *Tinospora crispa* extract (fraction 1 to 5 respectively), ascorbic acid, butylated hydroxyltoluene (BHT) and blank which is DPPH. 0.5 mL of the samples was prepared by adding respectively 5mg of the *Tinospora crispa* fraction 1 to 5, ascorbic acid and BHT into 1mL of absolute ethanol respectively. The mixtures were kept in dark at room temperature for 30 min. The absorbance of the free radical scavenging activity was measured by spectrophotometer at λ_{max} 517 nm. The inhibition rate was monitored for every 15 min interval for 2 h for each sample fractions and positive controls. The experiment was done in replicates of six for each sample fractions and positive controls. Ethanol was used as control in this experiment. The percentage of inhibition of the sample against DPHH radicals was calculated from the following equation (Eq. 1). Flow chart of DPPH assay is summarized in Figure 3.2.

$$\% \text{ Inhibition} = \frac{[\text{Absorbance of control} - \text{Absorbance of test sample}]}{\text{Absorbance of control}} \times 100 \qquad \text{(Eq. 1)}$$

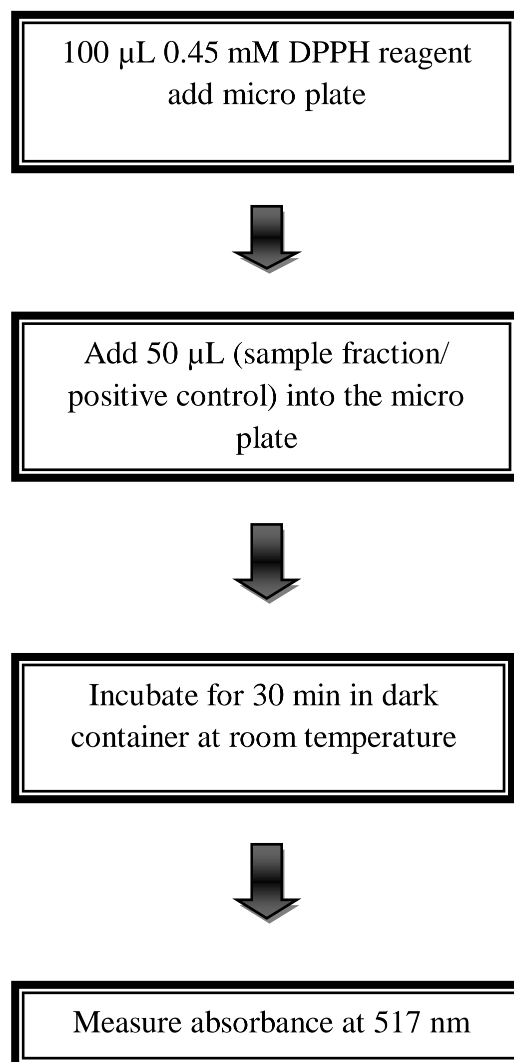


Figure 3.2: Flow chart of DPPH assay

3.5.3 Folin-Ciocalteu reagent method

The total phenolic content of the fractions of extract was determined using the Folin-Ciocalteau reagent method (Figure 3.3) following the procedure described by (Velioglu *et al.*, 1998) with slight modification. An aliquot of the fraction (1, 2, 3, 4 and 5) 50 µL respectively was mixed with 50 µL 10% Folin-Ciocalteau reagent in the microplate wells.

The aliquot of fractions were prepared by dissolving 1 mg of crude extract in 10 mL distill water. Dimethyl sulfoxide (DMSO) of 1 % (Ofokansi et al., 2008) was used to dilute the fractions. 10 % Folin-Ciocalteu reagent was prepared by diluting 1ml of Folin-Ciocalteu reagent to 9 mL and left to stand at room temperature incubated in a dark container. Following 3 min, 100 µL 10 % sodium bicarbonate is added to the microplate wells and left to stand at room temperature incubated in the dark container.

Following 90 min, the absorbance is read at λ_{max} 750 nm. 200 µL of 10 % Folin-Ciocalteu reagent is set as blank. Total phenolic content of the sample was calculated as gallic acid equivalent (GAE) from the standard curve of gallic acid standard solution (0, 2, 4, 6, 8 and 10) µg/mL and expressed as gallic acid equivalent µg (GAE) per every mg of sample. 100 µl of DMSO was diluted in 9.9 mL of distill water and was set as negative control. The experiment is repeated six times for each fraction. Total content of phenolic was calculated based on the equation (Eq. 2) below.

$$\text{Total content of phenolic} = \frac{\text{Concentration of fraction } (\mu\text{g GAE/ mL})}{0.1 \text{ mg/mL}} \qquad \text{(Eq. 2)}$$

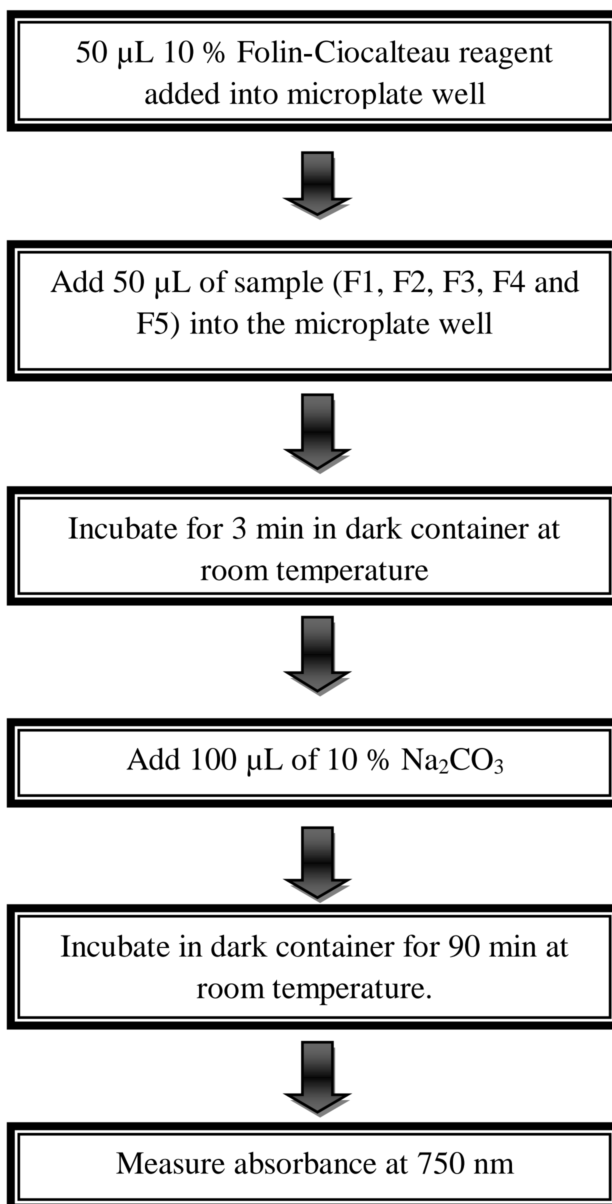


Figure 3.3: Flow chart of Folin-Ciocalteu reagent method

3.5.4 Identification of alkaloid compounds using Thin Layer Chromatography (TLC).

The alkaloid compounds were identified using TLC method as described by Harborne (1988) with slight modification. The dried fraction F4 obtained was diluted with solvent chloroform to methanol in ratio of (1:1). Then the fraction F4 extract was applied to the aluminium plate coated with silica gel by using a glass capillary. Before using these plates, they were marked with scale, fixing the distance to be travelled by solvent using sharp pin. The distance between two spots was kept at minimum of 1.0 cm.

The solvent chloroform to methanol the ratio of (8:2) was prepared and filled in the chamber to the depth of 1.0 cm. The chromatogram was developed by the ascending technique in which plate was immersed in the developing solvent to a depth of 0.5 cm. The chamber used was lined with sheets of filter paper which dip into solvent where, this ensures that the chamber is saturated with solvent vapor. Development was allowed to proceed until the solvent front traveled the required distance, then the plate was removed from the chamber and solvent front was immediately marked with pointed object. One side of the plate containing a spot was covered and the other spot was sprayed with spraying reagent Dragendorff. The plate was dried in the fume hood.

The compound was separated into bands with different retention factor (R_f) values. The R_f value is calculated using the equation (Eq. 3).

$$R_f = \frac{\text{Distance travelled by sample}}{\text{Distance travelled by solvent front}} \qquad \text{(Eq. 3)}$$

These bands were then detected under UV light at λ_{max} 254 nm and λ_{max} 366 nm.

3.5.5 Identification of phenolic compounds using Thin Layer Chromatography (TLC).

The phenolic compounds were identified using TLC method as described by Harborne (1988) with slight modification. The dried fraction F3 and F4 obtained were diluted with solvent methanol to chloroform in ratio of (1:1). Then the fraction F3 and F4 extracted were applied to the aluminium plate coated with silica gel by using a glass capillary. Before using these plates, they were marked with scale, fixing the distance to be travelled by solvent using sharp pin. The distance between two spots was kept at minimum of 1.0 cm.

The solvent chloroform to methanol to the ratio of (9:1) was prepared and filled in the chamber to the depth of 1.0 cm. The chromatogram was developed by the ascending technique in which plate was immersed in the developing solvent to a depth of 0.5 cm. The chamber used was lined with sheets of filter paper which dip into solvent where, this ensures that the chamber is saturated with solvent vapor. Development was allowed to proceed until the solvent front traveled the required distance, then the plate was removed from the chamber and solvent front was immediately marked with pointed object. Two spots were spotted on the plate. One side of the plate containing one spot was covered and the other spot was sprayed with spraying reagent Vanilin-sulphuric acid. The Vanilin-sulphuric acid reagent was prepared by diluting 1.0 g of vanillin in 100mL of 98% sulphuric acid to ethanol, ratio (2:3). Phenolics appears pink to reddish bands when sprayed with this reagent (Harborne, 1988). The plate was dried in the fume hood.

The compound was separated into bands with different retention factor (R_f) values. The R_f value is calculated using equation (Eq. 4).

$$R_f = \frac{\text{Distance travelled by sample}}{\text{Distance travelled by solvent front}} \qquad \text{(Eq. 4)}$$

These bands were then detected under UV light at λ_{max} 254 nm and λ_{max} 366 nm.

3.5.6 Statistical analysis

Statistical analysis was performed using Statistical Program for Social Sciences (SPSS) software version 15.0 generating One-way ANOVA. In all cases, $p < 0.05$ was considered significant. All values are expressed as means ± standard error.

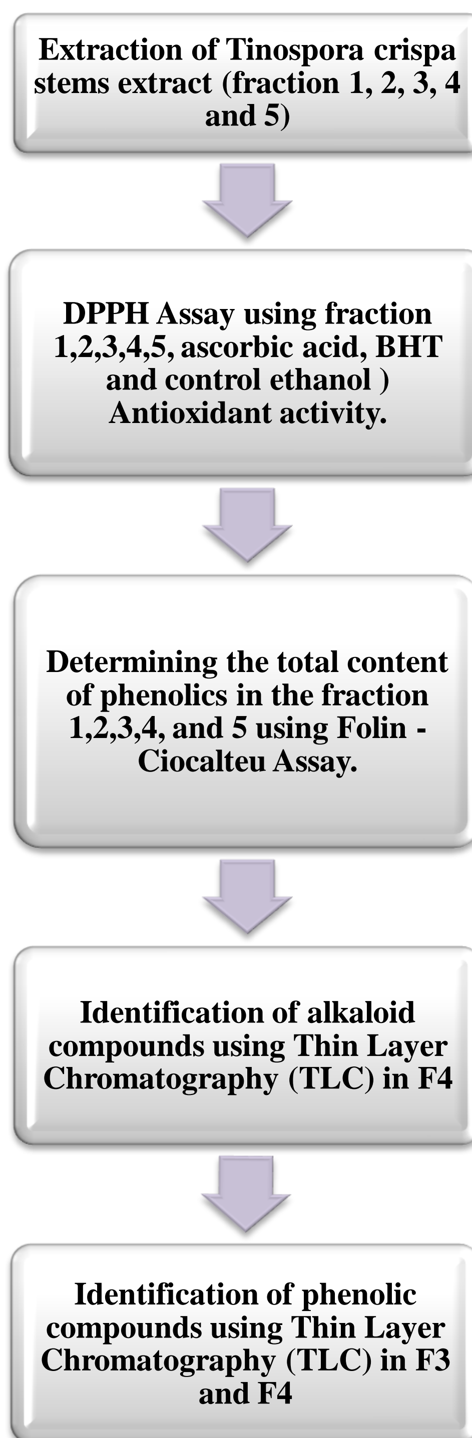


Figure 3.4: Flow chart of research methodology

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

In this section of this report, findings and data on the experiment conducted based on the general methodology in chapter 3 is discussed. This section comprises data findings and discussion on the yield of fractions extracted from 1 kg of grinded *Tinospora crispa* stems, antioxidant activity of the fractions and positive controls, Total content of phenolic compounds in the fractions extracted, identification of alkaloid compounds in fraction 4 and identification of phenolic compounds in fraction 3.

4.2 YIELD OF DRIED FRACTIONS

In this experiment 1kg of dried and grinded *Tinospora crispa* stems were used. The Yield and percentage of the respective fractions obtained are tabulated in the Table 4.1.

Table 4.1: Table of yield of fractions extracted

Fractions	Yield (g)	Yield percentage (%)
F1	8.74 ± 0.001	0.87
F2	Only screened	
F3	39.49 ± 0.001	3.95
F4	5.08 ± 0.001	0.51
F5	Only screened	

Based on the Table 4.1, fraction F3 had the highest yield percentage neglecting the other two uninvestigated fractions. About 39.49 ± 0.001 g of fraction F3, 8.74 ± 0.001 g of fraction F1 and 5.08 ± 0.001 g of fraction F4 was obtained from 1 kg of dried *Tinospora crispa* stems extracted. Fraction F3 had the highest yield percentage of 3.95 % extracted neglecting the fraction 2 and fraction 5 while F4 had the lowest yield percentage of about 0.51 %. Most of the weight loss from the initial plant source is due to fibers and residues of plant waste after filtration which was not used in this experiment.

Based on the Table 4.2, all fractions after drying formed a semi-solid substance which in case of fraction F1, F2, F3 and F4 was sticky substances while F5 was substance with crystals formation in it. The crystals formed are believed to be various forms of mineral salts present in the plant material.

Table 4.2: Table of physical properties of the fractions extracted

Fractions	Physical properties
F1	Dark brown-green sticky substance
F2	Dark brown sticky substance
F3	Dark brown-black substance
F4	Brownish substance
F5	Brownish substance with crystals formation

4.3 ANTIOXIDANT ACTIVITY OF *TINOSPORA CRISPA* STEMS FRACTIONS, POSITIVE CONTROLS BHT AND ASCORBIC ACID.

Based on the experiment carried out, the antioxidant activity of the Tinospora crispa stems fraction (F1, F2, F3, F4 and F5) is determined using the DPPH Assay. Ethanol was set as control to calculate the percentage of inhibition while ascorbic acid and BHT was used as positive control to compare the antioxidant strength of the

fractions extracted. Followings are the Table 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 4.10, 4.11 and Figure 4.1 of antioxidant activity from 0 min to 2 h.

Table 4.3: Percentage (%) of inhibition of fractions, BHT and ascorbic acid at 0 min

Samples	**0 min (%)**
F1	3.97 ± 1.19
F2	41.66 ± 0.85
F3	70.80 ± 0.24
F4	61.85 ± 0.48
F5	11.66 ± 1.53
BHT	83.73 ± 0.42
Ascorbic acid	83.76 ± 0.28

Based on the Table 4.3, F5 showed its maximum antioxidant capacity of about 11.66 ± 1.53 % at 0 min.

Table 4.4: Percentage (%) of inhibition of fractions, BHT and ascorbic acid at 15 min

Samples	**15 min (%)**
F1	4.39 ± 0.56
F2	41.62 ± 0.52
F3	70.99 ± 0.63
F4	61.49 ± 0.74
F5	10.88 ± 1.07
BHT	93.60 ± 0.15
Ascorbic acid	93.69 ± 0.10

Based on the Table 4.4, Ascorbic acid and BHT showed its maximum antioxidant activity about 93.69 ± 0.10 % and 93.60 ± 0.15 % respectively at 15 min.

Table 4.5: Percentage (%) of inhibition of fractions, BHT and ascorbic acid at 30 min

Samples	30 min (%)
F1	3.77 ± 1.78
F2	44.97 ± 1.71
F3	83.51 ± 0.63
F4	63.30 ± 0.94
F5	7.36 ± 2.23
BHT	93.34 ± 0.17
Ascorbic acid	93.35 ± 0.07

Table 4.6: Percentage (%) of inhibition of fractions, BHT and ascorbic acid at 45 min

Samples	45 min (%)
F1	3.76 ± 1.06
F2	47.46 ± 0.86
F3	86.34 ± 0.31
F4	64.91 ± 0.58
F5	7.56 ± 1.34
BHT	93.28 ± 0.06
Ascorbic acid	93.40 ± 0.10

Based on the Table 4.6, F4 showed its maximum antioxidant activity of about 64.91 ± 0.58 % at 45 min.

Table 4.7: Percentage (%) of inhibition of fractions, BHT and ascorbic acid at 1 h

Samples	1 hr (%)
F1	7.24 ± 1.92
F2	48.33 ± 1.14
F3	87.88 ± 0.45
F4	63.40 ± 1.02
F5	5.13 ± 1.90
BHT	92.85 ± 0.14
Ascorbic acid	92.95 ± 0.17

Based on the Table 4.7, F1 showed its maximum antioxidant activity of about 7.24 ±1.92 % at 1 h.

Table 4.8: Percentage (%) of inhibition of fractions, BHT and ascorbic acid at 1 h 15 min

Samples	1 h 15 min (%)
F1	2.63 ± 0.97
F2	45.56 ± 0.97
F3	88.40 ± 0.37
F4	62.13 ± 0.67
F5	3.79 ± 1.36
BHT	92.45 ± 0.13
Ascorbic acid	92.50 ± 0.13

Based on the Table 4.8, F3 showed its maximum antioxidant activity of about 88.40 ± 0.37 % at 1 h and 15 min.

Table 4.9: Percentage (%) of inhibition of fractions, BHT and ascorbic acid at 1 h 30 min

Samples	1 h 30 min (%)
F1	5.83 ± 1.79
F2	39.14 ± 2.02
F3	87.00 ± 0.37
F4	56.06 ± 0.96
F5	4.56 ± 1.96
BHT	91.02 ± 0.25
Ascorbic acid	91.21± 0.25

Table 4.10: Percentage (%) of inhibition of fractions, BHT and ascorbic acid at 1 h 45 min

Samples	1 h 45 min (%)
F1	2.34 ± 1.08
F2	48.87 ± 0.62
F3	86.18 ± 1.65
F4	60.25 ± 0.35
F5	1.91 ± 0.75
BHT	90.92 ± 0.16
Ascorbic acid	91.28 ± 0.10

Based on Table 4.10, F2 showed its maximum antioxidant activity of about 48.87 ± 0.62 % at 1 h and 45 min.

Table 4.11: Percentage (%) of inhibition of fractions, BHT and ascorbic acid at 2 hr

Samples	2 h (%)
F1	1.49 ± 0.42
F2	42.05 ± 0.34
F3	77.53 ± 2.30
F4	50.07 ± 2.13
F5	3.23 ± 0.32
BHT	84.73 ± 0.13
Ascorbic acid	84.78 ± 0.12

The detailed data obtained is tabulated in the tables in appendix A, titled Antioxidant activity of the *Tinospora crispa* stems extracts.

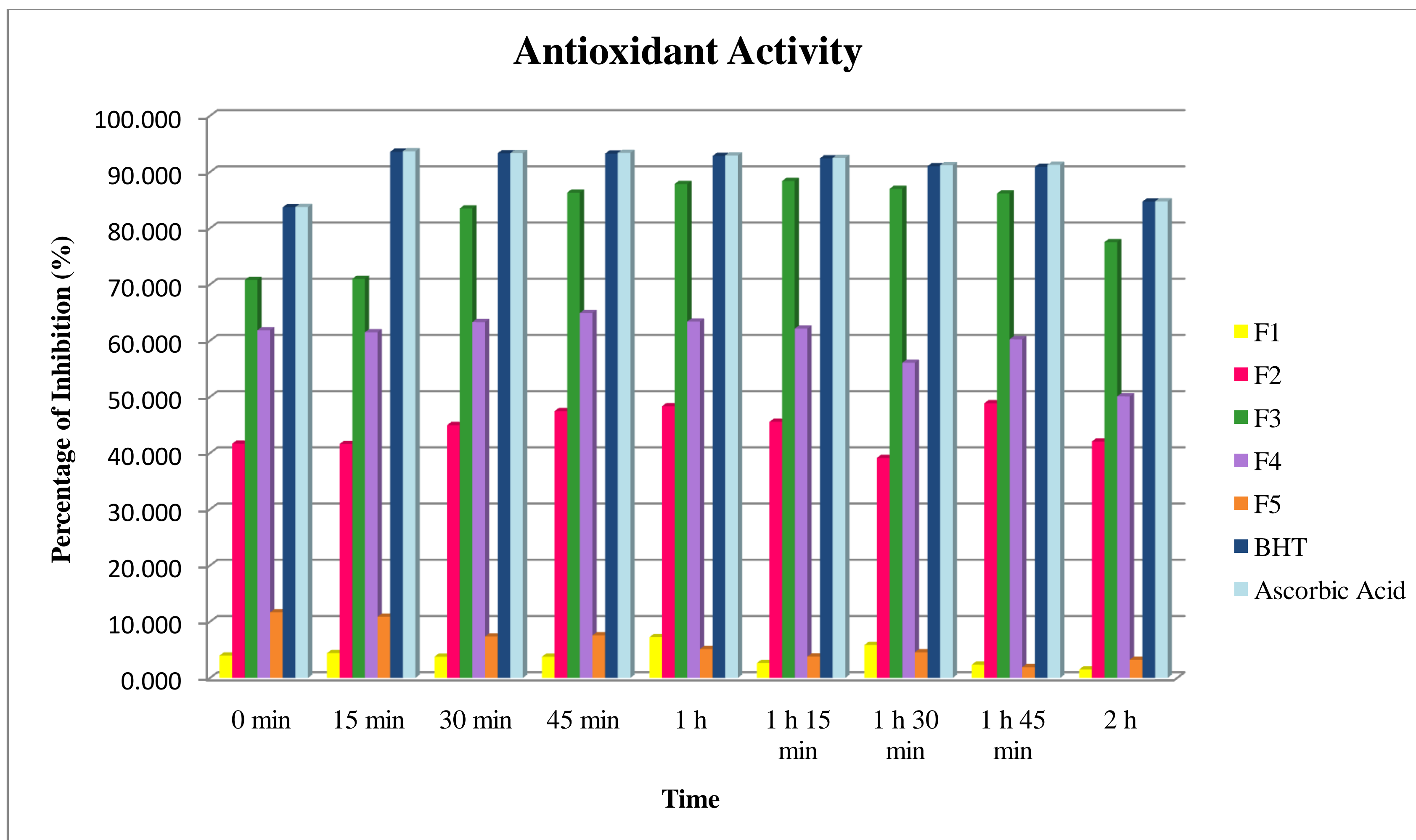


Figure 4.1: Graph of antioxidant activity of fraction (F1, F2, F3, F4 and F5), BHT and ascorbic acid

Based on the Table 4.3 up to Table 4.11 and Figure 4.1 above, ascorbic acid has the highest antioxidant activity followed by BHT, F3, F4, F2, F5 and F1 from 0 min to 2 h. The antioxidant activity of the samples tested can be categorized in the following order of increasing antioxidant activity (% inhibition).

Ascorbic acid > BHT > F3 > F4 > F2 > F5 > F1

Antioxidant activity increases

Ascorbic acid and BHT tested showed highest antioxidant activity at 15 min where the percentage of inhibition was found to be 93.69 ± 0.10 % and 93.60 ± 0.15 % respectively. On the other hand, F3 showed its highest antioxidant activity at 1 hr and 15 min (Table 4.8) with 88.40 ± 0.37 % of inhibition. The F3 appeared to have maximum reactivity at this point of time. Next, F4 showed maximum antioxidant activity at 45 min with 64.91 ± 0.58 % of inhibition which was relatively faster than F3. Besides that, F2 had maximum antioxidant activity at 1 h and 45 min with 48.87 ± 0.62 % of inhibition followed by F5 which had maximum antioxidant capacity at 11.66 ± 1.53 % of inhibition at 0 min. Finally, F1 had maximum antioxidant capacity of about 7.24 ± 1.92 % at 1 h.

The antioxidant activity of the fractions F1 and F5 was comparably lower than the other fractions and positive controls. This was found to be so because F1 essentially is the neutral extract which consists fats and waxes in large. On the other hand, F5 is the polar plant extract consisting quaternary alkaloids, N-oxides and mineral salts (Harborne, 1988) as it is reported by Amom et al. (2009) in the current study on *Tinospora crispa* plant material. Fraction F2 also showed considerable percentage of antioxidant activity. Through the research conducted it was found that F2 essentially comprises all the compounds in fraction F3, F4 and F5 before fractionation. Therefore, this clearly reveals the reason behind the antioxidant strength of the fraction F2. However, the fraction F2 relatively had lower antioxidant capacity than F3 and F4 because due to presence of mixture of components which may have caused the free radical scavenging strength of the fraction to be inhibited.

Ascorbic acid is a widely used industrial preservative which acts as a radical scavenger. Ascorbic acid too is a well noted antioxidant and free radical scavenger product is widely diffused in the plant kingdom, and it is a DPPH• (radical) scavenging agent of high strength (Schlesier et al., 2002). Its reaction is also very fast with respect to other scavenging molecules, such as polyphenols (Brand-Williams et al., 1995 and Zhang and Hamauzu, 2003). Ascorbic acid when added to violet solution of DPPH• (radical) immediately changes colour to bright pale yellow indicating the radical scavenging activity. Therefore, these studies clearly support the reason behind the percentage of inhibition of the ascorbic acid to be higher than all the other fractions and BHT.

On the other hand, the mechanism of scavenging activity of the ascorbic acid with DPPH• (radical) can be explained through the following reaction mechanism proposed by (Courtland et al., 1987). One molecule of ascorbic acid has the potential to scavenge two molecules of DPPH• (radical) as in the Figure 4.2.

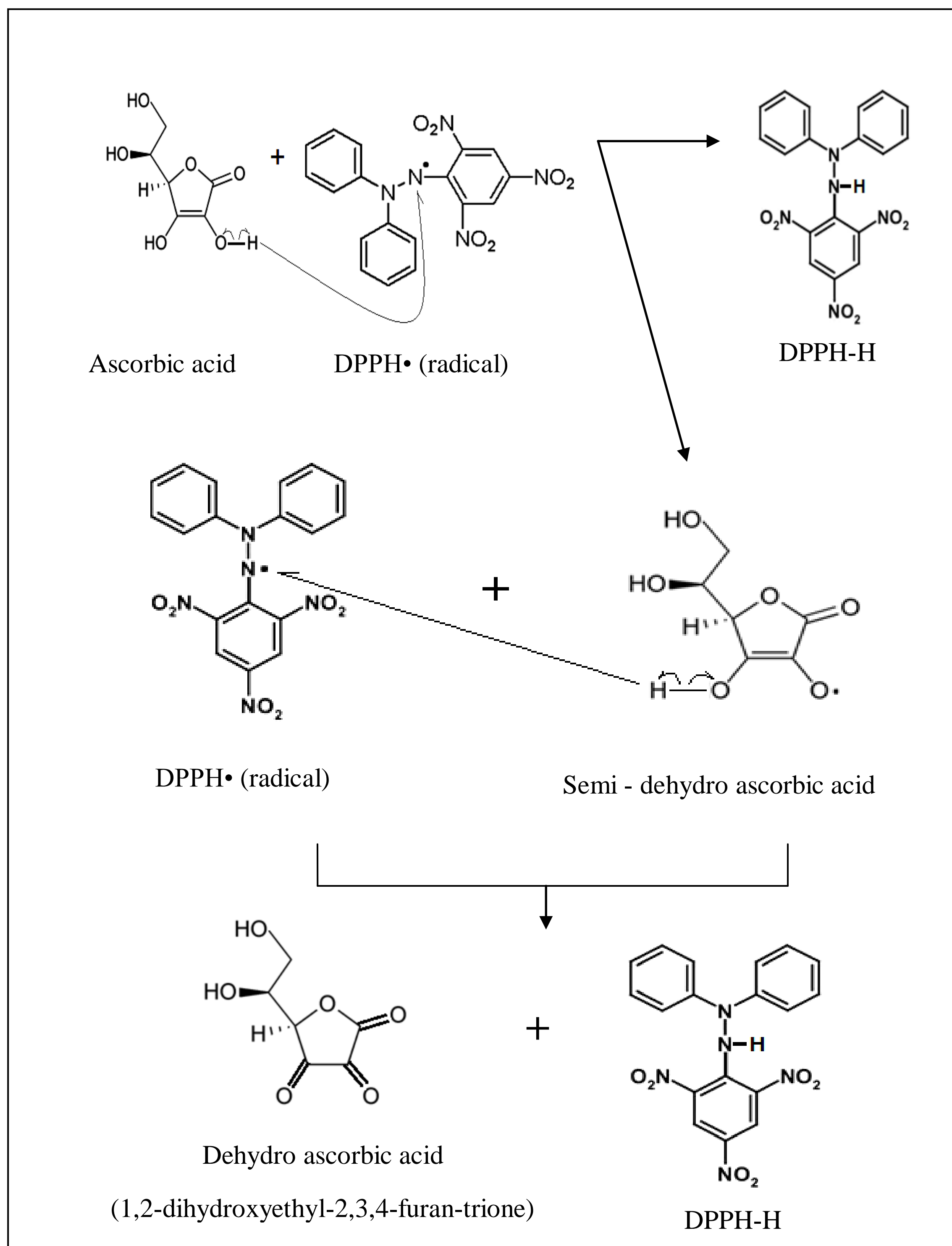


Figure 4.2: Proposed reaction mechanism of ascorbic acid with DPPH• (radical)

Source: Courtland et al. (1987)

Besides ascorbic acid, BHT also showed high antioxidant capacity almost similar to ascorbic acid. BHT or 2,6-di-*tert*-butyl-p-cresol is a synthetic antioxidant used extensively in the food industry. It is used in low-fat foods, fish products, packaging materials, paraffin, and mineral oils. BHT is also widely used in combination with other antioxidants such as BHA, propyl gallate, and citric acid for the stabilization of oils and high-fat foods (Donnelly and Robinson, 1991). BHT has the ability to donate its one molecule of hydrogen to scavenge the DPPH• (radical). The following is a proposed mechanism of BHT with DPPH• (radical) by Brand-Williams et al. (1995) as in Figure 4.3.

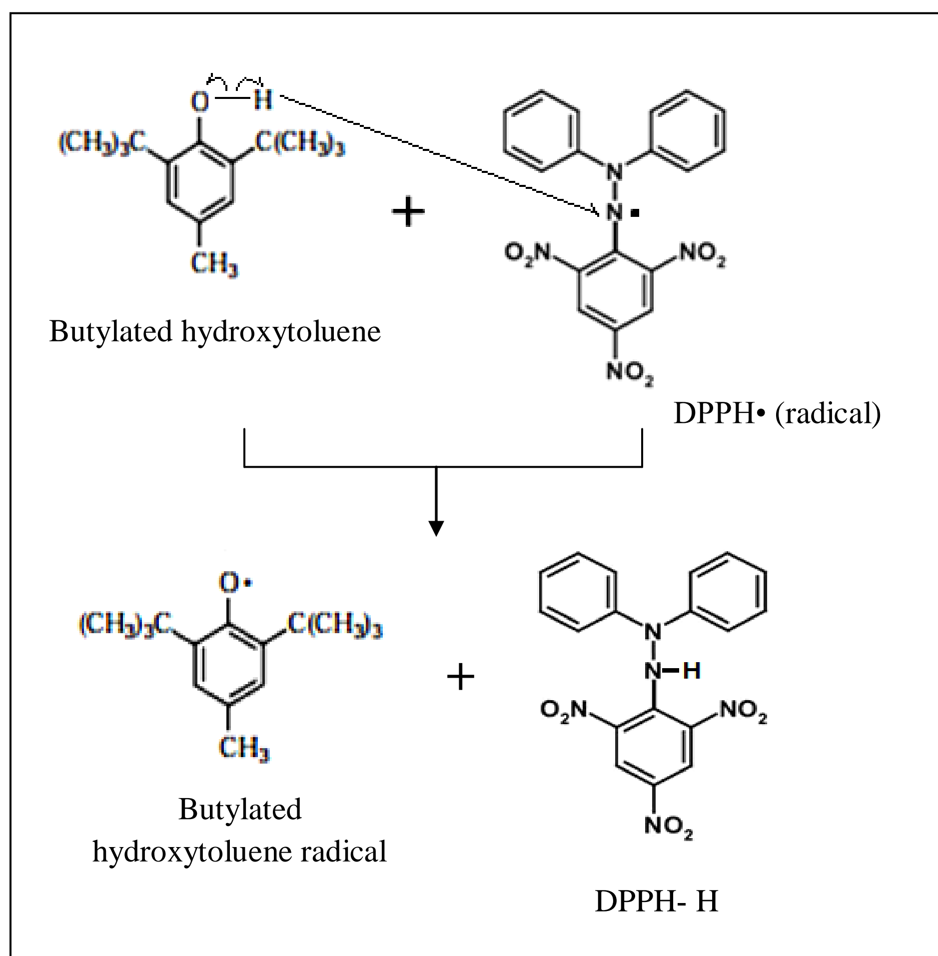


Figure 4.3: Proposed reaction mechanism for BHT and DPPH• (radical)

Source: Brand-Williams et al. (1995)

Fraction F3 also showed highest antioxidant activity compared to fraction F1, F2, F4 and F5. This is because fraction F3 is a moderately polar extract which generally contains terpenoids and phenolics (Harborne, 1988). In contrast, fraction F4 also had significant antioxidant activity. This is the basified extract which contains mostly alkaloids (Harborne, 1988). However, the significant antioxidant activity demonstrated by the fraction F4 could be due to incomplete separation of fraction F3 resulting in the presence of some phenolics in F4.

All plants produce an amazing diversity of secondary metabolites. One of the most important groups of these metabolites is phenolic compounds. Phenolics are characterized by at least one aromatic ring (C6) bearing one or more hydroxyl groups. Phenols are divided into several different groups, distinguished by the number of constitutive carbon atoms in conjunction with the structure of the basic phenolic skeleton (simple phenols, benzoic acids, phenylopropanoids and flavonoids) (Chaudiere and Ferrari-Iliou, 1999).

Antioxidant action of phenolic compounds is due to their high tendency to chelate metals. Phenolics possess hydroxyl and carboxyl groups, able to bind particularly iron and copper (Jung et al., 2003). They may inactivate iron ions by chelating and additionally suppressing the superoxide-driven Fenton reaction, which is believed to be the most important source of Reactive Oxygen Species, (ROS) (Rice-Evan et al., 1997).

The main characteristic of an antioxidant is it s ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. Antioxidant compounds like phenolic acids and polyphenols will scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases (Halliwell and Gutteridge, 1999).

There is another mechanism underlying their antioxidant ability. Metal ions decompose lipid hydroperoxide (LOOH) by the homolytic cleavage of the O-O bond and give lipid alkoxyl radicals, which initiate free radical chain oxidation. Phenolic antioxidants inhibit lipid peroxidation by trapping the lipid alkoxyl radical. This activity depends on the structure of the molecules, and the number and position of the hydroxyl group in the molecules (Millic et al., 1998). Hence, the presence of phenolic compounds in fraction F3 and F4 may have resulted in the antioxidant activity of the substance where they are believed to have reacted with DPPH• (radical) by acting as reducing agents by transferring a hydrogen atom to the DPPH• (radical) molecule.

The nitrogen-centered radical 2,2-diphenyl-1-picrylhydrazyl, DPPH• (radical) has been extensively employed in kinetic studies of hydrogen-atom abstractions from carbon, nitrogen, sulfur, and oxygen particularly from phenols. This radical is popular for such studies because it is monomeric in solution and air stable, commercially available, and is strongly colored. This last property allows the course of reaction to be monitored using conventional UV-Vis spectroscopy.

The DPPH is a stable lipophilic radical (Scalzo, 2008) that can readily undergo scavenging activity by antioxidant (Lu and Yeap, 2000). The principle of the assay is based on the colour change of the DPPH solution from purple to yellow as the radical is quenched by the antioxidant. The free radical scavenging effect was assessed by the discoloration of the DPPH solution (Sanchez *et al.,* 2001). Antioxidant reacts with DPPH due to its hydrogen donating ability (Chen and Ho, 1994). The interaction results in either the transfer of an electron or a hydrogen atom to DPPH, which neutralizes the free radical (McCune and Johns, 2007). The result from this study proved that T*inospora crispa* extract from fraction F3 and F4 is as effective as established antioxidants like BHT and vitamin C in scavenging DPPH• (radical). The following mechanism of reaction is suggested in this research where H atom from phenolic compound reacts to reduce the DPPH• (radical) to DPPH-H as in Figure 4.4.

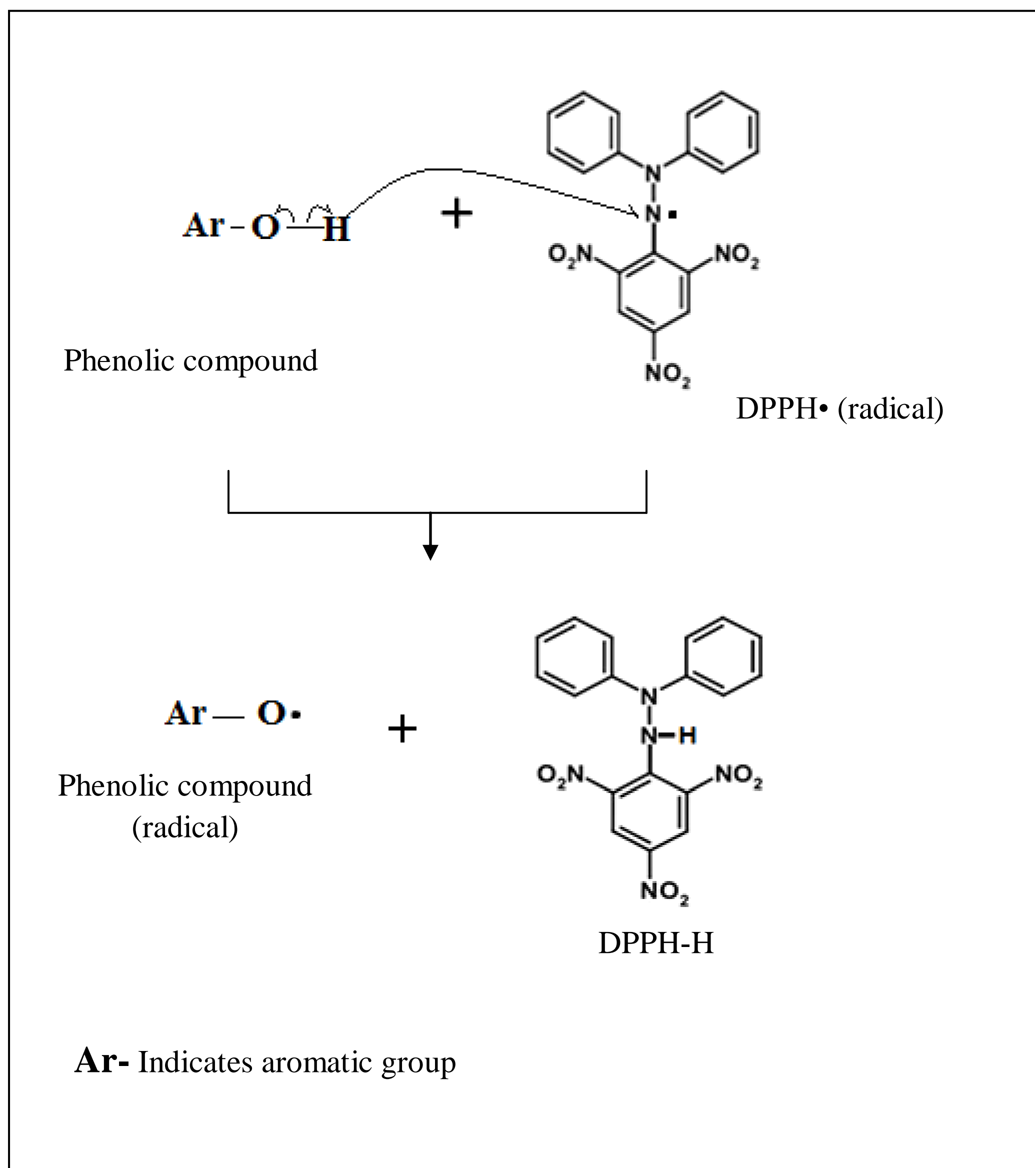


Figure 4.4: Proposed reaction mechanism of phenolic with DPPH• (radical)

Source: McCune and Johns (2007)

4.4 TOTAL CONTENT OF PHENOLICS IN *TINOSPORA CRISPA* STEMS FRACTIONS

The total content of phenolics in the fractions extracted from the Tinospora crispa plant material using solvent extraction method was determined using the Folin – Ciocalteu reagent method. The following is the graph of standard gallic acid equivalent (GAE) (Figure 4.5) and Table 4.12 obtained for the determination of the concentration of the fractions.

Table 4.12: Concentration and absorbance of gallic acid equivalent

Concentration (µg/mL)	Absorbance
0.00	0.054 ± 0.001
2.00	0.122 ± 0.001
4.00	0.189 ± 0.002
6.00	0.260 ± 0.003
8.00	0.315 ± 0.004
10.00	0.365 ± 0.002

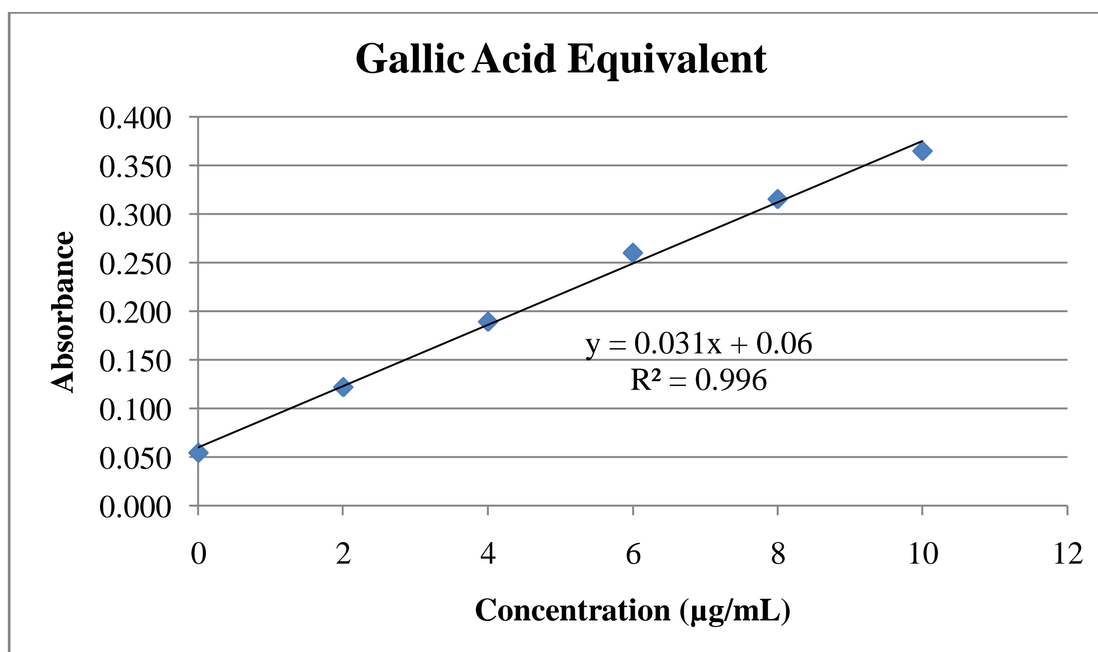


Figure 4.5: Graph of Gallic Acid Equivalent (GAE)

From the gallic acid equivalent the concentration and the total content of phenolic compounds in fraction F1, F2, F3, F4 and F5 from *Tinospora crispa* stems are tabulated in the Table 4.13 and Table 4.14.

Table 4.13: Concentration of fraction F1, F2, F3, F4 and F5 from gallic acid equivalent standard curve

Samples	Concentration (µg/ml)
F1	0.06 ± 0.01
F2	3.58 ± 0.09
F3	7.35 ± 0.22
F4	6.21 ± 0.14
F5	0.52 ± 0.02

Table 4.14: Total content of phenolic compounds in fraction F1, F2, F3, F4 and F5

Samples	(µg GAE/mg)
F1	0.59 ± 0.13
F2	35.78 ± 0.86
F3	73.54 ± 2.22
F4	62.10 ± 1.42
F5	5.19 ± 0.18

Based on the table 4.14, the total content of phenolic in F3 is found to be the highest with 73.54 ± 2.22 µg GAE per mg of sample, followed by F4 with 62.10 ± 1.42 µg GAE per mg of sample and F2 with 35.78 ± 0.86 µg GAE per mg of sample. F5 and F1 on the other hand was found to contain 5.19 ± 0.18 µg GAE per mg of sample and 0.59 ± 0.13 µg GAE per mg of sample respectively. The following order shows the order of increasing amount of phenolic content in the fractions extracted.

F3 > F4 > F2 > F5 > F1

Increasing amount of total phenolic content

The relationship between the antioxidant activity and total content of phenolic can be seen clearly where the increase in the total content of phenolic is directly proportional to the increase in antioxidant activity of the fraction. This can be seen in the case where F3 had the highest antioxidant activity when compared between the fractions from *Tinospora crispa* stems and F3 had the highest amount of total phenolic content. For detailed information on the data obtained refer to appendix B.

4.5 PRE-IDENTIFICATION OF ALKALOID COMPOUNDS

The alkaloid present in fraction 4, F4 was determined using the thin layer chromatography method. The following results were obtained based on the method described in chapter 3 on pre-identification of alkaloid compounds using Thin Layer Chromatography (TLC) as in Figure 4.4.

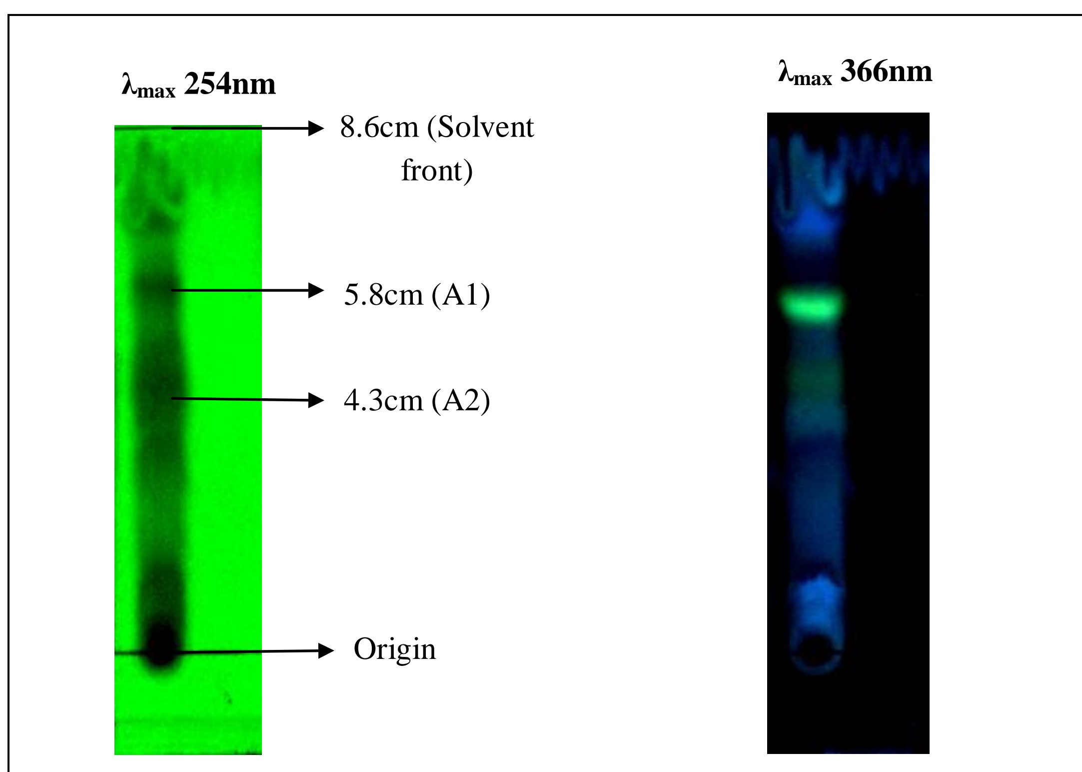


Figure 4.4: Pre-Identification of alkaloid using TLC Visualizer at λ_{max} 254nm and λ_{max} 366nm for fraction F4

Based on the Figure 4.4 the R_f value of the two visible alkaloids are 0.67 and 0.50 respectively for A1 and A2. The R_f value being > 0.2 simply shows that the compounds are separable. These compounds identified formed orange spots when sprayed with Dragendorff reagent indicating presence of alkaloid (Harborne, 1988). This TLC analysis carried out clearly supports that fraction 4 may contain alkaloids as reported by Harborne, 1988. In vitro studies have demonstrated that these alkaloids can act as scavengers of reactive oxygen species (ROS) and have effective antioxidant property (Tse et al., 1991).

4.6 PRE-IDENTIFICATION OF PHENOLIC COMPOUNDS

The phenolic present in fraction F3 and F4 was determined using the thin layer chromatography method. The following results were obtained based on the method described in chapter 3 on pre-identification of phenolic compounds using Thin Layer Chromatography (TLC) as in Figure 4.5.

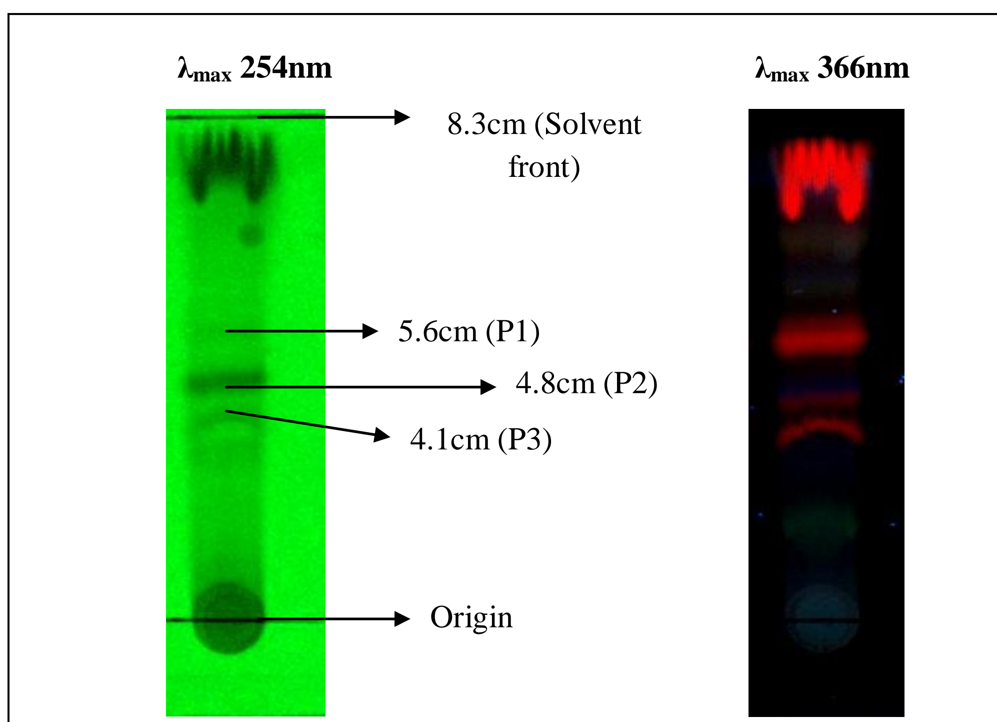


Figure 4.5: Pre-Identification of phenolic using TLC Visualizer at λ_{max} 254nm and λ_{max} 366nm for fraction F3

Based on the Figure 4.5 the R_f value of the three visible phenolics are 0.67, 0.59 and 0.49 respectively for P1, P2 and P3 in fraction F3. The R_f value being > 0.2 simply shows that the compounds are separable. These compounds identified formed pink to reddish bands when sprayed with Vanillin-Sulphuric acid reagent indicating presence of phenolic (Harborne, 1988). Plant phenolics are strong antioxidants (Halliwell and Gutteridge, 1999). Figure 4.6 shows the Pre-Identification of phenolic using TLC Visualizer at λ_{max} 254nm and λ_{max} 366nm for fraction F4.

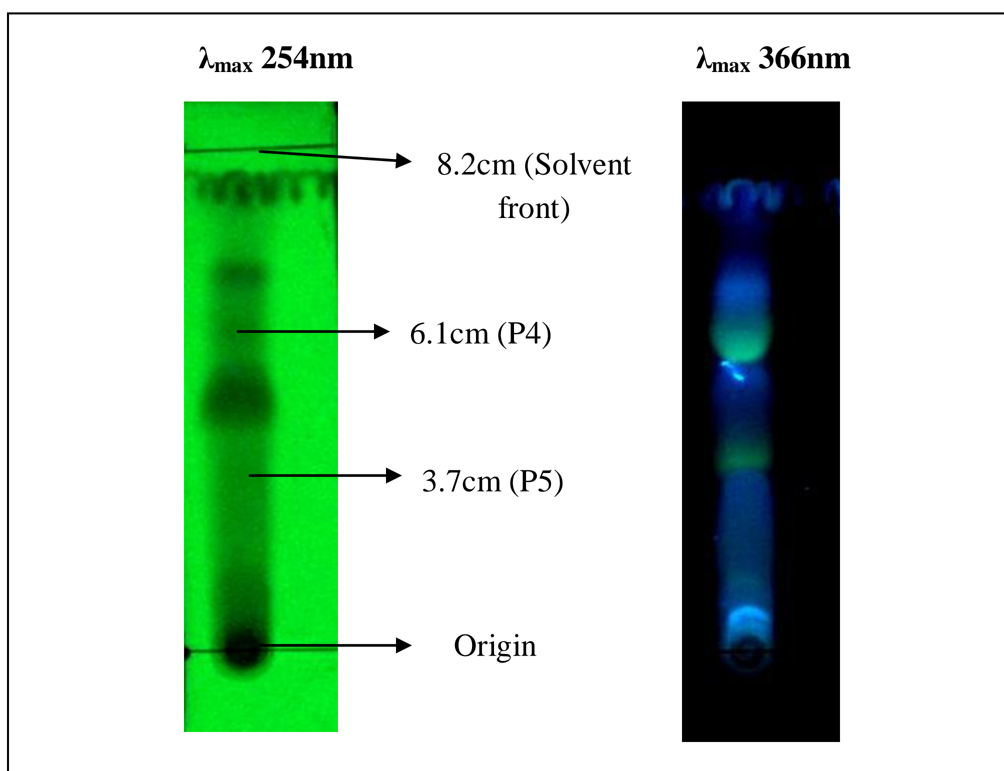


Figure 4.6: Pre-Identification of phenolic using TLC Visualizer at λ_{max} 254nm and λ_{max} 366nm for fraction F4.

Based on the Figure 4.6 the R_f value of the two visible phenolics are 0.45 and 0.74 respectively for P4 and P5 in fraction F4. The R_f value being > 0.2 simply shows that the compounds are separable. These compounds identified formed pink to reddish bands when sprayed with Vanillin-Sulphuric acid reagent indicating presence of phenolic (Harborne,

1988). Therefore, the result obtained clearly supports the amount of phenolic content to be significantly present in the fraction 4 from the total phenolic content evaluation using Folin-Ciocalteu reagent method.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 INTRODUCTION

In this chapter, the conclusion from the overall research is summarized. It includes the approach on whether research carried out met the objectives of the study. Other, than that it includes general significance of the research to industry and society based on the findings. It also includes some recommendations on further investigation on the current research that can be done.

5.2 CONCLUSION

Through the research carried out, I believe that all the objectives of this study had been met. This is because all five fractions from *Tinospora crispa* stems extracts was fractioned, the antioxidant activity of the respective fractions were determined and finally the total content of phenolics in the extracted fractions were determined. Based on the research carried out, it was found that fraction F3 and F4 from the extract of *Tinospora crispa* stems extracts possess antioxidant activity comparable with the established antioxidants, like ascorbic acid and BHT.

The antioxidant activity of *Tinospora crispa* extract might be attributed to its effective hydrogen-donating ability and effective as scavenger of free radicals due to the presence of phenolics in the fraction F3 and F4. The results suggest that *Tinospora crispa*

stems are a valuable source of natural antioxidant and can be potentially developed as herbal supplementary in combating free-radical mediated diseases such as cancer and aging. Other than that, the study provoke that due to the ready availability of the plant source widely distributed in Asia and the plant source being cheap will definitely make it viable for industrial applications to produce medicinal value products.

5.3 RECOMMENDATIONS

The bioassay carried out indicated that maybe the presence of phenolic compounds in the fractions extracted could have influenced the antioxidant activity of the fractions. Thus, further investigations on the plant extracts can be done to isolate the responsible phenolic compounds that possess free radical scavenging property. It is recommended that column chromatography for fractionation of the extract can be used. Next, the pure compounds obtained from the column chromatography can be used for further identification using High Performance Liquid Chromatography (HPLC) and Nuclear Magnetic Resonance (NMR) spectroscopy. Besides, other bioassay such as Ferric Reducing Antioxidant Power (FRAP) assay can be used to determine the reducing strength of the plant extract.

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APPENDICES

APPENDIX A

ANTIOXIDANT ACTIVITY OF TINOSPORA CRISPA STEMS

Appendix A1: DPPH Assay absorbance at 0 min

Replicates/Samples	F1	F2	F3	F4	F5	control	BHT	Ascorbic acid
1	1.5898	0.9704	0.4590	0.6398	1.4283	1.5906	0.2313	0.2399
2	1.5609	0.9637	0.4629	0.6236	1.4407	1.6207	0.2575	0.2641
3	1.5219	0.9218	0.4911	0.6374	1.3991	1.6649	0.2726	0.2708
4	1.5288	0.9107	0.4536	0.5981	1.4613	1.5859	0.2598	0.2560
5	1.5497	0.9476	0.4734	0.5935	1.4549	1.5865	0.2806	0.2601
6	1.5792	0.9536	0.4986	0.6152	1.3961	1.6722	0.2798	0.2885
Mean	1.5551	0.9446	0.4731	0.6179	1.4301	1.6201	0.2636	0.2632

Appendix A2: Percentage (%) of inhibition at 0 min

Replicates/Samples	F1	F2	F3	F4	F5	BHT	Ascorbic acid
1	0.05	38.99	71.14	59.78	10.20	85.46	84.92
2	3.69	40.54	71.44	61.52	11.11	84.11	83.71
3	8.59	44.63	70.50	61.72	15.96	83.63	83.74
4	3.60	42.58	71.40	62.29	7.86	83.62	83.86
5	2.32	40.27	70.16	62.59	8.29	82.31	83.61
6	5.56	42.97	70.18	63.21	16.51	83.27	82.75
Mean	3.97	41.66	70.80	61.85	11.66	83.73	83.76

Appendix A3: DPPH Assay absorbance at 15 min

Replicates/Samples	F1	F2	F3	F4	F5	control	BHT	Ascorbic acid
1	1.5022	0.9311	0.4439	0.6321	1.3987	1.5456	0.0915	0.0975
2	1.5100	0.9211	0.4221	0.6101	1.4230	1.6062	0.0980	0.0954
3	1.4992	0.9199	0.4733	0.6299	1.3772	1.5509	0.0992	0.1022
4	1.4788	0.8998	0.4511	0.5786	1.4332	1.5347	0.1002	0.0988
5	1.5114	0.9322	0.4625	0.5867	1.4119	1.6082	0.1117	0.0984
6	1.5331	0.9125	0.4886	0.6003	1.3764	1.6065	0.1046	0.1037
Mean	1.5058	0.9194	0.4569	0.6063	1.4034	1.5754	0.1009	0.0993

Appendix A4: Percentage (%) of inhibition at 15 min

Replicates/Samples	F1	F2	F3	F4	F5	BHT	Ascorbic acid
1	2.81	39.76	71.28	59.10	9.50	94.08	93.69
2	5.99	42.65	73.72	62.02	11.41	93.90	94.06
3	3.33	40.69	69.48	59.38	11.20	93.60	93.41
4	3.64	41.37	70.61	62.30	6.61	93.47	93.56
5	6.02	42.03	71.24	63.52	12.21	93.05	93.88
6	4.57	43.20	69.59	62.63	14.32	93.49	93.54
Mean	4.39	41.62	70.99	61.49	10.88	93.60	93.69

Appendix A5: DPPH Assay absorbance at 30 min

Replicates/Samples	F1	F2	F3	F4	F5	Control	BHT	Ascorbic acid
1	1.4431	0.8207	0.2337	0.5320	1.3771	1.4453	0.0930	0.0969
2	1.4361	0.8118	0.2552	0.5431	1.3992	1.4533	0.0980	0.0962
3	1.4429	0.8099	0.2342	0.5259	1.3543	1.5822	0.0992	0.1016
4	1.4168	0.8795	0.2243	0.5444	1.4001	1.4436	0.0943	0.0989
5	1.4339	0.8108	0.2752	0.5772	1.3879	1.4443	0.1078	0.0977
6	1.4256	0.7798	0.2500	0.5553	1.3546	1.5819	0.1030	0.1031
Mean	1.4331	0.8188	0.2454	0.5463	1.3789	1.4918	0.0992	0.0991

Appendix A6: Percentage (%) of inhibition at 30 min

Replicates/Samples	F1	F2	F3	F4	F5	BHT	Ascorbic acid
1	0.15	43.22	83.83	63.19	4.72	93.57	93.30
2	1.18	44.14	82.44	62.63	3.72	93.26	93.38
3	8.80	48.81	85.20	66.76	14.40	93.73	93.58
4	1.86	39.08	84.46	62.29	3.01	93.47	93.15
5	0.72	43.86	80.95	60.04	3.91	92.54	93.24
6	9.88	50.70	84.20	64.90	14.37	93.49	93.48
Mean	3.77	44.97	83.51	63.30	7.36	93.34	93.35

Appendix A7: DPPH Assay absorbance at 45 min

Replicates/Samples	F1	F2	F3	F4	F5	Control	BHT	Ascorbic acid
1	1.4100	0.7801	0.1887	0.5100	1.3331	1.4222	0.0934	0.0969
2	1.4006	0.7887	0.1957	0.5003	1.3231	1.4351	0.0987	0.0961
3	1.4009	0.7702	0.1876	0.4988	1.3432	1.5072	0.1015	0.0927
4	1.4012	0.7535	0.2008	0.5004	1.3987	1.4406	0.0948	0.0957
5	1.4009	0.7558	0.2023	0.5372	1.3543	1.4382	0.0959	0.0979
6	1.4010	0.7448	0.2202	0.5213	1.3298	1.5051	0.1041	0.0977
Mean	1.4024	0.7655	0.1992	0.5113	1.3470	1.4581	0.0981	0.0962

Appendix A8: Percentage (%) of inhibition at 45 min

Replicates/Samples	F1	F2	F3	F4	F5	BHT	Ascorbic acid
1	0.86	45.15	86.73	64.14	6.26	93.43	93.19
2	2.40	45.04	86.36	65.14	7.80	93.12	93.30
3	7.05	48.90	87.55	66.91	10.88	93.27	93.85
4	2.73	47.70	86.06	65.26	2.91	93.42	93.36
5	2.59	47.45	85.93	62.65	5.83	93.33	93.19
6	6.92	50.51	85.37	65.36	11.65	93.08	93.51
Mean	3.76	47.46	86.34	64.91	7.56	93.28	93.40

Appendix A9: DPPH Assay absorbance at 1 h

Replicates/Samples	F1	F2	F3	F4	F5	Control	BHT	Ascorbic acid
1	1.2221	0.6988	0.1553	0.4991	1.2991	1.3027	0.0940	0.0937
2	1.2345	0.6954	0.1587	0.4871	1.2671	1.2998	0.0983	0.0943
3	1.2407	0.6898	0.1498	0.4685	1.2982	1.4362	0.0996	0.0923
4	1.2321	0.7007	0.1768	0.4914	1.2567	1.2973	0.0954	0.0990
5	1.2987	0.6938	0.1713	0.5123	1.2763	1.3169	0.0956	0.0936
6	1.2776	0.7008	0.1678	0.5008	1.2780	1.4548	0.0955	0.0975
Mean	1.2510	0.6966	0.1633	0.4932	1.2792	1.3513	0.0964	0.0951

Appendix A10: Percentage (%) of inhibition at 1 h

Replicates/Samples	F1	F2	F3	F4	F5	BHT	Ascorbic acid
1	6.19	46.36	88.08	61.69	0.28	92.78	92.81
2	5.02	46.50	87.79	62.53	2.52	92.44	92.75
3	13.61	51.97	89.57	67.38	9.61	93.07	93.57
4	5.03	45.99	86.37	62.12	3.13	92.65	92.37
5	1.38	47.32	86.99	61.10	3.08	92.74	92.89
6	12.18	51.83	88.47	65.58	12.15	93.44	93.30
Mean	7.24	48.33	87.88	63.40	5.13	92.85	92.95

Appendix A11: DPPH Assay absorbance at 1 h 15 min

Replicates/Samples	F1	F2	F3	F4	F5	Control	BHT	Ascorbic acid
1	1.2024	0.6818	0.1243	0.4862	1.2001	1.2421	0.0947	0.0923
2	1.2331	0.6831	0.1477	0.4767	1.2131	1.2658	0.0945	0.0939
3	1.2807	0.6729	0.1491	0.4728	1.2233	1.3038	0.0952	0.0962
4	1.1866	0.6817	0.1536	0.4614	1.1954	1.1971	0.0950	0.0947
5	1.1922	0.6848	0.1412	0.4776	1.1936	1.1966	0.0943	0.0934
6	1.2331	0.6895	0.1571	0.4733	1.2115	1.3260	0.0944	0.0932
Mean	1.2214	0.6823	0.1455	0.4747	1.2062	1.2552	0.0947	0.0940

Appendix A12: Percentage (%) of inhibition at 1 h 15 min

Replicates/Samples	F1	F2	F3	F4	F5	BHT	Ascorbic acid
1	3.20	45.11	89.99	60.86	3.38	92.38	92.57
2	2.58	46.03	88.33	62.34	4.16	92.53	92.58
3	1.77	48.39	88.56	63.74	6.17	92.70	92.62
4	0.88	43.05	87.17	61.46	0.14	92.06	92.09
5	0.37	42.77	88.20	60.09	0.25	92.12	92.19
6	7.01	48.00	88.15	64.31	8.63	92.88	92.97
Mean	2.63	45.56	88.40	62.13	3.79	92.45	92.50

Appendix A13: DPPH Assay absorbance at 1 h 30 min

Replicates/Samples	F1	F2	F3	F4	F5	Control	BHT	Ascorbic acid
1	1.0011	0.6612	0.1231	0.4687	1.0009	1.0313	0.0942	0.0879
2	0.8971	0.6422	0.1294	0.4578	0.9811	0.9941	0.0941	0.0932
3	1.0501	0.6527	0.1358	0.4575	1.0201	1.1128	0.0921	0.0916
4	0.9665	0.6411	0.1397	0.4443	0.9781	0.9822	0.0923	0.0921
5	0.9698	0.5991	0.1383	0.4483	0.9776	0.9957	0.0943	0.0917
6	1.0016	0.5989	0.1457	0.4668	1.0010	1.1443	0.0928	0.0917
Mean	0.9810	0.6325	0.1353	0.4572	0.9931	1.0434	0.0933	0.0914

Appendix A14: Percentage (%) of inhibition at 1 h 30 min

Replicates/Samples	F1	F2	F3	F4	F5	BHT	Ascorbic acid
1	2.93	35.89	88.06	54.55	2.95	90.87	91.48
2	9.76	35.40	86.98	53.95	1.31	90.53	90.62
3	5.63	41.35	87.80	58.89	8.33	91.72	91.77
4	1.60	34.73	85.78	54.76	0.42	90.60	90.62
5	2.60	39.83	86.11	54.98	1.82	90.53	90.79
6	12.47	47.66	87.27	59.21	12.52	91.89	91.99
Mean	5.83	39.14	87.00	56.06	4.56	91.02	91.21

Appendix A15: DPPH Assay absorbance at 1 h 45 min

Replicates/Samples	F1	F2	F3	F4	F5	Control	BHT	Ascorbic acid
1	0.9788	0.5012	0.1111	0.3841	0.9802	0.9813	0.0932	0.0850
2	0.9565	0.5031	0.1012	0.3925	0.9870	0.9981	0.0907	0.0834
3	0.9577	0.4887	0.1100	0.3865	0.9234	0.9769	0.0908	0.0871
4	0.9554	0.5121	0.1176	0.3952	0.9435	0.9580	0.0825	0.0821
5	0.9871	0.4911	0.1889	0.3997	0.9756	0.9932	0.0854	0.0872
6	0.9234	0.5189	0.1871	0.3863	0.9762	0.9905	0.0928	0.0897
Mean	0.9598	0.5025	0.1360	0.3907	0.9643	0.9830	0.0892	0.0858

Appendix A16: Percentage (%) of inhibition at 1 h 45 min

Replicates/Samples	F1	F2	F3	F4	F5	BHT	Ascorbic acid
1	0.25	48.92	88.68	60.86	0.11	90.50	91.34
2	4.17	49.59	89.86	60.68	1.11	90.91	91.64
3	1.97	49.97	88.74	60.44	5.48	90.71	91.08
4	0.27	46.54	87.72	58.75	1.51	91.39	91.43
5	0.61	50.55	80.98	59.76	1.77	91.40	91.22
6	6.77	47.61	81.11	61.00	1.44	90.63	90.94
Mean	2.34	48.87	86.18	60.25	1.91	90.92	91.28

Appendix A17: DPPH Assay absorbance at 2 h

Replicates/Samples	F1	F2	F3	F4	F5	Control	BHT	Ascorbic acid
1	0.5138	0.3013	0.0999	0.2971	0.5002	0.5252	0.0801	0.0803
2	0.5250	0.2993	0.0981	0.2895	0.5117	0.5273	0.0782	0.0782
3	0.5114	0.3120	0.0992	0.2465	0.5123	0.5281	0.0788	0.0791
4	0.5124	0.3002	0.0987	0.2677	0.5045	0.5204	0.0805	0.0810
5	0.5123	0.3023	0.1572	0.2411	0.5006	0.5166	0.0804	0.0803
6	0.5100	0.2998	0.1493	0.2226	0.5012	0.5142	0.0801	0.0777
Mean	0.5142	0.3025	0.1171	0.2608	0.5051	0.5220	0.0797	0.0794

Appendix A18: Percentage (%) of inhibition at 2 h

Replicates/Samples	F1	F2	F3	F4	F5	BHT	Ascorbic acid
1	2.17	42.63	80.98	43.43	4.76	84.75	84.71
2	0.44	43.24	81.40	45.10	2.96	85.17	85.17
3	3.16	40.92	81.22	53.32	2.99	85.08	85.02
4	1.54	42.31	81.03	48.56	3.06	84.53	84.44
5	0.83	41.48	69.57	53.33	3.10	84.44	84.46
6	0.82	41.70	70.96	56.71	2.53	84.42	84.89
Mean	1.49	42.05	77.53	50.07	3.23	84.73	84.78

APPENDIX B

TOTAL CONTENT OF PHENOLICS *TINOSPORA CRISPA* STEMS

Appendix B1: Concentration of fraction F1, F2, F3, F4 and F5 from gallic acid equivalent standard curve

Replicates/samples	F1	F2	F3	F4	F5
1	0.07	3.22	6.95	5.72	0.51
2	0.08	3.52	6.97	6.53	0.45
3	0.05	3.76	7.02	5.82	0.54
4	0.05	3.51	7.11	6.42	0.55
5	0.09	3.70	7.88	6.43	0.56
6	0.01	3.76	8.20	6.35	0.51
Mean	0.06	3.58	7.35	6.21	0.52

Appendix B2: Total content of phenolic compounds in fraction F1, F2, F3, F4 and F5

Replicates/samples	F1	F2	F3	F4	F5
1	0.68	32.19	69.45	57.19	5.10
2	0.77	35.19	69.74	65.26	4.48
3	0.55	37.61	70.19	58.16	5.36
4	0.48	35.07	71.07	64.23	5.48
5	0.94	37.03	78.81	64.26	5.65
6	0.11	37.61	81.97	63.48	5.07
Mean	0.59	35.79	73.54	62.10	5.19

Appendix B3: Absorbance of F1, F2, F3, F4, F5 and control DMSO at 750 nm

Replicates/samples	F1	F2	F3	F4	F5	DMSO
1	0.0621	0.1598	0.2753	0.2373	0.0758	0.0023
2	0.0624	0.1691	0.2762	0.2623	0.0739	0.0021
3	0.0617	0.1766	0.2776	0.2403	0.0766	0.0022
4	0.0615	0.1687	0.2803	0.2591	0.077	0.0022
5	0.0629	0.1748	0.3043	0.2592	0.0775	0.0023
6	0.0633	0.1766	0.3141	0.2568	0.0757	0.0024