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EVALUATION OF *IN VITRO* METHODS OF ANTIOXIDANT ACTIVITY DETERMINATION USING CAFFEIC ACID

BY

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DECLARATION

I hereby declare that this submission is my own work towards the Master of Science degree in Biochemistry and that, to the best of my knowledge, it contains no material previously published by another person or material which has been accepted for the award of another degree of the University, except where due acknowledgement has been made in the text.

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Abstract

The differences in chemical composition of natural antioxidants provides challenges at separating, detecting, and quantifying individual non-enzymatic antioxidants in complex food/biological systems. Consequently, the antioxidant capacity is a better approach at assaying beneficial health effects that can result from the combined actions of individual antioxidant species. Antioxidant assay methods are based either on electron transfer (ET) or hydrogen atom transfer (HAT) mechanisms. The results obtained are different because of the mechanisms, redox potentials, pH and solvent requirements of the various assay methods.

Antioxidant activities of caffeic acid were evaluated and compared with that of Ascorbic acid using different *in vitro* methods of 1, 1-diphenyl-2-picryl-hydrazyl free radical (DPPH) scavenging, hydrogen peroxide scavenging and Phosphomolybdenum antioxidative power methods. At tested concentrations of 1mM and 2mM, caffeic acid showed an inhibition of 84.63% and 84.93% on the DPPH radical respectively, while Ascorbic acid gave 83.43% and 85.43% inhibition respectively. Both substances showed significant antioxidant activities in the tested analytical methods. The study showed that the two substances are good scavengers of the DPPH radical. However caffeic acid demonstrated a better H_2O_2 radical scavenging ability with ascorbic acid ranking better in the scavenging assay of the Phosphomolybdenum radical.

DEDICATION

To: Moyinoluwa, Fehintoluwa and Funmilola.

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List of Abbreviations

AAPH: 2, 2'-Azobis (2-amidinopropane) hydrochloride

ABAP: 2, 2'-Azobis (2-aminopropane)

ABTS: 2, 2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)

AO: Antioxidant

AOC: Antioxidant capacity

AUC: Area under curve

BDE: Bond dissociation energy

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoluene

BUC: Bucillamine

CAT: Catalase

CD: Cyclodextrin

CUPRAC: Cupric reducing antioxidant capacity

DCM: Dichloromethane

DPPH: 2, 2-Di (4-tert-octylphenyl)-1-picrylhydrazyl

DMPD: N, N-dimethyl-1, 4-diaminobenzene

ET: Electron transfer

EtOH: Ethanol

FC: Folin-Ciocalteu

FCR-Folin Ciocalteu Reagent

FRAP: Ferric reducing antioxidant power

GSH: Glutathione

HAT: Hydrogen atom transfer

KMBA: α-keto-γ-methiolbutyric acid

LDL: Low-density lipoprotein

LG: Lauryl gallate

M-β-CD: Methyl-β-cyclodextrin

MDA: Malondialdehyde

MeOH: Methanol

NAC: N-acetyl cysteine

NBT: Nitro blue tetrazolium

ORAC: Oxygen radical absorbance capacity

PAP-Phosphomolybdenum Antioxidative Assay

PG: Propyl gallate

RPM: Revolutions per minute

ROS: Reactive oxygen species

RNS: Reactive nitrogen species

RSS: Reactive sulfur species

SOD: Superoxide dismutase

TAC: Total antioxidant capacity

TBA: Thiobarbituric acid

TBARS: Thiobarbituric acid–reactive substances

TBHQ: Tert-butyl hydroquinone

TEAC: Trolox equivalent antioxidant capacity

TPTZ: Tripyridyltriazine

TRAP: Total peroxyl radical-trapping antioxidant parameter

XO: Xanthine oxidase

CHAPTER ONE

INTRODUCTION

1.0 Background

The importance of oxidation of foodstuffs in the human body cannot be underestimated as it is essential for the survival of cells (Huang *et al*., 2005). A side effect of this oxidation is the production of free radicals and other reactive oxygen species (ROS) such as superoxide anion (O_2•$^-$), hydroxyl (•OH), peroxyl (ROO•), alkoxyl radicals (RO•), hydrogen peroxide (H_2O_2), singlet oxygen ($O_2{}^1\Delta g$) and reactive nitrogen species (RNS) mainly NO (nitric oxide), ONOO (peroxy nitrate), NO_2 (nitrogen dioxide) and N_2O_3 (dinitrogen trioxide). These may attack biological macromolecules, giving rise to protein, lipid, and DNA damage, cell aging, oxidative stress-originated diseases (e.g. cardiovascular and neurodegenerative diseases), and cancer (Ames *et al.*, 1993; Halliwell *et al*., 1995; Ghiselli *et al*., 2000; Gulcin *et al*., 2010). These free radicals affect the adjoining stable molecules by electron transfer. When this happens, the attacked molecule itself becomes another free radical setting up a chain of reactions, resulting in the destruction of the living cell (Frenkel and German, 2006). Antioxidants scavenge or quench ROS and RNS products of respiration that can cause oxidative damage to cells (Helmut 2007; Cao *et al*., 1993). There is increasing evidence linking the involvement of such species with a variety of normal *in vivo* regulatory systems (Pulido *et al*., 2000). When ROS and RNS accumulates, they can disrupt the activities of protective enzymes such as superoxide dismutase, catalase and peroxidase and cause lethal cellular effects (e.g. senescence and apoptosis) by altering the normal structures of membrane lipids, cellular proteins, DNA and enzymes, thus down regulating cellular respiration (Hooper and Cassidy, 2006). Furthermore, ROS have been shown to influence cell signaling pathways (Vinson *et al*., 1998; Kaneyuki *et al*., 1999; Lee *et al*., 2005). Food items can

also be oxidized resulting in chemical spoilage that leads to rancidity and /or deterioration of the nutritional quality, color, flavor, texture and eventual safety of foods (Oyaizu, 1986; Dinis *et al*., 1994; Fuleki and Francis, 1968). Evidence exists that half of the world's fruit and vegetable are lost due to postharvest deteriorations caused by oxidative reactions (Zhu *et al*., 2002; Brand *et al*., 1995; Badarinath *et al*., 2010). Defense mechanisms against the effects of excessive oxidations are augmented by the action of various natural antioxidants and thus the measurement of antioxidant activity is imperative (Milan *et al*., 2010; Verica *et al*., 2007; Andreas *et al*., 2001). Antioxidant activity relates to the kinetics of a reaction that takes place between an antioxidant and the prooxidant or the radical it reduces or scavenges, whereas antioxidant capacity determines the thermodynamic conversion efficiency of an oxidant probe upon reaction with an antioxidant (Max Romeo *et al*., 2010; Alam *et al*., 2013).

Caffeic acid (3, 4-dihydroxycinnamic acid, 3-(3, 4-dihydroxyhenyl) propenoic acid, 3-(3, 4-dihydroxyhenyl)-2-propenoic acid) is among the major hydroxycinnamic acids, a subgroup of phenolic compounds, present in plant materials including food products of plant origin, it is a potent antioxidant and an integral part of human and animal diets (Bennick, 2002; Robards *et al*., 1997; Balasundram *et al*., 2006; Gulcin, 2006).

Any substance whose presence, at relatively low concentration, in comparison to those of the oxidizable substrate, reduces the oxidation of the substrate is referred to as an antioxidant (Apak *et al*., 2007; Garry *et al*.; 2013; Heng-Yuan *et al*., 2007). They are classified as either primary/ chain-breaking antioxidants, or secondary/preventative antioxidants (Doughari, 2012).

The methods of assessing antioxidant activity fall into two broad categories based on either activity in foods or bioactivity in humans (Rong Tsao, 2010). There is the increasing need to assess the ability of the antioxidant(s) to provide protection for the food against spoilage that may

result from oxidation and reduction reactions. The principles of other assay methods of determination of antioxidant activity involves measurement of activity in foods, particularly fruits, vegetables and beverages, bearing in mind the need to access the dietary burden as a result of *in vivo* activity of the active antioxidants present (Devanand *et al*., 2006). According to Terao *et al*. (1993) and Jiang and Ho (1997), oxidative stress in humans arises from an imbalance in the antioxidant status (ROS and RNS levels) in reference to defense and repair mechanisms. The body's endogenous defense machinery include enzymes such as superoxide dismutase, catalase and glutathione peroxidase, vitamin E, uric acid and serum albumins (Stalikas, 2007; Gupta, 2015). In addition to these, food-derived antioxidants has been found to be important as it complements the body's endogenous defense machinery (Mellina *et al*., 2011; Moon and Shibamoto, 2009). The sum of endogenous and food-derived antioxidants represents the total antioxidant activity of the extracellular fluid (Khoddami *et al*., 2013). Cooperation of all the different antioxidants provides greater protection against attack by reactive oxygen or nitrogen radicals, than any single compound alone. The overall antioxidant capacity gives a more relevant biological information compared to that obtained by the measurement of individual components, as it considers the cumulative effect of all antioxidants present in plasma and body fluids (MacDonald-Wicks *et al*., 2006).

Antioxidants can deactivate radicals by two major mechanisms: Hydrogen Atom Transfer (HAT) and Single Electron Transfer (SET) (Ronald *et al*., 2005; Michael *et al*., 2002; Apak *et al*., 2013). Regardless of mechanism, the end result is the same, differing only in kinetics and potential for side reactions (Jayanthi and Subash, 2010; Charalampos *et al*., 2013). Both reactions can occur in parallel, with the mechanism dominating a given system being determined by antioxidant structure and properties, solubility and partition coefficient, and solvent system (Tchinda *et al*., 2014; Simona *et al*., 2010). Ultimately, bond dissociation energy (BDE) and ionization potential (IP) are

major factors that determine the mechanism and the efficacy of antioxidants (Rabeta *et al*., 2013; Jagtap *et al*., 2010).

1.1 Problem Statement

Studies in recent years confirm that bioactive compounds have properties that are beneficial to human health, with potential physiological effects such as anticancer, vasoprotective, anti-inflammatory and hepatoprotective, among others. On the other hand, the reported harmful effects of synthetic antioxidant compounds including butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) which are often used in the food industry to prevent or retard oxidative deterioration in foods has led the World Health Organization to impose restrictions on their use. The awareness of related ecological issues has stimulated research in food, drug and cosmetic industries. The increasing need to search for antioxidant compounds from vegetables, fruits, herbs and spices which are less harmful alternative to synthetic antioxidants underscores the importance of suitable parameters to accessing their capabilities and activity. It is therefore imperative that screening programs for antioxidants, based on the assumption that a combination of data would provide a better description of antioxidant activity than obtained from the employment of a single assay method.

1.2 Significance of study

This project will achieve the following:

- Aid the identification and quantification of properties and mutual effects of antioxidants.
- Provide a rational basis for the classification of antioxidant assays with their constraints and challenges, and make the results more comparable and understandable.

- It will give an insight into the principles and methodologies for the development of standard antioxidant capacity assays for use in food, nutraceutical, and dietary supplement industries.

1.3 OBJECTIVES

The main purpose of this research is to evaluate the various *in-vitro* methods of antioxidant activity determination using caffeic acid and ascorbic acid with the aim of achieving the following specific objectives:

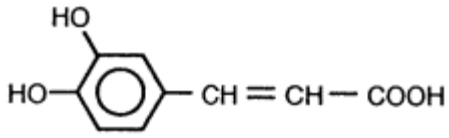
- Examination of the antioxidative properties of individual active substances or complex mixtures, such as plant extracts for the direct use as cosmetic agent for the skin or for the antioxidative stabilization of cosmetic products food complements or for the antioxidative stabilization of drugs.
- Detection of the effectiveness of antioxidative components and complex mixtures such as plant extracts for the use as food complement – design of Novel Food with antioxidants as protection against cancer cardiovascular diseases.
- Comparison of the results obtained from different assay methods used for the measurement of antioxidant activity.

CHAPTER TWO

LITERATURE REVIEW

2.1 Caffeic acid

Caffeic acid (3, 4-dihydroxycinnamic acid, 3-(3, 4-dihydroxyhenyl) propenoic acid, 3-(3, 4-dihydroxyhenyl)-2-propenoic acid) is among the major hydroxycinnamic acids, a subgroup of phenolic compounds, present in plant materials including food products of plant origin, it is a potent antioxidant, a α-tocopherol protectant in low-density lipoprotein (LDL) (Laranjinha *et al*., 1995) and an integral part of human and animal diets (Bennick, 2002; Robards *et al*., 1997; Balasundram *et al*., 2006; Gulcin, 2006).

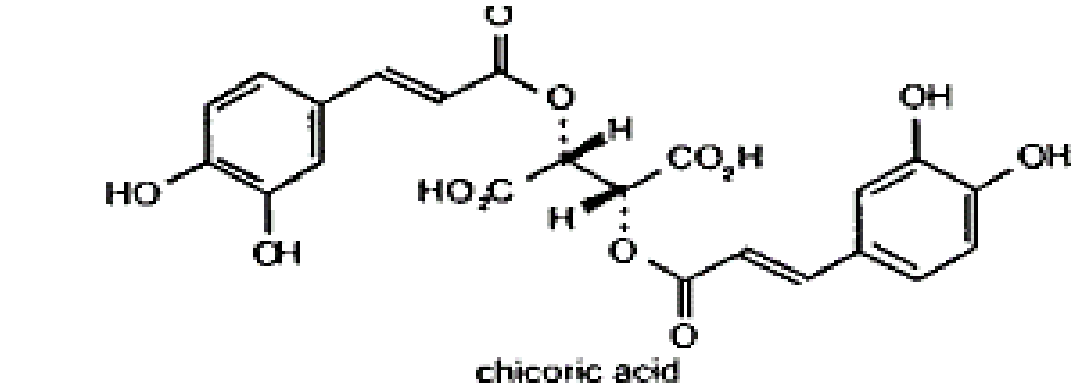


($C_9H_8O_4$, Mol.wt. 180.15).

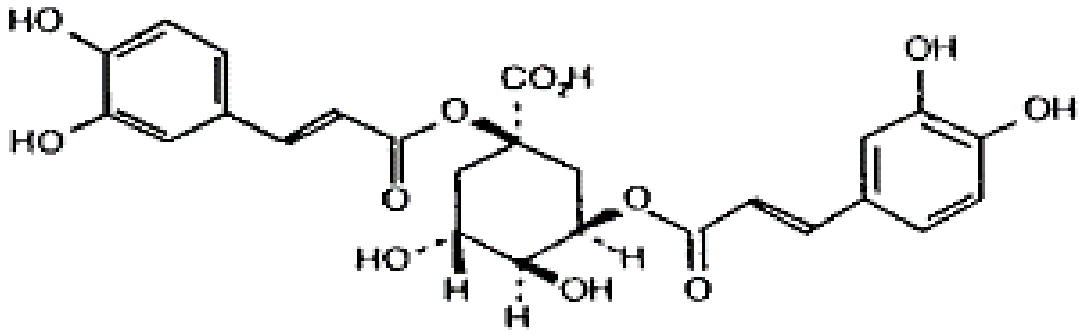
Figure 2.1 Structure of Caffeic acid

Caffeic acid occurs naturally in a wide range of plants, free and in various combined forms (Conkerton and Chapital, 1983), as a multifunctional naturally organic substance which plays a significant role in binding metal ions from natural environment, food substances and beverages such as coca cola, mineral water etc. The ligand has two complexing sites in competition: the catechol group (dihydroxybenzene) and the carboxylic function (Abebe, 2012). It is a constituent of numerous species, including Umbelliferae, Cruciferae, Cucurbitaceae, Polygonaceae, Compositae, Labiatae, Solanaceae, Leguminosae, Saxifragaceae, Caprifoliaceae, Theaceae and Valerianaceae (Zubaida *et al*., 2013). Caffeic acid has been identified in plants used for medicinal purposes, it is present in a variety of fruits, vegetables and seasonings, predominantly

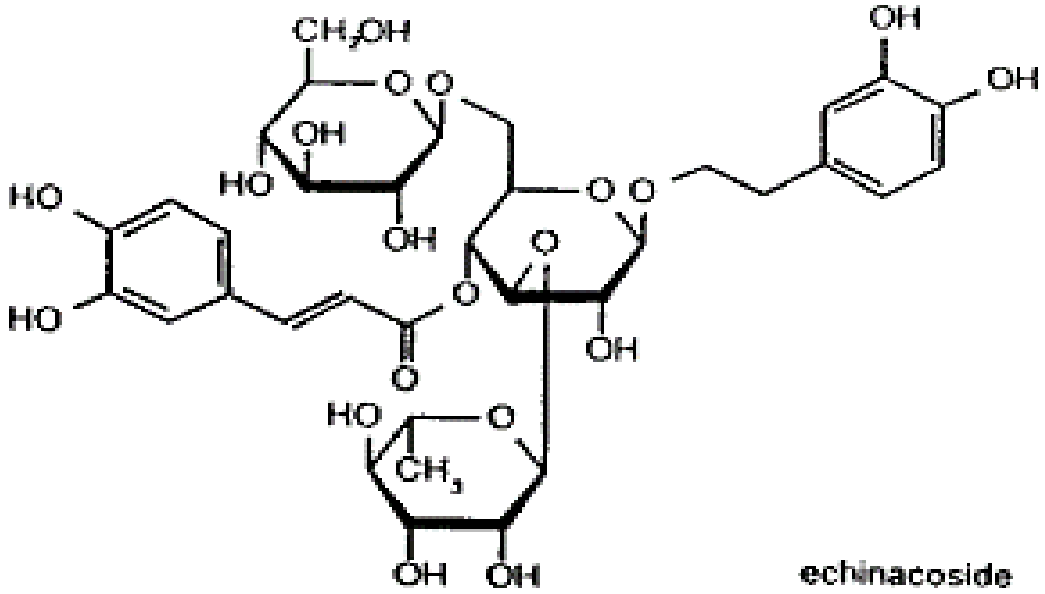
in the form of ester conjugates (Fig.2.2), including chlorogenic acids (esters of caffeic acid and quinic acid) and related compounds. Upon ingestion, the conjugates are hydrolyzed and may lead to variable uptake of caffeic acid (Table 2.1).



chicoric acid



cynarin (1,5-dicaffeylquinic acid)



echinacoside

Figure 2.2 Caffeic acid Conjugates

Caffeic acid effectively inhibits lipoxygenase and thus the biosynthesis of leukotrienes, which are implicated in immunoregulation and in a variety of diseases, including asthma. It has been implicated in the process of platelet aggregation as a result of its ability to inhibit the production of thromboxane A2, which can cause bronchoconstriction (Koshihara *et al*., 1984; Murota and Koshihara, 1985). Caffeic acid, together with related hydroxycinnamic acid compounds are important in chain-breaking reactions involved in radical scavenging activities, in relation to their hydrogen or electron donating capacity, thereby resulting in their ability to delocalize/stabilize the resulting phenoxyl radical included in their structure (Teixeira *et al*., 2013). According to Terao *et al*. (1993), the presence of a second phenolic hydroxyl group in caffeic acid may enhance the radical scavenging activity due to additional resonance stabilization and o-quinone formation. Phenolics operate as secondary oxidants due to their ability to chelate potentially pro-oxidative transition metal ions in a manner dependent on their relative stabilization energies, using the 5-OH and/or 3-OH moiety with a 4-oxo group in the A /C ring structure or a large number of hydroxyl groups (Rice-Evans *et al*., 1997), forming a complex incapable of promoting oxidation (Mira *et al*., 2002).

The assertion that phenolics and polyphenolics (polymeric phenolics) can alleviate complications associated with certain physical ailments and degenerative diseases in humans, including the reduction of cardiovascular disease and certain cancers by Scalbert *et al*. (2002); Arts and Hollman (2005); led to extensive studies into the extraction and analysis of phenolics from plants and other food sources. They are added to functional foods and nutraceuticals to provide additional targeted health benefits to consumers and are associated with many aspects of food quality including color, flavor properties, and nutrition (Naczk and Shahidi, 2004; Dai and Mumper, 2010). Caffeic acid

occur as phenyl-propanoids and Hydroxycinnamate conjugates in Citrus fruit (Rice-Evans *et al*., 1997).

Table 2.1 Occurrence of caffeic acid in selected food plants, therapeutic use and method of analysis.

Family and species name	Method of Analysis	Therapeutic use	Reference
Lamiaceae (*Salvia officinalis)*	HPLC	Common cold/abdominal pain	Dorman *et al. (*2004)
Lamiaceae (*Satureai cuneifolia)*	FC/HPLC	Common cold/abdominal pain	Dorman *et al.* (2004)
Lamiaceae (*Origonium onites)*	FC/DPPH	Stomach/bowels/gallbladder aches	Everest *et al.* (2005)
Linaceae (*Linum usitatissimum)*	HPLC	For abscesses	Lorenc-Kakula *et al.* (2005)
Punicaceae (*Punica granatum)*	HPLC/TRAP	For diarrhea	Li *et al.* (2006)
Solanum Torvum	DPPH/FC	Antidiabetic/Antioxidant	Takahashi *et al.* (2010)
Nut-meg *(Myristica fragans and M. argentea)*	HPLC	Flavoring	Brewer (2011)
Asteraceae (*Artemisia absinthium)*	UHPLC	Flavoring	Luo *et al.* (2002)
Crassulaceae (*Kalonchoe pinnata)*	FCR/FRAP	Astringent	Rohan and Anup (2014)
Monodora myristica	HPLC	Spice	Bruno *et al.* (2015)
Piper guineens	HPLC	Spice	Bruno *et al.* (2015)

2.2 Biosynthesis

The biosynthesis of caffeic acid occurs via the shikimate pathway metabolism of phenylalanine, which is converted by the enzyme ammonia lyase to trans-cinnamic acid. Hydroxylation at the position-4 of the aromatic ring yields 4-hydroxycinnamic acid or p-coumaric acid (Fig.1.3). Further hydroxylation at position-3 of aromatic ring results in the formation of caffeic acid (Rodrigues *et al*., 2015).

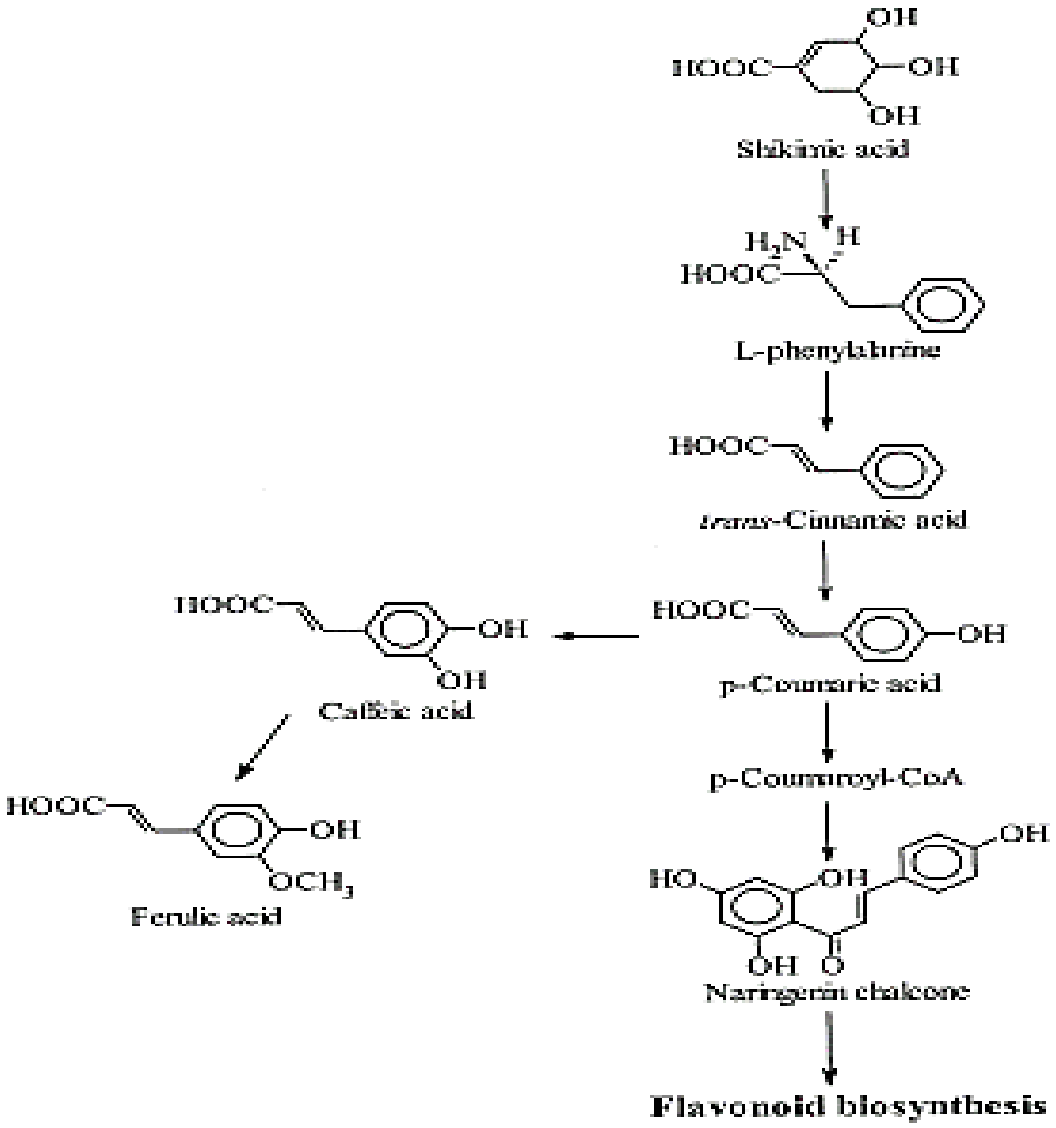


Figure 2.3 Biosynthetic pathway of Caffeic acid (Shahidi, 2000; Wu *et al*., 2008)**.**

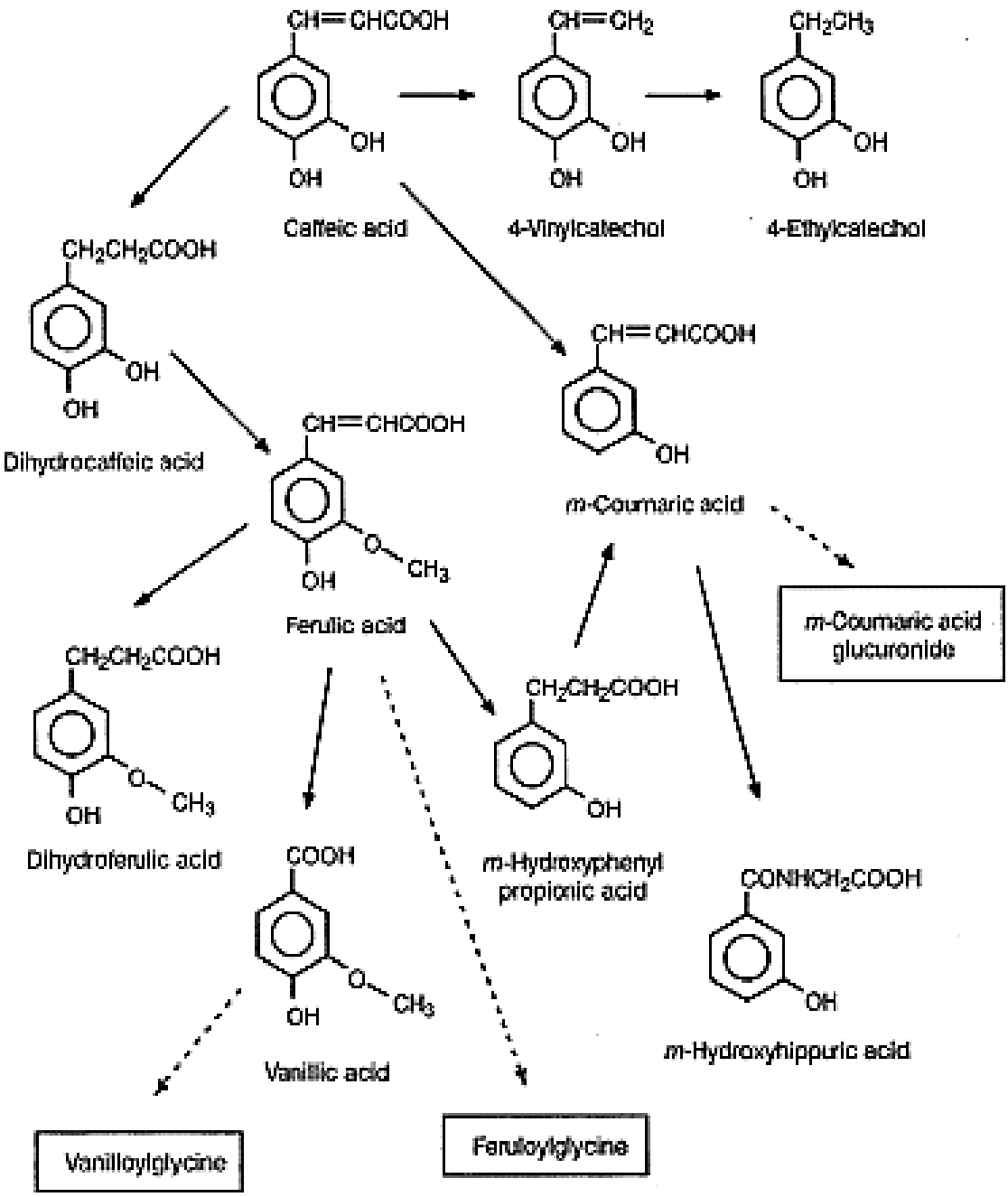


Figure 2.4 Pathway for the metabolism of caffeic acid in Man (Arnaud, 1988).

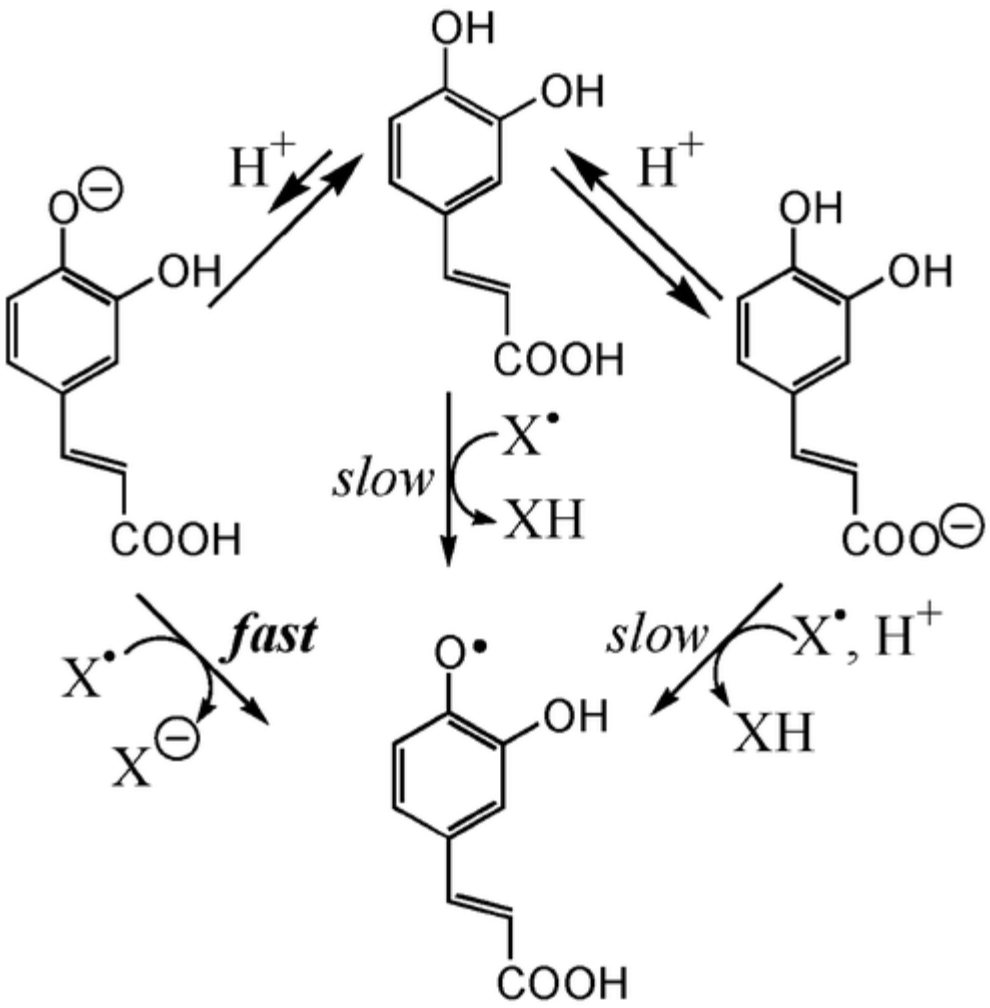


Figure 2. 5 Mechanism of Caffeic Acid antioxidant Action (Wu *et al.*, 2008).

2.3 Free Radicals

Free radicals are reactive chemical species having one or more unpaired electrons in the valence shell (Halliwell, 2006). They are the result of homolytic scission of covalent bonds and occur most frequently between two atomic elements of similar electronegativity (Fig.2.6). It often involve the O-O bond in peroxide species or O-N bonds. Some of these radicals are produced inside the human body as a natural byproduct of oxidative metabolism and have been shown to effect cell-cell signaling (Adjimani and Prince, 2015). These are known as reactive oxygen species (ROS),

reactive nitrogen species (RNS) and reactive sulfur species (RSS) easily formed by the reaction of ROS with thiols (Brewer *et al*., 2011). They are extremely unstable and reactive, tending to capture electron from other surrounding molecules in order to attain a stable electronic configuration. Consequent upon this, their presence in high concentrations can result in a situation of oxidative damage to normal cells (Somogyi *et al*., 2007). They include hydroxyl radicals (•OH), superoxide anions (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxides (H_2O_2), organic peroxides (R-OOH), nitric oxide and peroxynitrite. The main targets of ROS, RNS and RSS are proteins, DNA (Deoxyribonucleic acid) and RNA (Ribonucleic acid) molecules, sugars and lipids (Lü *et al*., 2010; Craft *et al*., 2012). The modification of proteins can be achieved either by oxidation of specific amino acids, free radical-induced peptide cleavage or the formation of protein cross-linkage as a result of reactions with lipid peroxidation products (Lobo *et al*., 2010).

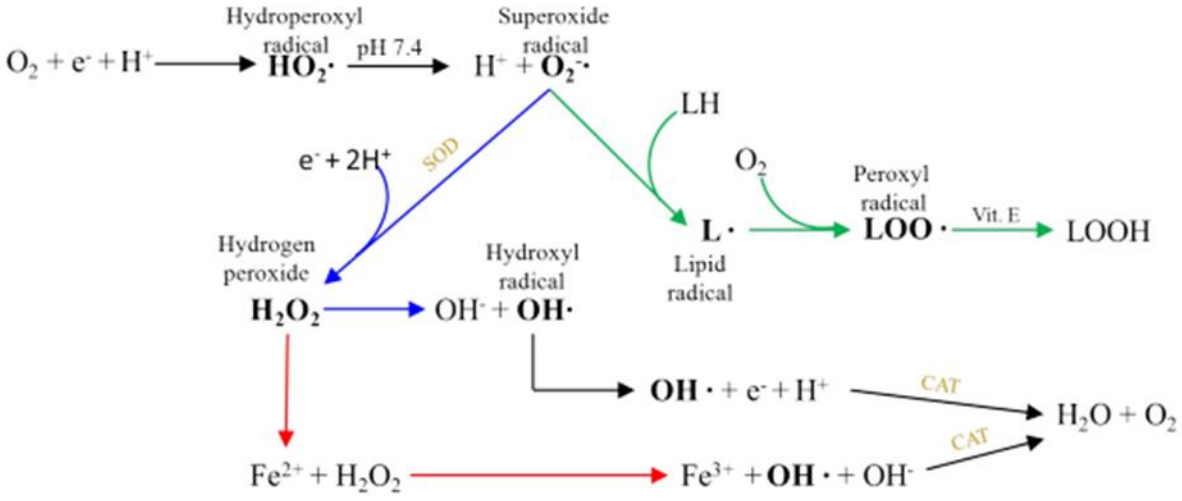


Figure 2.6 Overview of reactions leading to the production of reactive species (Brewer, 2011).

Free radical-induced damage to DNA leads to the production of bases with free sites, deletions, base modifications, broken strands, DNA-protein cross-links and chromosomal aberrations.

An important reaction involved in DNA damage is the production of the hydroxyl radical through the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^-$).

There is increasing evidence that this radical reacts with all the components of the DNA molecule: the purine and pyrimidine bases as well as the deoxyribose backbone, with the peroxyl and OH^- radicals intervening in DNA oxidation (Dizdaroglu *et al*., 2002; Valko *et al*., 2004). The formation of oxygen free radicals in the process of glycation leading to the formation of sugars, could result in glyco-oxidative damage. In the early stages of non-enzymatic glycosylation, the fragmentation of sugar produces short chain species like glycoaldehyde which may be unable to cyclize and therefore susceptible to autoxidation, to yield the superoxide radical. This radical further propagates a chain reaction resulting in the formation of α- and β-dicarbonyls, which are well known mutagens (Hulya and Gulcin, 2011). The mechanisms by which free radicals interact with surrounding molecules include electron donation, reducing radicals, electron acceptance or oxidizing radicals manifested by (a), hydrogen abstraction (b), addition reactions (c), self-annihilation reactions (d) disproportionation (Etim *et al*., 2013). There is a growing body of evidence that ROS may not only regulate apoptotic signal transduction, but also activate apoptotic death pathways (Leist and Jaattela, 2001).

2.4 Role of Antioxidants

An organism's defense against the attack of ROS and RNS is provided by antioxidants and lack of equilibrium between free radicals and antioxidants leads to oxidative stress (Sies, 1991) which is a disturbance in the prooxidant‐antioxidant balance in favor of the prooxidant that leads to potential damage. The term antioxidant is reserved for any compound, whose presence in low concentrations, has the ability to block or significantly delay the reaction of a substrate with molecular oxygen or ROS/RNS (Halliwell and Gutteridge, 2007).

ROS, RNS and RSS that result from the respirative cycle of oxidative phosphorylation may attack biological macromolecules like cellular DNA, giving rise to single- and double-strand breaks that may eventually cause cell ageing, cardiovascular diseases, mutagenic changes and cancerous tumor growth. When natural defenses of the organism (of enzymatic, non-enzymatic or dietary origin) are overwhelmed by an excessive generation of ROS/RNS, a situation of oxidative stress occurs, in which cellular and extracellular macromolecules (proteins, lipids and nucleic acids) can suffer oxidative damage, causing tissue injury. It has been established that the consumption of foods rich in natural antioxidants provides an efficient way of combating tissue injuries, undesired transformations and prevent health risks (Halliwell and Gutteridge, 2007).

Some other compounds, known as retarder molecules, have the ability to reduce the rate of oxidation only when they are present in high concentrations and are often wrongly reported as antioxidants (Velioglu *et al*., 2006). Consequently, in addressing the assessment of antioxidant effect, the following factors are important:

- ✓ The difference between antioxidant capacity and antioxidant activity. Considering antioxidant activity as the reaction between a single antioxidant species and the free radical, the antioxidant capacity is the reaction between an antioxidant solution, containing a mixture of antioxidant compounds and the radical (Prior and Cao, 1999). While antioxidant activity is defined as the rate constant of the reaction between a unique antioxidant and a given free radical, antioxidant capacity is the number of moles of free radical scavenged by an antioxidant testing solution that could lead to a different result for the same radical (Shon *et al*., 2004).
- ✓ The potential self-reactivity of radicals derived from parent antioxidants, versus the reaction products (Pollyanna *et al*., 2014).

- ✓ The specificity of the antioxidant compound towards the free radical as there are no universal antioxidants able to efficiently quench any type of reactive oxygen species (Simona *et al*., 2010).

Many studies have been conducted in the field of free radicals, oxidative stress and antioxidant activity of food. This gives antioxidants a prominent beneficial role, and regardless of the quantity ingested, the absorption is very limited, and in some cases prooxidants have been shown to be beneficial to human health (Rice-Evans, 1997; Gupta, 2015). The determination of antioxidant activity and specific antioxidant compounds can be achieved with the use of a large number of different assays, all of them with advantages and disadvantages (Mohammed *et al.,* 2013).

Interest in antioxidants, particularly those intended to prevent the presumed deleterious effects of free radicals in the human body, as well as the deterioration of fats and other constituents of foodstuffs has increased in recent years (Pisoschi and Negulescu, 2011; Gupta, 2015) .

2.5 Natural sources of antioxidants

Plants and vegetable materials have proven to be very important sources of antioxidant compounds. Antioxidant phytochemicals such polyphenols, flavonoids and isoflavones, anthocyanins etc. have been the subject of many recent studies and are used as food additives (Tchinda *et al*., 2014). Plant polyphenols are mainly aromatic hydroxylated compounds, 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 (Thomas, 1997). Phenolic derivatives represent the largest group known as 'secondary plant products' usually synthesized by plants. This maybe 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. For example, by providing defense against microbial attacks and by preventing herbivorous animals from feeding on them (Reyes *et al*., 2003). The most widely employed antioxidant phytoderivatives in food industry are compounds derived from hydroxybenzene (phenol) and are commonly known as polyphenols.

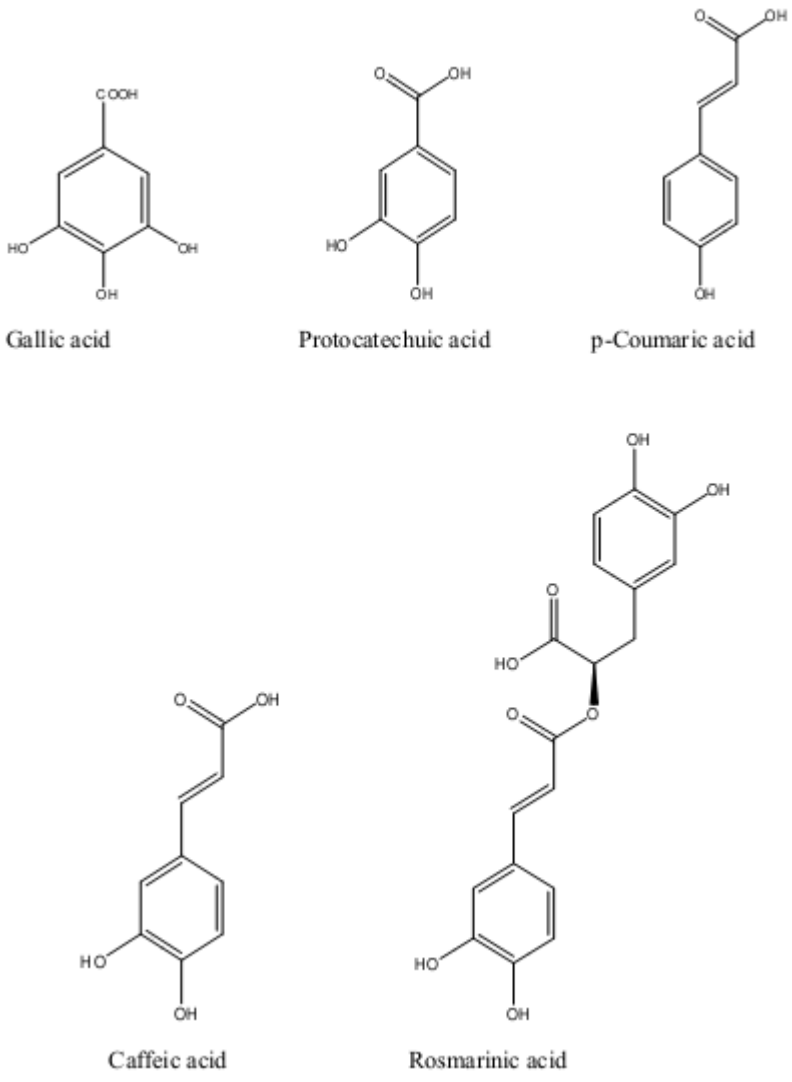


Figure 2.7 Some Naturally-Occurring Phenolics

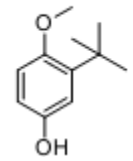
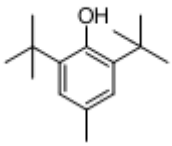
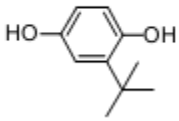
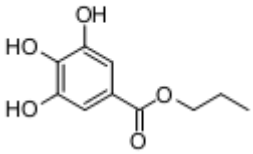
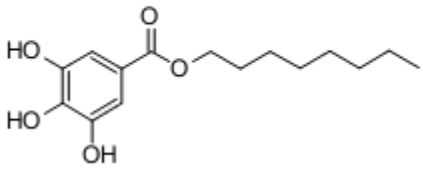
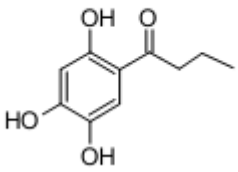
The plant phenols being regarded as those substances derived from the shikimate pathway and phenylpropanoid metabolism, following the phosphoenolpyruvate → phenylalanine → cinnamate → 4-coumarate course, leading to chalcone, flavanone, dihydroflavonol, and anthocyanin (Huang *et al*., 2005). Natural antioxidants are not liable to the toxicity problems which may arise from the use of synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) (Tavasalka *et al*., 2012). The antioxidant effect of these compounds is related to their chemical structure and their capability to redistribute electrons over their aromatic ring, with the acrylic functional groups also making significant contributions (Karadag *et al*. 1999). When these compounds react with a free radical the captured electron is delocalized leading to stabilization by the resonance effect of the aromatic nucleus thereby preventing free radical chain reactions (Cao *et al*., 1995). This is often called radical scavenging, but polyphenolic compounds inhibit oxidation by various other mechanisms, depending on the source of the material and possible presence of synergists or antagonists. Significant antioxidant, antitumor, antiviral and antibiotic activities are frequently reported for plant phenols (Burda *et al.* 2001). 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. Plant phenols and polyphenols are thought to play important roles in long term health and reduction in the risk of chronic and degenerative diseases (Robarbs, 1997). 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 (Seeram *et al*., 2002). These substances contain at least one aromatic ring with one or more attached –OH groups, in addition to other substituents. Antioxidants are known to play a key role in the protective influence exerted

by plant foods and have been traditionally divided into two classes; primary or chain-breaking antioxidants, and secondary or preventative antioxidants (Rong, 2010; Alkasim *et al.,* 2015).

2.6 Synthetic Antioxidants

These include butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT) and propyl gallate (PG). Reports revealing that BHA and BHT could be toxic, and the higher manufacturing costs and lower efficiency together with the increasing consciousness of consumers with regard to food additive safety, has created a need for identifying alternative natural and probably safer sources of food antioxidants (Tavasalka *et al*., 2012). Synthetic antioxidants function by interrupting the free radical chain reaction, like natural ones, as they contain aromatic or phenolic rings, they donate H• to the free radicals formed during oxidation becoming a radical themselves which are stabilized by the resonance delocalization of the electron within the aromatic ring and formation of Quinone structures (Zheng *et al.,* 2013). In addition, many of the phenolics lack positions suitable for molecular oxygen attack. Table2.2 shows some natural antioxidants and their applications.

Table 2.2 Chemical structure and Applications of some synthetic antioxidants

Name/Structure	Applications	Reference
BHA (butylated hydroxyanisole) 	Food Antioxidant	Wanasundara *et al*., 2005
BHT (butylated hydroxytoluene) 	Food Antioxidant	Wanasundara *et al*., 2005
TBHQ (*tert*-butylhydroquinone) 	Animal-processed food Antioxidant	Tavasalka *et al*., 2012
PG (Propyl gallate) 	Food Antioxidant	Maestri *et al*., 2006
OG (Octyl gallate) 	Food and Cosmetic Antioxidant/Antifungal	Craft *et al*., 2012
2,4,5-Trihydroxy butyrophenone 	Food Antioxidant	Schlesier *et al*., 2002

2.7 Mechanisms of action of antioxidants

Antioxidants slow down the oxidation rates of foods by a combination of scavenging free radicals, chelating prooxidative metals, quenching singlet oxygen and photosensitizers, and inactivating lipoxygenase (Huang *et al*., 2005;Schlesier *et al*., 2002). They scavenge free radicals of foods by donating hydrogen to them, and they produce relatively stable antioxidant radicals with low standard reduction potential (Lee, 2013). The higher stability of antioxidant radicals than that of food radicals is due to resonance delocalization throughout the phenolic ring structure (Choe and Min, 2005). The activity of an antioxidant is determined by : (a) Its reactivity as a hydrogen or electron-donating agent (which relates to its reduction potential), (b) The fate of the resulting antioxidant-derived radical, which is governed by its ability to stabilize and delocalize the unpaired electron, (c) Its reactivity with other antioxidants and (d) The transition metal-chelating potential (Shahidi, 2000).

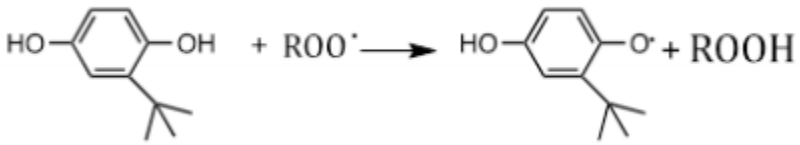


Figure 2.8a Antioxidant mechanism of a radical terminator via the donation of an electron from a phenolic hydroxyl group

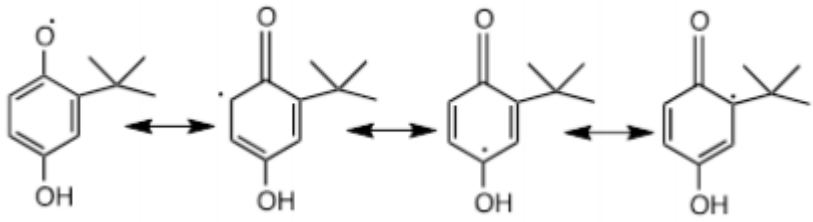


Figure 2.8b Resonance stabilization of a phenolic antioxidant

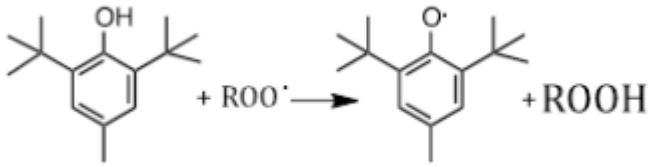


Figure 2.8c Oxidation of BHA via donation of an electron from a phenolic hydroxyl group

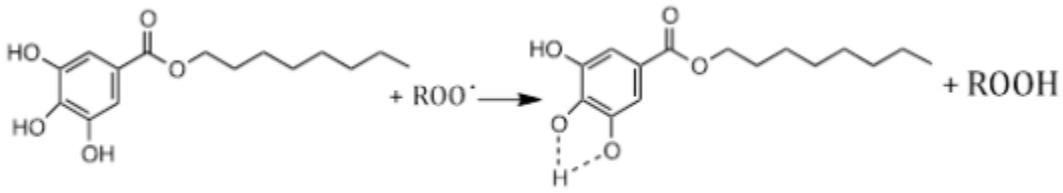


Figure 2.8d Generation of phenoxy radical showing intramolecular hydrogen bond formation.

2.8 *In vitro* Assay Methods

There are a number of analytical methods for the determination of antioxidant capacity and these methods belongs to different classes and these are given below:

- ❖ Spectroscopic Techniques
- ❖ Electrochemical Techniques
- ❖ Chromatographic Techniques

(Emphasis here will be on the spectroscopic methods).

Several methods have been developed for the evaluation of the antioxidant effect of molecules but since many variables have to be taken into account when measuring the antioxidant characteristics of a compound, the results have to be treated with caution. There is no universal system able to provide information about the 'true' antioxidant power or capacity of a single antioxidant or

complex mixture of antioxidant phytochemicals (Frenkel, 1993; Ou *et al*., 2002), because the activity is dependent on the substrate, the reaction medium, the oxidation conditions, interfacial phenomena and the antioxidant partitioning properties between the phases (Mohammed *et al.,* 2013). The possibility of generating selectively and quantitatively, reactive oxygen, nitrogen, and sulfur species by employing methods available from the radiation chemistry of aqueous solutions allows time dependent changes in the concentration of these radicals during the course of their reaction making it possible to quantify the reaction kinetics, radical lifetimes, diffusion length and one-electron redox potentials reliably (Laura *et al*., 2013). These parameters can then be used to establish the likely and unlikely reactions and competitive reactions of antioxidant substances under suitable biological conditions. The need to use standardized methods has resulted in a sort of bioanalytical protocol to assess the antioxidant efficacy of phenolic compounds from food and raw materials that includes some common basic features (Becker *et al*., 2004) namely:

- The quantification and possibly, the identification of the phenolic compounds;
- The quantification of the radical scavenging activity and the determination of the formal reduction potential;
- The evaluation of the inhibition or ending point of lipid oxidation in biological model systems;
- The study of the efficiency against relevant oxidative markers.

A good radical scavenging activity does not necessarily imply a good antioxidant activity and thus not all the compounds showing a high radical scavenger effect show good antioxidant properties (Meyer and Isaksen, 1995; Thomas, 1997). In ascribing antioxidant properties to a compound, it is also necessary to determine its efficacy at preventing the oxidation of relevant substrates such

as lipids, lipoproteins, DNA etc. against relevant free radical species such as the peroxyl, superoxide or hydroxyl radical (Andressa, 2013).

Evaluation of antioxidant activity is complicated by the prooxidative effect of antioxidants in the presence of unsequestered metal ions such as iron and copper. Most especially, the lower oxidation states of these metals (i.e., Fe (II) and Cu (I)) should not be present at significant levels in tests measuring antioxidant status so as not to initiate Fenton-type reactions (Sajid *et al*., 2010).

HAT−based assays, oxygen radical absorbance capacity (ORAC) assay (Cao *et al*., 1995) applies a competitive reaction scheme in which antioxidant and substrate kinetically compete for thermally generated peroxyl radicals through the decomposition of azo compounds such as ABAP (2,2'-azobis (2-aminopropane) dihydrochloride) (Huang *et al*., 2005). ORAC measures inhibition of peroxyl radical induced oxidations by antioxidants and thus reflects classical radical chain-breaking antioxidant activity by H-atom transfer (Ou *et al*., 2002). Other HAT−based assays include total peroxyl radical-trapping antioxidant parameter (TRAP assay) using R-phycoerythrin as the fluorescent probe, developed by Ghiselli *et al*. (2000), Crocin bleaching assay using AAPH as the radical generator (Bors *et al*., 1984) and β-carotene bleaching assay, although the latter bleaches not only by peroxyl radical attack but by multiple pathways (Prior *et al*., 2005). Generally, HAT reactions are relatively independent of solvent and pH effects, and are completed in a short time (Khoddami *et al*., 2013).

Depending on these assumptions, the mechanism involved and the type of assessment, antioxidant capacity assays can be divided in two main categories. The first category is an assessment of antioxidant efficacy in relation to free radical species. This category includes different reaction mechanisms models such as:

- Hydrogen atoms transfer reactions model (HAT) based on the transfer of hydrogen atoms;
- Single electron transfer reactions model (SET) based on the transfer of a single electron;
- Hydrogen‑electron transfer reactions model combining the two mechanisms HAT and SET (Bondet *et al.*,, 1997).

The second category is an assessment of antioxidant efficacy using biological significant markers and substrates (Prior 2001; Griffiths and Cooney, 2002; Roginsky and Lissi, 2005). This involves the determination of antioxidant efficacy by evaluating the damaging effects on a biological substrate produced by ROS or RNS when reacting with lipids, lipoproteins and DNA.

- ❖ Radical scavenging activity toward either reactive species (e.g., ROS) such as $OH^{\cdot}$, $O_2^{\cdot}$, $^1O_2$, or toward lipid peroxide radicals such as R, $RO^{\cdot}$, and $ROO^{\cdot}$; radical scavenging action generally proceeds via hydrogen atom transfer or electron donation;
- ❖ Prevention of the transition metal-catalyzed production of reactive species (i.e., via Fenton-type reactions) through metal chelation; $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$
- ❖ Interaction with other antioxidants (such as cooperative actions), localization, and mobility of the antioxidant at the microenvironment (Alkasim *et al*., 2015).

2.9 Hydrogen Atom Transfer (HAT) based assays

The HAT reaction is a key step promoting radical chain reactions. Nevertheless, as reported by Prior *et al*. (2005), monitoring the reaction pathway, it becomes very difficult to distinguish between the hydrogen atom and the electron transfer reactions. HAT assays are based on kinetics, involving a competitive reaction scheme in which both antioxidant and substrate compete for thermally-generated peroxyl radicals resulting from the decomposition of azo compounds (Saeed

et al. 2014). The two reactions can occur simultaneously with the mechanism being determined by the structure, solubility and the partition coefficient of the antioxidant. The polarity of the solvent and the pH of the medium are also important factors (Jafri *et al*., 2014). The reactions are very fast and generally occur through the formation of peroxyl radical intermediates. Thus hydrogen atom transfer reaction can be depicted as follows: X* + AH → XH + A*, X*—Free Radical; AH—any presumed antioxidant compound able to act as hydrogen atom donor. HAT−based assays measure the capability of an antioxidant to quench free radicals (generally peroxyl radicals) by H-atom donation. Included in this group of *in vitro* assay are Oxygen Radical Absorbance Capacity (ORAC) method, Lipid Peroxidation Inhibition Capacity (LPIC) assay, Total radical trapping antioxidant parameter (TRAP), inhibited oxygen uptake (IOC), Crocin bleaching nitric oxide radical inhibition activity, Hydroxyl radical scavenging activity by p-NDA (p-butrisidunethyl aniline), Scavenging of H_2O_2 radicals, ABTS radical scavenging method and Scavenging of super oxide radical formation by alkaline (SASA) (Huang *et al.,* 2005).

2.9.1 Oxygen Radical Absorbance Capacity (ORAC) method

When a free-radical generator such as an azo-initiator compound is added to a fluorescent molecule such as β-phycoerythrin or fluorescein and heated, the azo-initiator produces peroxyl free radicals, which damage the fluorescent molecule, resulting in the loss of fluorescence. Curves of fluorescence intensity against time are recorded, and the area under the curves (AUC) with and without addition of an antioxidant is calculated and compared to a standard curve generated using the antioxidant (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (Fig. 2.9), a water-soluble analog of vitamin E.

This assay is based on generation of free radical using AAPH (2, 2-azobis 2-amidopropane dihydrochloride) and measurement of decrease in fluorescence in the presence of free radical

scavengers. Prior *et al*. (2005) reported an automated ORAC assay in which β-phycoerythrin (β-PE) is used as target free radical damage, AAPH as a peroxy radical generator and Trolox as a standard control. After addition of AAPH to the test solution, the fluorescence is recorded and the antioxidant activity is expressed as Trolox equivalent (Cao *et al*., 1993; Bruno *et al*., 2015).

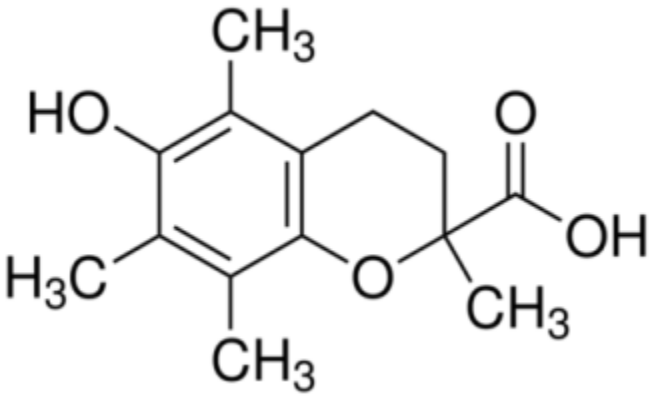


Figure 2.9 Molecular structure of Trolox, an analogue to vitamin E (*Adapted from Sigma-Aldrich*)

The assay can be carried out according to Prior *et al*. (2005) in 96-well polypropylene fluorescence plates with a final volume of 200µL. Assays are conducted at pH 7.0 with Trolox (6.25, 12.5, 25, and 50µmol/L for lipophilic assays; 12.5, 25, 50 and 100µmol/L hydrophilic assays) as the standard and 75mM/L phosphate buffer as the blank. After the addition of AAPH, the plate is placed immediately in a multi label counter preheated to 37ºC. The plate is shaken in an orbital manner for 10s and the fluorescence is read at 1min intervals for 35minutes at the excitation wavelength of 485nm and emission wavelength of 520nm. Area-under-the-curve is calculated for each sample using Wallac Workout 1.5 software. Final computation of results is made by taking the difference of areas-under-the-decay curves between blank and sample and/or standard (Trolox) and expressing this in µM of Trolox equivalents (TE) per g dry weight of sample (µMTE/g).

2.9.2 Total Radical-Trapping Antioxidant Parameter (TRAP) method.

This method uses a luminescence spectrometer to measure the fluorescence decay of R-phycoerythrin during a controlled peroxidation reaction. TRAP values are calculated from the length of the lag-phase caused by the antioxidant compared to that of Trolox. According to Ghiselli *et al*. (2000),120 µL of diluted sample is added to 2.4mL of phosphate buffer (pH 7.4), 375µL of bidistilled water, 30µL of diluted R-PE and 75µL of ABAP; the reaction kinetics at 38ºC is recorded for 45 min by a luminescence spectrometer. TRAP values are calculated from the length of the lag-phase due to the sample compared with standard. ABAP (2, 2' -azo–bis (2 amidino-propane) hydrochloride).

2.9.3 Hydroxyl radical averting capacity (HORAC) method.

The HORAC assay described by Ou *et al*. (2002) measures the metal-chelating activity of antioxidants in the conditions of Fenton-like reactions employing a Co (II) complex and hence the protecting ability against formation of hydroxyl radical. Hydrogen peroxide solution of 0.55M is prepared in distilled water and 4.6mM Co (II) is prepared by dissolving 15.7mg of $CoF_2.4H_2O$ and 20mg of picolinic acid in 20mL of distilled water. Fluorescein –170µL (60nM, final concentration) and 10µL of sample are incubated at 37ºC for 10 min. directly in the plate reader. After incubation 10µL H_2O_2 (27.5mM, final concentration) and 10µL of Co (II) (230µM final concentration) solutions are added subsequently. The initial fluorescence is measured after which the readings are taken every minute after shaking. For the blank sample, phosphate buffer solution is used. 100, 200, 600, 800 and 1000 µM standard antioxidant solutions (in phosphate buffer 75mM, pH 7.4) are used for building the standard curve. The final HORAC values are calculated using a regression equation between the standard antioxidant concentration and the net area under the curve. One

HORAC unit is assigned to the net protection area provided by 1µM standard antioxidant and the activity of the sample is expressed as µM standard antioxidant equivalents per gram of fresh weight of the samples. Gallic acid can be used as standard antioxidant.

2.9.4 Nitric oxide scavenging activity

NO• is generated in biological tissues by specific nitric oxide synthases, which metabolize arginine to citrulline with the formation of NO• via a five electron oxidative reaction (Bruno *et al.,* 2001). The compound sodium nitroprusside is known to decompose in aqueous solution at physiological pH (7.2) producing NO•. Under aerobic conditions, NO• reacts with oxygen to produce stable products (nitrate and nitrite), the quantities of which can be determined using Griess reagent (MacDonald-Wicks *et al*., 2006).

Sodium nitroprusside solution, (2mL of 10mM) in 0.5mL phosphate buffer saline (pH 7.4) is mixed with 0.5mL of sample at various concentrations (0.2–0.8mg/mL). The mixture is incubated at 25ºC for 150 minutes after which 0.5mL of the incubated solution is withdrawn and mixed with 0.5mL of Griess reagent [(1.0mL Sulfanilic acid reagent (0.33% in 20% glacial acetic acid at room temperature for 5 min with 1mL of naphthylethylenediamine dichloride (0.1% w/v)]. The mixture is again incubated at room temperature for 30minutes and its absorbance measured at 546nm. The amount of nitric oxide radical inhibition is calculated thus:

% inhibition of NO radical = $[A_0-A_1]/A_0 \times 100$

where A_0 is the absorbance before reaction and A_1 is the absorbance after reaction has taken place with Griess reagent.

2.9.5 Xanthine oxidase method.

The assay is based on the principles that Coumarins acting to inhibit XO enzyme activity and scavenge free radical (Lü *et al*., 2010). The XO enzyme activity can be measured spectrophotometrically by continuously measuring uric acid formation at 295 nm with xanthine as the substrate. The assay is initiated by adding the enzyme to the reaction mixture containing xanthine, EDTA and 3-(cyclohexylamino)-1-propane-sulfonic acid, without or with inhibitors at 37°C.

According to Cicco *et al*. (2009), the extract (500µL of 0.1mg/mL) and allopurinol (100µg/mL) (in methanol) are mixed with 1.3mL phosphate buffer (0.05 M, pH 7.5) and 0.2mL of 0.2 units/ mL xanthine oxidase solution. After 10minutes of incubation at 25ºC, 1.5mL of 0.15M xanthine substrate solution is added to this mixture and again incubated for 30minutes at the same temperature of 25ºC. The absorbance is measured at 293nm using a spectrophotometer. The solution of 0.5mL methanol, 1.3mL phosphate buffer, 0.2mL xanthine oxidase and 1.5mL xanthine substrate is used as a control. Percentage of inhibition is calculated using the formula:

[1-(As / Ac)] x 100**.**

As and Ac are the absorbance values of the test sample and control, respectively.

2.9.6 Ferric thiocyanate (FTC) method

As performed by Gyamfi *et al*. (1999), a mixture of 4mg of sample (final concentration of 0.02% w/v) in 4mL ethanol, 4.1mL of 2.51% linoleic acid in ethanol, 8.0mL of 0.02M phosphate buffer (pH 7.0) and 3.9mL of distilled water contained in screw cap vial is placed in an oven at 40ºC in the dark. Reaction mixture (0.1ml) is transferred to a test tube and 9.7mL of 75% (v/v) aqueous ethanol, followed by 0.1ml of 30% aqueous ammonium thiocyanate and 0.1ml of 0.02M ferrous

chloride in 3.5% hydrochloric acid were added. Three minutes after the addition of ferrous chloride to the reaction mixture, the absorbance of the resulting mixture (red color) is measured at 500nm every 24hours until the absorbance of the control reached its maximum. During the linoleic acid oxidation, peroxides are formed and that leads to oxidation of Fe^{2+} to Fe^{3+}. The latter ions form a complex with thiocyanate and this complex has a maximum absorbance at 500nm. This step is repeated every 12hours until the control reaches its maximum absorbance value, high absorbance indicating high linoleic acid emulsion oxidation. All data on total antioxidant activity are the average of duplicate experiments. The inhibition percentage of lipid peroxidation in linoleic acid emulsion is calculated by the following equation: inhibition of lipid peroxidation (%) =

$$100 - [(Ac / As)] \times 100$$

where Ac is the absorbance of control reaction and As is the absorbance in the presence of the sample or standard compounds (Gulcin *et al*., 2010).

Standard antioxidant (final concentration of 0.02% w/v) is used as positive control, and the mixture without the sample is used as the negative control.

2.9.7 Thiobarbituric acid (TBA) method.

This test quantifies malonaldehyde and malonaldehyde-type products (such as trans−2, 4−heptadienal, trans−2−heptenal, trans−2 hexenal, and hexanal), as well as secondary oxidation decomposition products of polyunsaturated fatty acids. These products react with 2thiobarbituric acid (TBA) to form a stable pink chromophore, with a λ max of 532nm, and can therefore be quantified spectrophotometrically (Frankel, 1993; Sendra *et al.,* 2006). Because the malonald-ehyde-type compounds are highly reactive, the TBARS test only measure products temporarily occurring in the steps of oxidation.

A sample concentration of 0.02% w/v was used in this method (Matthaus, 2002). Two millilitres of 20% trichloroacetic acid and 2mL of 0.67% of thiobarbituric acid are added to 1mL of sample solution. The mixture is placed in a boiling water bath for 10 min and then centrifuged after cooling at 3000rpm for 20min. The absorbance activity of the supernatant is measured at 552nm and recorded after reaching its maximum.

2.9.8 β-carotene linoleic acid method/conjugated diene assay

β-carotene (0.5mg) in 1mL of chloroform is added to 25µL of linoleic acid and 200mg of tween-80 emulsified mixture. Chloroform is evaporated at 40ºC, 100mL of distilled water saturated with oxygen is slowly added to the residue and the solution is vigorously agitated to form a stable emulsion (Miroslav *et al*., 2012). Four millilitres of this mixture is transferred into test tubes containing 200µL of samples prepared in methanol at final concentrations (25, 50, 100, 200 and 400µg/mL). As soon as the emulsified solution is added to the tubes, zero time absorbance is measured at 470nm. The tubes are incubated for 2hours at 50ºC. Vitamin C can be used as standard. Antioxidant activity is calculated as percentage of inhibition (I %) relative to the control using the following equation: $1\% = [1 - (As - As_{120}) / (Ac - Ac_{120})]$

where As = initial absorbance, As_{120} = the absorbance of the sample at 120min. Ac = initial absorbance of negative control and Ac_{120} = the absorbance of the negative control at 120min.

2.9 Single Electron Transfer (SET) based assays

SET-based assays are used to assess the capability of an antioxidant to reduce a specific oxidant. These reactions are usually slow, require a long period and are multi-step processes that can be schematically presented thus: $X^* + AH \rightarrow X^- + AH^{*+}$,

$AH + H_2O \rightarrow A^* + H_3O^+$,

$X^- + H_3O^+ \rightarrow XH + H_2O$,

$M(III) + AH \rightarrow AH^+ + M(II)$.

X*—Free Radical; AH—any presumed antioxidant able to transfer an electron; M—a 3d metal able to initiate a chain reaction.

ET−based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes color, absorbance or fluorescence when reduced, each using different chromogenic redox reagents with different standard potentials (Ramirez-Sanchez *et al.,* 2010). This group includes Trolox equivalent antioxidant capacity (TEAC) decolorization, Ferric reducing antioxidant power (FRAP), DPPH free radical scavenging assay, Copper (II) reduction capacity, Total phenols by Folin-Ciocalteu, and N,N-dimethyl-p-Phenylenediamine (DMPD) assay. The CUPRAC (cupric ion reducing) offer distinct advantages over other ET−based assays, namely the selection of working pH at physiological pH (as opposed to the FC and FRAP methods, which work at alkaline and acidic pH respectively), applicability to both hydrophilic and lipophilic antioxidants (unlike FC and DPPH), completion of the redox reactions for most common flavonoids (unlike FRAP), selective oxidation of antioxidant compounds without affecting sugars and citric acid commonly contained in foodstuffs and the capability to assay –SH bearing antioxidants unlike FRAP (Ruch *et al.,*1989). Other similar ET–based antioxidant assays that have been developed or modified for phenolics are the Fe (III) − and Ce (IV) − reducing capacity methods.

2.9.1 Trolox equivalent antioxidant capacity (TEAC) method/ABTS radical cation decolorization

This method, which is similar in principle to ORAC, uses a diode-array spectrophotometer to measure the loss of color when an antioxidant is added to the blue-green chromophore $ABTS^{+}$, 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Prior *et al*., 2005). The antioxidant reduces $ABTS^{+}$ to ABTS, decolorizing it. $ABTS^{+}$ is a stable radical not found in the human body (Pollyanna *et al.*, 2014).

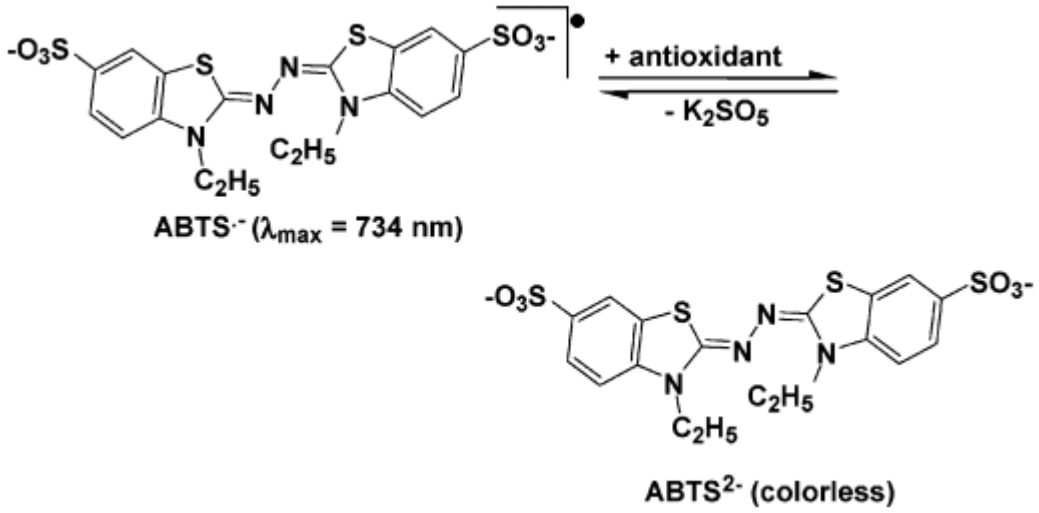


Figure 2.10 Mechanism of ABTS radical reaction

ABTS radical cations are prepared by adding solid manganese dioxide (80mg) to a 5mM aqueous stock solution of ABTS (20mL using a 75mM Na/K buffer of pH 7). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of vitamin E, can be used as an antioxidant standard. A standard calibration curve is constructed for Trolox at 0, 50,100, 150, 200, 250, 300, and 350µM concentrations (Schlesier *et al.,* 2002). Samples are diluted appropriately according to antioxidant activity in Na/K buffer pH 7. Diluted samples are mixed with 200µL of $ABTS^{+}$ radical cation solution in 96-well plates, and absorbance is read (at 750nm) after 5minutes

in a microplate reader. TEAC values can be calculated from the Trolox standard curve and expressed as Trolox equivalents (in mM) (Abebe *et al.,* 2012)

2.9.2 Total Oxyradical Scavenging Capacity (TOSC) method

This method is based on the reaction between peroxyl radicals and α-keto-γ-methiolbutyric acid (KMBA), which is oxidized to ethylene (Winston *et al*., 1998). The method allows the quantification of the absorbance capacity of antioxidants with specificity towards three potent oxidants i.e. hydroxyl radicals, peroxyl radicals, and peroxynitrite thereby addressing the important issue of being able to evaluate different antioxidants from different biologically relevant radical sources. The added antioxidant competes with KMBA for the peroxyl radicals, reducing the production of ethylene, generally monitored by gas chromatography (Bondet *et al.,* 1997).

2.9.3 Ferric Reducing/Antioxidant Power (FRAP) method

This method determines the ability of antioxidants to reduce the ferric iron. It is based on the reduction of the complex of ferric iron and 2, 3, 5-triphenyl-1, 3, 4-triaza-2-azoniacyclopenta-1, 4-diene chloride (TPTZ) to the ferrous form at low pH (Prior *et al*., 2005). This reduction is monitored by measuring the change in absorption at 593nm, in a diode-array spectrophotometer.

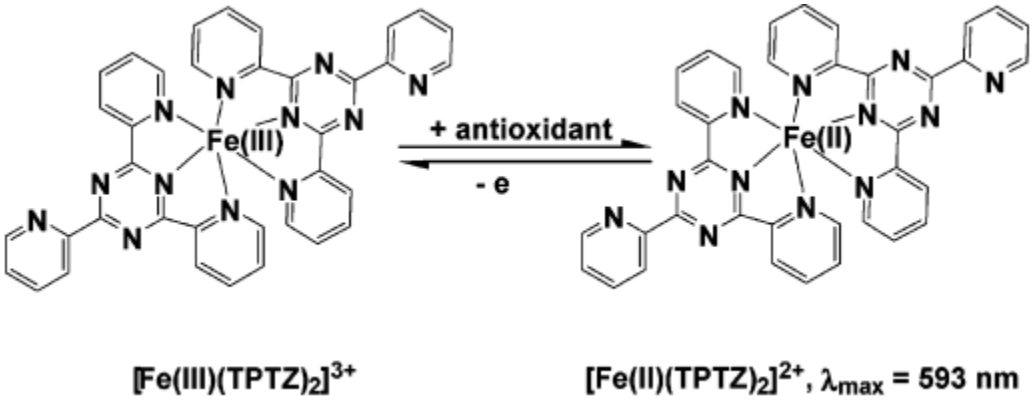


Figure 2.11 Mechanism of Iron chelation

Three mL of prepared FRAP reagent is mixed with 100µL of diluted sample; the absorbance at 593nm is recorded after a 30minutes incubation at 37ºC. FRAP values can be obtained by comparing the absorption change in the test mixture with those obtained from increasing concentrations of Fe^{3+} and expressed as mM of Fe^{2+} equivalents per kg (solid food) or per L (beverages) of sample. The simultaneous use of ferricyanide and ferric ions as chromogens makes the FRAP assay more favorable for use in a greater variety of antioxidants (Rohan and Anup, 2014). Other advantages include:

- FRAP assay gives an immediate result of a large range of individual antioxidants in dose-response manner.
- The higher the degree of color formation, the higher the reducing power of the analyte.
- The procedure is simple and reproducible.

2.9.4 Cupric ion reducing antioxidant capacity (CUPRAC) method

This assay is based on the reduction of Cu (II) to Cu (I) by the combined action of all antioxidants (reducing agents) in a sample yielding a chromophore with maximum absorbance at 490 nm (Apak *et al*., 2007). 1mL of 10^{-2}M of $CuCl_2$, 1mL of 7.5 x 10^{-3} M neocuproine and 1M NH_4 CH_3COOH solution are added into the glass test tube. Then, 400µL of freshly prepared standard solution is added and diluted to the final volume of 4.1mL with deionized water. This procedure is repeated for 400µL, 300µL, 200µL, 100µL and 50µL additions of freshly prepared solutions of the sample.

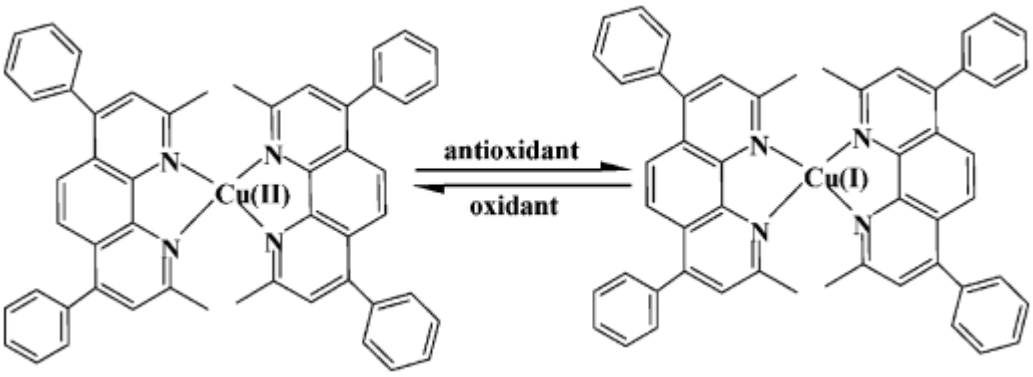


Figure 2.12 Mechanism of the CUPRAC assay

The prepared solutions are mixed and incubated at room temperature for 30min. The absorbance at 450nm is determined against a reagent blank by spectrometer. CUPRAC involves both complexometric and redox reactions. It has specific features distinct from FRAP.

- The redox reaction giving rise to a colored chelate of Cu (I)-Nc is relatively not affected by many parameters such as air, sunlight, solvent type, and pH.
- The CUPRAC reagent is reasonably selective, stable, easily accessible, and sensitive comparing with the FRAP method.
- The reaction is carried out at normal pH as opposed to acidic pH of FRAP.

2.9.5 Superoxide radical scavenging activity (SOD)

The superoxide anion scavenging activity can be measured as described by Apak *et al.* (2007). The superoxide anion radicals are generated in 3.0mL of Tris–HCl buffer (16mM, pH 8.0), containing 0.5mL of nitroblue tetrazolium (NBT) (0.3mM), 0.5mL NADH (0.936mM) solution, 1.0mL extract and 0.5mL Tris–HCl buffer (16mM, pH 8.0). The reaction is initiated by adding 0.5mL phenazine methosulfate (PMS) solution (0.12mM) to the mixture, incubated at 25ºC for 5 minutes and then the absorbance is measured at 560nm against a blank sample.

2.9.6 Reducing power method (RP)

In the method described by Oyaizu (1986) 2.5 mL of 0.2M phosphate buffer (pH 6.6) and 2.5 mL of $K_3Fe(CN)_6$ (1% w/v) are added to 1.0mL of sample dissolved in distilled water. The resulting mixture is incubated at 50ºC for 20 minutes, followed by the addition of 2.5mL of Trichloro acetic acid (10% w/v). The mixture is centrifuged at 3000rpm for 10minutes to collect the upper layer of the solution (2.5mL), mixed with distilled water (2.5mL) and 0.5mL of $FeCl_3$ (0.1%, w/v).

Ferricyanide/Prussian blue: $Fe(CN)_6^{3-} + ArOH \rightarrow Fe(CN)_6^{4-} + ArO\bullet + H^+$

$Fe(CN)_6^{4-} + Fe^{3+} + K^+ \rightarrow K\,Fe[Fe(CN)_6]$, where $K\,Fe[Fe(CN)_6]$: Prussian blue with λ max at 700nm. The absorbance is then measured at 700nm against blank.

2.9.7 Metal chelating activity

Ferrozine forms a complex with a red color when chelated with Fe^{2+}. This reaction is restricted in the presence of other chelating agents and results in a decrease of the red color of the ferrozine-Fe^{2+} complexes. The intensity of color reduction determines the chelating activity to compete with ferrozine for the ferrous ions (Soler-Rivas *et al*., 2000).

An aliquot (0.1mL) of the extract is added to a solution of 0.5mL ferrous chloride (0.2mM). The reaction is started by the addition of 0.2mL of ferrozine (5mM) and incubated at room temperature for 10minutes and then the absorbance is measured at 562nm. EDTA or citric acid (Dinis *et al*., 1994) can be used as a positive control.

2.9.8 DMPD (N, N-dimethyl-p-phenylene diamine dihydrochloride) Method

DMPD radical cation decolorization method has been developed for the measurement of the antioxidant activity in food and biological samples. This assay is based on the reduction of buffered solution of colored DMPD in acetate buffer and ferric chloride. The procedure involves measurement of decrease in absorbance of DMPD in the presence of scavengers at its absorption maximum of 505 nm. The activity is expressed as percentage reduction of DMPD (Alam *et al*., 2013).

The compound N, N-dimethyl-1, 4-diaminobenzene (DMPD) is converted in solution to a relatively stable purple radical form by the action of ferric salt. After addition of a sample containing free radicals, these are scavenged and as a result of this scavenging, the colored solution is decolorized (Fogliano *et al*., 1999).

The assay is based on the following electron-transfer reaction:

probe (oxidant) + e^- (from antioxidant) → reduced probe + oxidized antioxidant.

The probe is an oxidant that abstracts an electron from the antioxidant, resulting in color changes to the probe. Absorbance is measured at 505nm. The degree to which the color change is proportional to the concentration of the antioxidant. The end- point of the reaction is reached when color change stops.

% inhibition calculated as: Scavenging effect (%) = [(OD of control-OD of sample) / (OD of control)] ×100.

Reagents for this assay are prepared thus: Solution 1: acetate buffer (0.2mol/L, pH 5.25); 1a) 2.17 g of sodium acetate trihydrate is dissolved in 80mL of water; 1b) 300µL of concentrated acetic

acid (>99.5 %, v/v) is diluted to a volume of 20mL with distilled water. These two solutions are mixed to reach pH = 5.5. Solution 2: 0.74Mm ferric chloride: 1mg of $FeCl_3 \cdot 6H_2O$ is dissolved with distilled water to a volume of 5ml. Solution 3: 36.7mM DMPD: 25mg of DMPD is dissolved in 5mL of distilled water. This solution must be prepared at the time of use due to its low stability. These three solutions (solutions No. 1, 2 and 3) are mixed in a 20:1:1 (v/v/v) ratio.

2.9.9 Folin-Ciocalteu (Total Phenolic Content) Assay

The assay is done colorimetrically as modified by Singleton and Rossi (1965) and Hoff and Singleton (1977). 2.5ml of 10-fold diluted Folin-Ciocalteu reagent (FCR) is added to 2ml of a 7.5% solution of sodium carbonate, and 0.5ml of sample solution in varying concentrations of 10µg/L, 15µg/L and 20µg/L are mixed well. The absorbance is measured at 765nm after a 15-minutes heating at 45°C; a mixture of water and reagents is used as a blank. This is the dual reagent method (Agbor *et al*., 2014). Because the FCR reacts with all reducing species it is not very sensitive and is used as preliminary approach (Huang *et al*., 2005). The maximum absorption of the chromophores depends on the alkaline solution and the concentration of phenolic compounds. However, this reagent rapidly decomposes in alkaline solutions, which makes it necessary to use an enormous excess of the reagent to obtain a complete reaction. This excess can result in precipitates and high turbidity, making spectrophotometric analysis impossible. To solve this problem, lithium salts are included in the reagent to prevent turbidity (Andressa *et al*., 2013).

Fundamentally, the assay is based on the oxidation of phenolic group compounds in alkaline (carbonate) solution with a molybdotungstophosphate heteropolyanion reagent ($3H_2O$-P_2O_5 - $13WO_3$ -$5MoO_3$ -$10H_2O$), yielding a green-blue product with an absorbance maximum (λ max) at 765nm. Since most phenolic compounds are in dissociated form (as conjugate bases, mainly phenolate anions) at the working pHp of the assay (pH ~ 10), they can be more easily oxidized

with the FCR, possibly giving rise to an overestimated TAC value (Huang *et al*., 2005; Apak *et al*., 2007; Kadriye *et al*., 2013). The molybdenum center in the complex reagent is reduced from Mo (VI) to Mo (V) with an electron donated by an antioxidant to produce a blue color according to : $H_3PW_{12}O_{40}$ + $H_3MO_{12}O_{40}$ + Phenolic compound → W_3O_{23} + MO_3O_{23}.

The method has been widely used for this kind of determination in food since it is simple and standardized, and the reagent is commercially available (Ramirez-Sanchez *et al*., 2010; Magalhães *et al*., 2010; Kalava *et al*., 2012). It is however limited by its lack of specificity, as other products of oxidation including sugars, aromatic amines, sulphur dioxide, organic acids and bases can react with the FCR resulting in an over estimation of the polyphenol content of the sample (Ramirez-Sanchez *et al*., 2010). The products present a blue coloration absorbing UV-vis radiation in wavelength range of 700-760 nm (Cicco *et al*., 2009) which can be measured. Other assay methods based on application of both HAT and SET mechanisms are Total oxidant scavenging capacity (TOSC), Inhibition of Briggs – Rauscher oscillation reaction, Chemiluminescence, Electrochemiluminescence, Fluorometric Analysis, Enhanced chemiluminescence (ECL), TLC bioautography, Cellular antioxidant activity (CAA) assay and Dye-substrate oxidation method (Badarinath *et al*., 2010).

Tables 2. 3 and 2.4 gives a summary of some *in vitro* assay methods, the radicals and measuring principles.

Table 2.3 Assay method, their radicals/oxidants and the measurement principles.

Assay	Radical/Oxidant	Measurement Principle	Measurement Media/Instrument
DMPD	$DMPD^{\bullet+}$	absorbance (505nm)	Spectrophotometer
DPPH	DPPH•	absorbance (515nm)	Spectrophotometer
FRAP	Chelated Fe^{3+}	absorbance (595nm)	microplate reader
FC	Mo(VI)	absorbance (765nm)	Spectrophotometer
ORAC	AAPH•	Fluorescence	microplate reader
TEAC	$ABTS^{\bullet+}$	absorbance (734nm)	Spectrophotometer
PCL	$O_2^{\bullet-}$	Chemiluminescence	Illuminometer

Table 2.4 Summary of Analytical Methods for Antioxidant Assay

Antioxidant capacity assay	**Principle of the method**	**End-product determination**
Spectrometry		
DPPH	Antioxidant reaction with an organic radical	Colorimetry
ABTS	Antioxidant reaction with an organic cation radical	Colorimetry
FRAP	Antioxidant reaction with a Fe(III) complex	Colorimetry
PFRAP	Potassium ferricyanide reduction by antioxidants and subsequent reaction of potassium ferricyanide with Fe3+	Colorimetry
CUPRAC	Cu (II) reduction to Cu (I) by antioxidants	Colorimetry
ORAC	Antioxidant reaction with peroxyl radicals, induced by AAPH (2,2'-azobis-2-amidino-propane)	Loss of fluorescence of fluorescein
HORAC	Antioxidant capacity to quench OH radicals generated by a Co(II) based Fenton-like system	Loss of fluorescence of fluorescein
TRAP	Antioxidant capacity to scavenge luminol-derived radicals, generated from AAPH decomposition	Chemiluminescence quenching
Fluorimetry	Emission of light by a substance that has absorbed light or other electromagnetic radiation of a different wavelength	Recording of fluorescence excitation/ emission spectra

Electrochemical Techniques		
Cyclic voltammetry	The potential of a working electrode is linearly varied from an initial value to a final value and back, and the respective current intensity is recorded	Measurement of the intensity of the cathodic/ anodic peak
Amperometry	The potential of the working electrode is set at a fixed value with respect to a reference electrode	Measurement of the intensity of the current generated by the oxidation/reduction of an electroactive analyte
Biamperometry	The reaction of the analyte (antioxidant) with the oxidized form of a reversible indicating redox couple	Measurement of the current flowing between two identical working electrodes, at a small potential difference and immersed in a solution containing the analyzed sample and a reversible redox couple
Chromatography		
Gas chromatography	Separation of the compounds in a mixture is based on the repartition between a liquid stationary phase and a gas mobile phase	Flame ionization or thermal conductivity detection
High performance liquid chromatography	Separation of the compounds in a mixture is based on the repartition between a solid stationary phase and a liquid mobile phase with different polarities, at high flow rate and pressure of the mobile phase	UV-VIS (e.g. diode array) detection, fluorescence, mass spectrometry or electrochemical detection

Pisoschi and Negulescu (2011).

CHAPTER THREE

MATERIALS AND METHODS

A number of *in vitro* spectrophotometric methods for quantification of phenolic compounds in plant materials have been developed. Based on different principles, the following assays will be employed in the present study.

- ❖ DPPH free radical scavenging activity
- ❖ Phosphomolybdenum antioxidative power assay
- ❖ Hydrogen peroxide scavenging (H_2O_2) assay.

With reference to these three methods, antioxidant activity of Caffeic acid will be compared to that of Ascorbic acid.

3.1 Chemicals and Instrumentation

The chemical substances used in the experiments were all of analytical reagent grade. The visible spectra and absorption measurements were recorded in matched cuvettes using *Genesys 10S UV-VIS* spectrophotometer.

3.2 Preparation of solutions

The stock solution of Caffeic acid (CA) was prepared by dissolving a weighed amount in a total volume of 10ml ethanol to give 10mM concentration, while Ascorbic acid was prepared in water. Working solutions of 1mM and 2mM concentrations were prepared from the 10mM stock solutions for each assay (Gulcin, 2006; Adjimani *et al*., 2015). All other reagents used were prepared by accurate dilutions from stock solutions. Reactions were carried out in triplicate.

3.3 DPPH radical scavenging activity

The free radical scavenging activity of caffeic acid was measured by the 1, 1-diphenyl-2-picryl-hydrazil (DPPH•). This activity was measured by following the methodology described by Gulcin, (2006) wherein the bleaching rate of a stable free radical, DPPH• is monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH• absorbs at 517nm, but upon reduction by an antioxidant or a radical species its absorption decreases. Briefly, 0.1mM solution of DPPH• in ethanol was prepared and 1ml of this solution was added to 3ml of caffeic acid solution in ethanol at different concentrations (1mM and 2mM). After 30minutes, the absorbance was measured at 517nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity.

% inhibition calculated as: Scavenging effect (%) = [(OD of control-OD of sample) / (OD of control)] ×100.

The DPPH• radical displays an intense UV-VIS absorption spectrum (Fig.3.1). In this test, a solution of radical is decolorized after reduction with an antioxidant (AH) or a radical (R•) in accordance with the following scheme: DPPH• + AH → DPPH•-H + A•, DPPH• + R• → DPPH•-R.

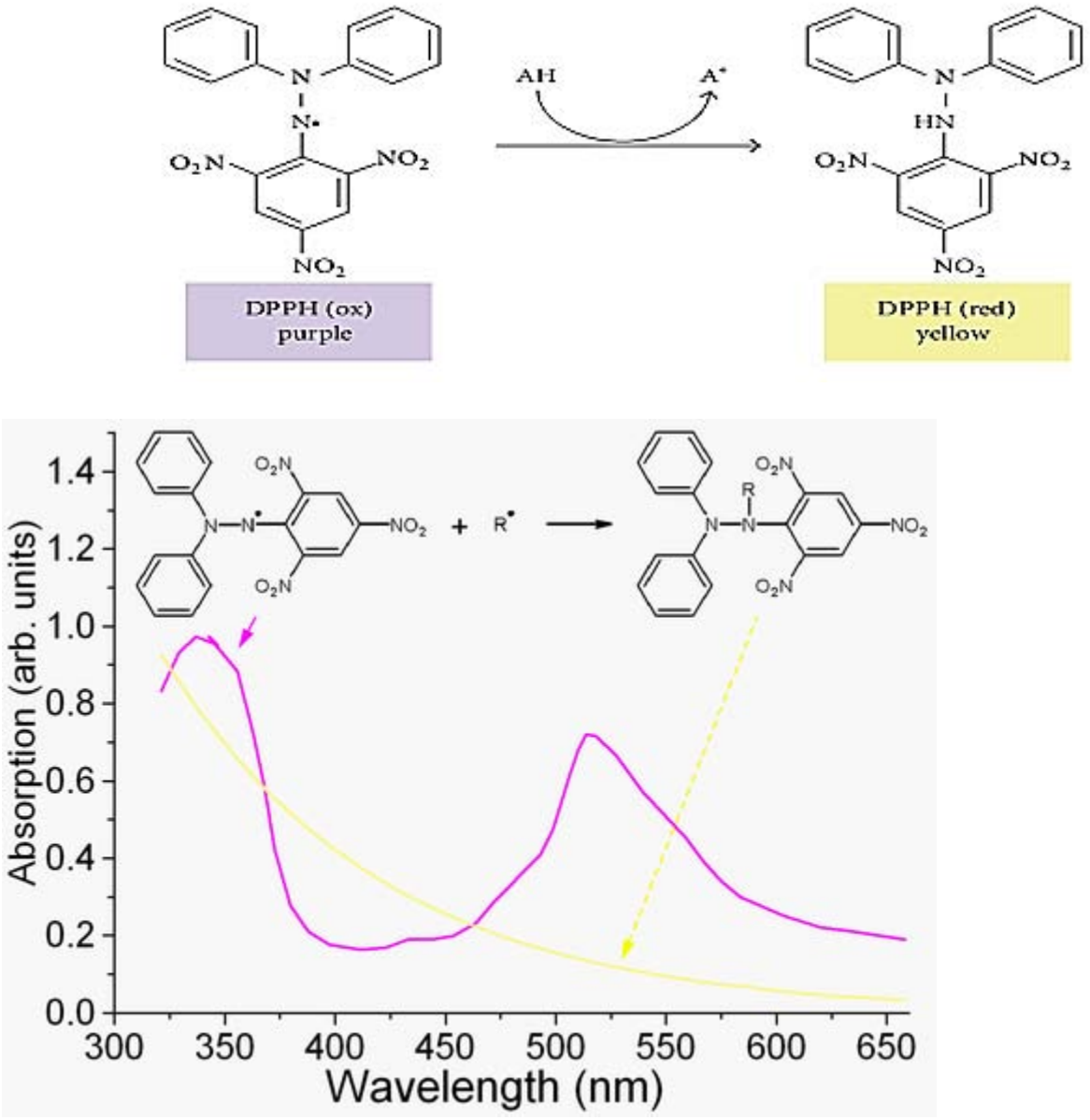


Figure 3.1 Reaction of the DPPH radical with an antioxidant.

3.4 Phosphomolybdenum antioxidative power assay

Phosphomolybdenum assay was conducted by the method of Prieto *et al*., (1999). This method is based on the reduction of phosphomolybdic acid to phosphomolybdenum blue complex by sodium sulfide. The Phosphomolybdenum blue complex obtained is oxidized by the addition of nitrite and this causes a reduction in intensity of the blue color.

Caffeic acid, 0.1ml (1mM - 2mM) was mixed with the Phosphomolybdenum reagent (1ml), (containing 0.6M H_2SO_4, 28mM Na_2SO_4 and 4mM ammonium molybdate). The mixture was incubated in a water bath for 90minutes at 95°C. Samples were cooled after the incubation and OD measured at 695nm against a blank in triplicate and percentage inhibition calculated according to:

Inhibition % = (1- OD of sample / OD of control) × 100

3.5 Hydrogen peroxide scavenging (H_2O_2) assay

Scavenging of hydrogen peroxide was assayed according to the method of Ruch *et al*. (1989). A solution of H_2O_2 (40mM) was prepared in phosphate buffer. Samples were added to the H_2O_2 solution (0.6ml) and the total volume was made up to 3ml. The absorbance of the reaction mixture is recorded at 230nm in a spectrophotometer. A blank solution of phosphate buffer, without H_2O_2 was used as control. The extent of H_2O_2 scavenging is calculated as:

% Scavenging = $(A_o - A_1) \times 100/A_o$

A_o =Absorbance of control, A_1 = Absorbance in presence of sample.

CHAPTER FOUR

RESULTS

The three *in vitro* methods presented in this study are based on the ability of antioxidants to reduce a radical cation / anion which can be monitored spectrophotometrically by determining decrease in absorbance.

The ions monitored included 1, 1-diphenyl-2-picryl-hydrazil (DPPH), Phosphomolybdenum and Hydrogen Peroxide (H_2O_2).

4.1 DPPH free radical scavenging activity

Radical scavenging activities are very important due to the destructive role of free radicals in foods and in biological systems. The DPPH radical scavenging activity is generally quantified in terms of inhibition percentage of the pre-formed free radical by antioxidants. In the DPPH assay, the samples were able to reduce the stable DPPH radical to the yellow colored, diamagnetic diphenyl-picrylhydrazine, indicating their abilities to scavenge DPPH radical. The color variation undergone by the DPPH radical is depicted in Figure 3.1. Figure 4.2 shows the percentage DPPH scavenging activity of 84.63% for caffeic acid and 83.43% for ascorbic acid at 1mM concentration. At 2mM concentration, the values obtained are 84.93% for Caffeic acid and 85.44% for Ascorbic acid.

4.2 Phosphomolybdenum Antioxidative Power (PAP) Assay

PAP assay measures the reduction degree of Mo (VI) to Mo (V). It is a quantitative method to investigate the reduction reaction rate among antioxidant, oxidant and molybdenum ligand involving thermal generation of auto-oxidation during prolonged incubation period at higher temperature and acidic pH. It gives a direct estimation of reducing capacity of an antioxidant.

Being distinctive from FRAP and CUPRAC assays it remains intact irrespective of concentration of free metal ions and unlike CUPRAC and FRAP, it forms a green Phosphomolybdenum complex without induction of free metal ions in solution thus making it unique among *in vitro* antioxidant assays methods (Bruno *et al.,* 2015).

The result of this assay is presented in Fig.4.3. Caffeic and ascorbic acids at 1mM concentration shows percentage inhibition of the phosphomolybdate ion of 67.81% and 71.90% respectively. At 2mM concentrations, the percentage values are 56.02% for Caffeic acid and 65.37% for Ascorbic acid, an indication that ascorbic acid is a better scavenger of the phosphomolybdate radical than caffeic acid with their abilities decreasing with increasing concentration.

4.3 Hydrogen peroxide scavenging activity

Hydrogen peroxide accepts protons (H^+) or electrons from antioxidants and is consequently reduced to H_2O. In this assay, the antioxidant scavenges hydrogen peroxide(H_2O_2) by donating hydrogen atoms thus reducing it to water, H_2O (Niki, 2010).

A significantly higher percentage hydrogen peroxide scavenging values of 98.82%, 98.77% were obtained for 1mM and 2mM caffeic acid respectively as against 51.46%, 45.42% obtained for 1mM and 2mM ascorbic acid respectively (Figure 4.4).

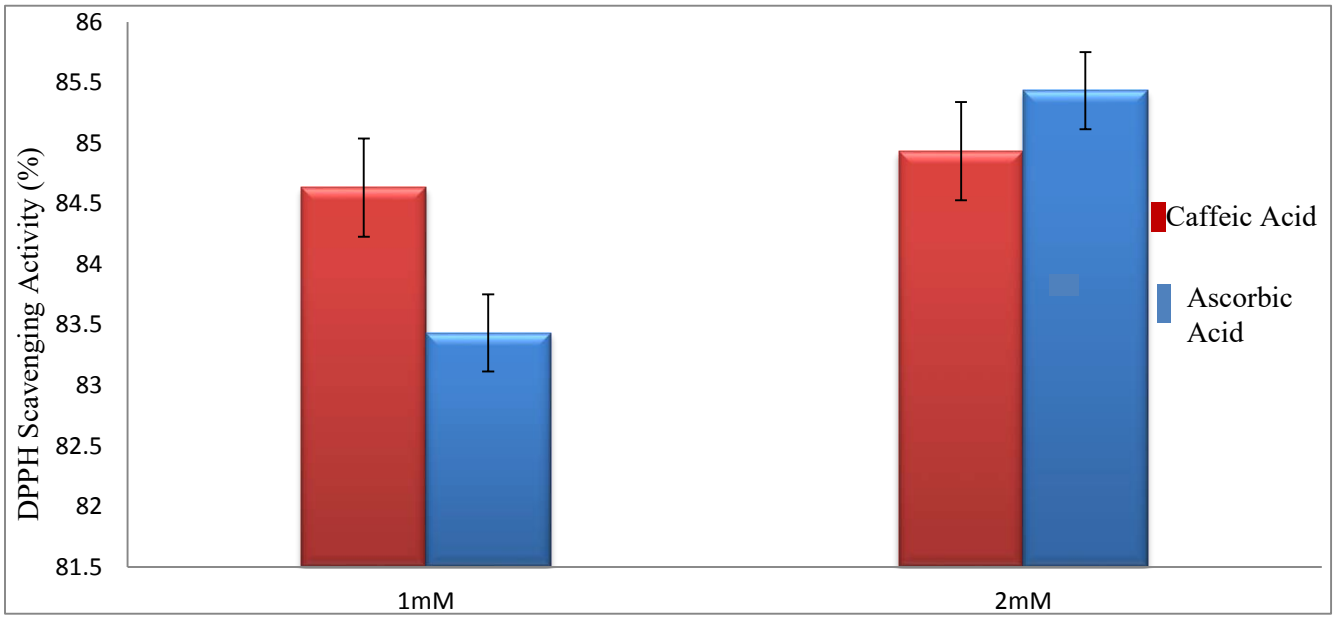


Figure 4.1 DPPH free radical scavenging activity of caffeic acid and ascorbic acid.

Solutions were prepared at 1mM and 2mM concentrations. DPPH (1ml, 0.1mM) solution was added to 3ml each of the aliquot of the solutions to make a total volume of 4ml reaction mixture. Each value is the average of three experiments with error bars indicating SEM.

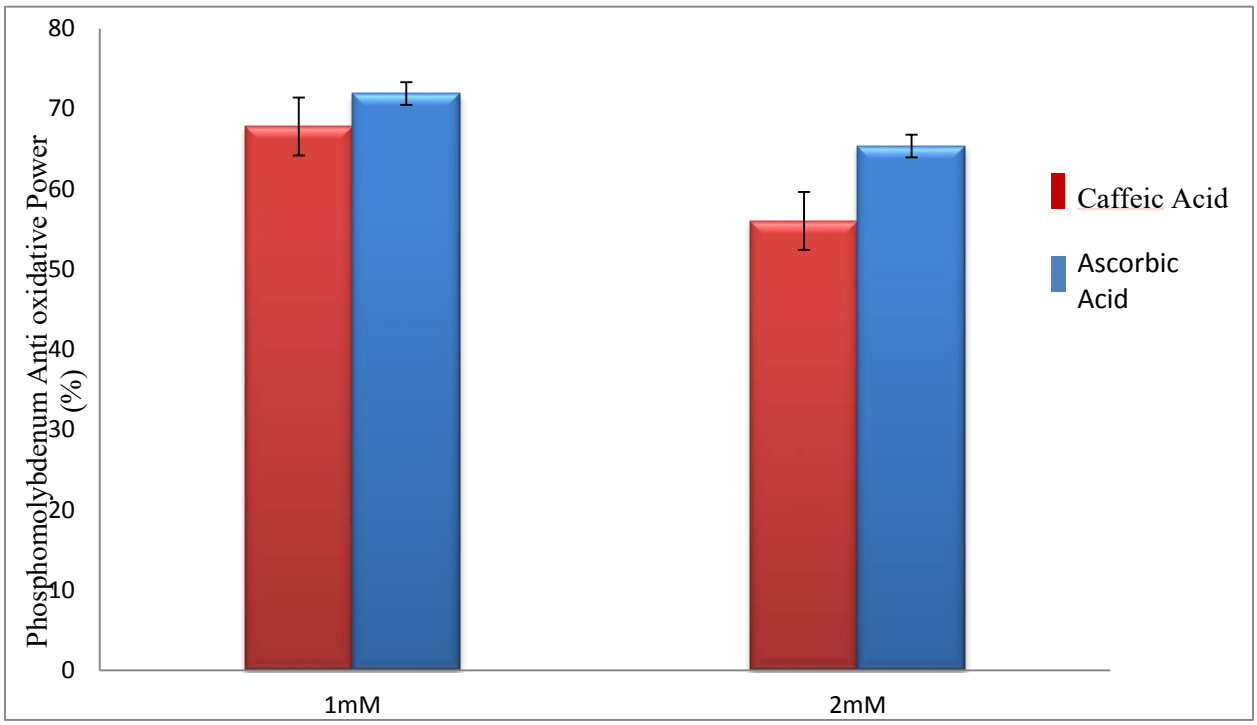


Figure 4.2 Phosphomolybdenum antioxidative power of caffeic acid and ascorbic acid.

Phosphomolybdenum solution, (1ml), was added to 0.1ml of Caffeic acid and Ascorbic acid at molar concentrations of 1mM and 2mM. Each bar represents the mean ± SEM of experiments carried out in triplicates.

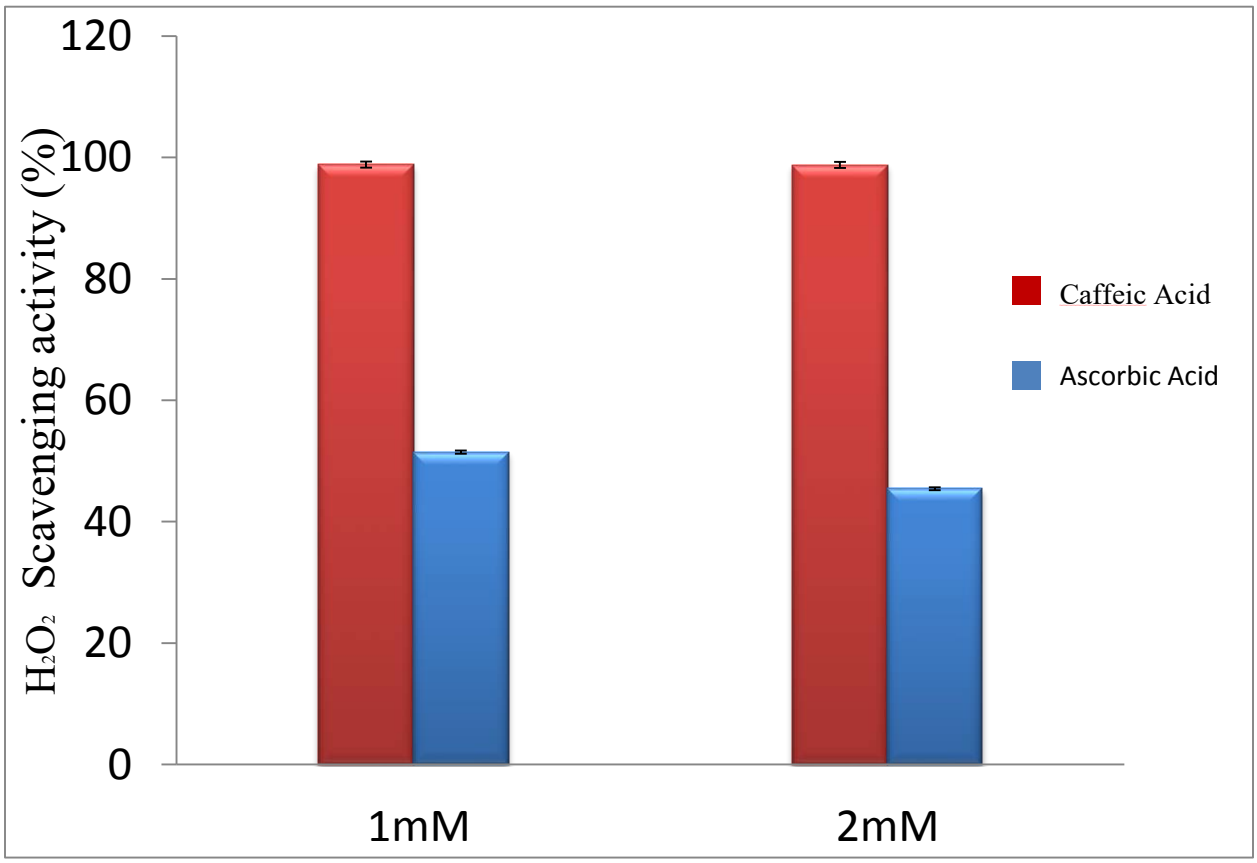


Figure 4.3. Hydrogen peroxide scavenging activity of caffeic and ascorbic acids.

H_2O_2 in pH 7.4 phosphate buffer (2.4ml) was added to 0.6ml sample solution at 1mM and 2mM concentrations. The total volume was made up to 3ml. Each bar is the mean ± SEM with all determinations conducted in triplicates.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 Discussion

Phenolic compounds exhibit free radical scavenging potential as their phenyl and hydroxyl groups confer scavenging properties (Yildirim *et al.*, 2000; Balasundram *et al.*, 2006); a positive relationship has been shown to exist between plant phenolics, their antioxidant and antimutagenic potentials (Shon *et al*., 2004; Sahreen *et al*., 2010).

Caffeic acid is a hydroxycinnamic acid with phenyl and acrylic functional groups, while L-ascorbic acid is a water-soluble dibasic acid with an enediol group built into a five membered heterocyclic lactone ring (Fig.5.1a).

(a) (b)

Figure 5.1 Structures of Ascorbic Acid (a), and Caffeic Acid (b).

The DPPH radical scavenging activity is manifested in the ability of an antioxidant to scavenge free radicals by Hydrogen Atom Transfer (HAT) and Single Electron Transfer (SET) reaction mechanisms thus neutralizing the generated free radical leading to the observable color characterizing the reaction as depicted in Fig. 4.1. A significant difference of 1.00 ± 0.4 in value was observed at 1mM of caffeic acid and ascorbic acid. The difference was nullified at a higher concentration of 2mM. This may be due to the fact that scavenging activity increases with

concentration in consonance with the observations of others including Oyaizu, 1986, Malki *et al*. 2013, Moon and Shibamoto, 2009, Mellina *et al.,* 2011, Villano *et al.,* 2007 and Robbins, 2013.

The radical scavenging activities of both caffeic and ascorbic Acids for the Phosphomolybdate ion is achieved via the reduction, by transfer of hydrogen atom from the antioxidant, to the phosphomolybdate blue complex leading to an eventual decrease in the measured intensity of the blue color. From Fig. 4.3, it is evident that ascorbic acid is a better scavenger of the phosphomolybdate ion than caffeic acid at the stated concentrations with a noticeable decrease in scavenging activity with gradually-increasing concentrations. This may be due to the high water solubility of ascorbic acid in water, resulting in the formation of strong hydrogen bonds (Fig.5.1a), which has been reported by Huang *et al.* (2005), which may increase the rate of scavenging of the phosphomolybdate radical, as opposed to caffeic acid which is only sparingly soluble in water.

Figure 4.4 shows the relative abilities of caffeic and ascorbic acids to scavenge the hydrogen peroxide radical by the donation of hydrogen ions or electrons which is consequently reduced to water. A higher ability to reduce hydrogen peroxide was shown by caffeic acid than ascorbic acid at both concentrations used. It can be observed (Fig.4.4) that caffeic acid is a better hydrogen peroxide scavenger than ascorbic acid at the concentrations used. This is expected as the resonance stabilization effect of the phenyl group (Fig.2.5, 2.8 a, 2.8 d), in caffeic acid coupled with the double bond of the acrylic group in reference to the lactone ring of ascorbic acid provides better stabilization of delocalized electrons abstracted from the free radicals over the aromatic ring preventing free radical chain reactions. The high solubility of ascorbic acid in cold water as opposed to the low water-solubility of caffeic acid could also be a factor as hydrogen peroxide scavenging activity is pH-dependent.

5.2 Conclusion and Recommendation

The study is consistent with the findings of Rice-Evans *et al*. (1997), that polyphenols such as caffeic acid, possess an ideal structural chemistry for free radical-scavenging activities. The presence of – C = C− CO_2H in addition to the phenyl ring and the hydroxyl substituent groups, play important roles in stabilizing free radicals by resonance (Fig.2.8 b), radical-quenching mechanisms through electron donation and singlet oxygen quenching. Substituent groups on the aromatic ring also affect the stabilization and therefore influence the radical-quenching ability showing the polyphenols to be more effective antioxidants *in vitro* than vitamins E and C on a molar basis. With reference to the different mechanisms involved in the antioxidant action of each group of natural products and the different rates of reactions in the underlying radical-scavenging reactions, the choice of assay can influence the overall results obtained. It is noteworthy to state here that not all methods and antioxidant sources are compatible, and the same antioxidant species can yield dissimilar results in different assays as observed in this work. This suggests a need to complete a multitude of antioxidant assessment assays on potential antioxidant sources, with particular attention paid to the chemistry involved and the important factors regarding the assays.

Understandably, the knowledge of antioxidant capacities will provide valuable *in vitro* data into the potential capabilities of antioxidant compounds *in vivo*.

With the new emphasis on functional food and nutraceuticals, there is the need to constantly create new antioxidant assessment methods for application in fast and cost-efficient assay of extracts and food products with observed biological activities. Each of these assays is made of a variety of critical steps that must be followed in order to maintain accuracy and precision. Additionally, the chemistry behind the assays must be critically assessed in order to run the assays and troubleshoot

effectively. Thus the need to standardize the methods to ensure uniformity in the scientific literature for better comparison between sources for determining the total amount of antioxidant present in the food stuff.

The development of standard protocols for evaluation of antioxidant activity, the complex nature of these methods and their compatibility with certain antioxidant sources must be a source of concern for future research and, thus, great care should be exercised when formulating methods for the measurement of antioxidant capabilities. One major drawback of the *in vitro* assay methods is the fact that the artificial radicals being tested are not always present *in vivo*. Nevertheless, the techniques are useful in ranking antioxidant activity of substances and food containing them and may serve in evaluating if a preservation technique influence a food's antioxidant activity. This may consequently be an indicator of the antioxidant potential before the consumption of such foods.

The correlation in the observable trends of antioxidant activities here measured is based on the principles of the assay methods i.e. HAT and SET mechanisms of antioxidant action.

The similarity in structure of the two samples compared (Fig.5.1), with reference to the propensities of hydroxyl groups and resonance stabilization of the lactone/phenyl groups, is responsible for the observable trend. As both substances are naturally-occurring, the methods employed in this study have shown a good correlation between structure and antioxidant activity in a concentration-dependent manner and will be a good addition to the available resources in the field of antioxidant /free radical research.

The antioxidant potential measurement assays can help in choosing naturally occurring antioxidant -rich food containing ascorbic acid, vitamin E, carotenoids and natural polyphenols for preservation of food and decreasing the incidence of ROS in body

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APPENDICES

Appendix 1 Descriptive Analysis DPPH Assay

Caffeic acid (%) - 1mM		*Caffeic acid (%) - 2mM*	
Mean	84.63333333	Mean	84.93333
Standard Error	0.405517502	Standard Error	0.868588
Median	84.7	Median	85.7
Mode	#N/A	Mode	#N/A
Standard Deviation	0.702376917	Standard Deviation	1.504438
Sample Variance	0.493333333	Sample Variance	2.263333
Kurtosis	#DIV/0!	Kurtosis	#DIV/0!
Skewness	-0.42327316	Skewness	-1.69768
Range	1.4	Range	2.7
Minimum	83.9	Minimum	83.2
Maximum	85.3	Maximum	85.9
Sum	253.9	Sum	254.8
Count	3	Count	3
Confidence Level (95.0%)	1.744800987	Confidence Level (95.0%)	3.737231

Vit C (%) - 1mM		*Vit C (%) - 2mM*	
Mean	83.43333333	Mean	85.43333333
Standard Error	0.437162568	Standard Error	0.317979734
Median	83.1	Median	85.4
Mode	#N/A	Mode	#N/A
Standard Deviation	0.757187779	Standard Deviation	0.550757055
Sample Variance	0.573333333	Sample Variance	0.303333333
Kurtosis	#DIV/0!	Kurtosis	#DIV/0!
Skewness	1.597096993	Skewness	0.271354762
Range	1.4	Range	1.1
Minimum	82.9	Minimum	84.9
Maximum	84.3	Maximum	86
Sum	250.3	Sum	256.3
Count	3	Count	3
Confidence Level (95.0%)	1.880958718	Confidence Level (95.0%)	1.36815637

Appendix 2 Descriptive Analysis of Phosphomolybdenum Antioxidative Assay

Caffeic Acid (%) - 1mM		*Caffeic Acid (%) - 2mM*	
Mean	67.81666667		56.02667
Standard Error	0.208832735	Standard Error	3.610916
Median	68	Median	58.7
Mode	#N/A	Mode	#N/A
Standard Deviation	0.361708907	Standard Deviation	6.254289
Sample Variance	0.130833333	Sample Variance	39.11613
Kurtosis	#DIV/0!	Kurtosis	#DIV/0!
Skewness	-1.694891426	Skewness	-1.57205
Range	0.65	Range	11.62
Minimum	67.4	Minimum	48.88
Maximum	68.05	Maximum	60.5
Sum	203.45	Sum	168.08
Count	3	Count	3
Confidence Level (95.0%)	0.898534736	Confidence Level (95.0%)	15.53652

Vit. C (%) - 1mM		*Vit C (%) - 2mM*	
Mean	71.90333	Mean	65.37333
Standard Error	0.838458	Standard Error	1.424796
Median	71.81	Median	65.8
Mode	#N/A	Mode	#N/A
Standard Deviation	1.452251	Standard Deviation	2.46782
Sample Variance	2.109033	Sample Variance	6.090133
Kurtosis	#DIV/0!	Kurtosis	#DIV/0!
Skewness	0.288012	Skewness	-0.75476
Range	2.9	Range	4.88
Minimum	70.5	Minimum	62.72
Maximum	73.4	Maximum	67.6
Sum	215.71	Sum	196.12
Count	3	Count	3
Confidence Level (95.0%)	3.607592	Confidence Level (95.0%)	6.130404

Appendix 3 Descriptive Analysis of Hydrogen Peroxide Scavenging Assay

Caffeic Acid (%) - 1mM		*Caffeic Acid (%) - 2Mm*	
Mean	98.82	Mean	98.77333
Standard Error	0.508625599	Standard Error	0.54566
Median	98a.98	Median	98.79
Mode	#N/A	Mode	#N/A
Standard Deviation	0.88096538	Standard Deviation	0.94511
Sample Variance	0.7761	Sample Variance	0.893233
Kurtosis	#DIV/0!	Kurtosis	#DIV/0!
Skewness	-0.790326725	Skewness	-0.07933
Range	1.74	Range	1.89
Minimum	97.87	Minimum	97.82
Maximum	99.61	Maximum	99.71
Sum	296.46	Sum	296.32
Count	3	Count	3
Confidence Level (95.0%)	2.188439322	Confidence Level (95.0%)	2.347784

Vit C (%) - 1mM		*Vit C (%) - 2mM*	
Mean	51.46333333	Mean	45.42667
Standard Error	0.243743946	Standard Error	0.311252
Median	51.65	Median	45.35
Mode	#N/A	Mode	#N/A
Standard Deviation	0.422176898	Standard Deviation	0.539104
Sample Variance	0.178233333	Sample Variance	0.290633
Kurtosis	#DIV/0!	Kurtosis	#DIV/0!
Skewness	-1.600705331	Skewness	0.627008
Range	0.78	Range	1.07
Minimum	50.98	Minimum	44.93
Maximum	51.76	Maximum	46
Sum	154.39	Sum	136.28
Count	3	Count	3
Confidence Level (95.0%)	1.048745554	Confidence Level (95.0%)	1.339209