ASSESSMENT OF *ULAM RAJA* (*COSMOS CAUDATUS*) EXTRACT AS A FUNCTIONAL INGREDIENT IN BEEF PATTY AND GREEN TEA

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By

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LIST OF SYMBOLS / ABBREVIATIONS

Symbol/Abbreviation	Caption
ABTS	2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)
DPPH	1,1-diphenyl-2-picrylhydrazyl
UREX	*Ulam Raja* Extract
HUREX	Higher level of UREX
LUREX	Lower level of UREX
GT	Green Tea
GTE	GT Extract
HGT	Higher level of GTE
LGT	Lower level of GTE
TEAC	Trolox Equivalent Antioxidant capacity
GA	Gallic Acid
GAE	GA Equivalent
TPC	Total Phenolic Content
FRAP	Ferric Reducing Antioxidant Power
HPLC	High Performance Liquid Chromatography
TSS	Total Soluble Solids

°Brix	Degree Brix
ORAC	Oxygen Radical Absorbance Capacity
TCA	Trichloroacetic Acid
TBA	2-thiobarbituric acid
TEP	1,1,3,3-tetraethoxypropane
TBARS	Thiobarbituric Acid Reactive Substances
db	Dried base
MDA	Malondialdehyde
T	Transmittance
NTU	Nephelometric Turbidity Units
L*	Lightness
a*	Redness
b*	Yellowness
ANOVA	One-way Analysis of Variance
WHO	World Health Organization
ITC	International Tea Committee
USDA	U.S. Department of Agriculture

LIST OF PUBLICATIONS AND SEMINARS

Seminar & Exhibition

1. Reihani, S.F.S. & Easa M.A. (2014). Utilization of high-ORAC extract of Ulam Raja (*Cosmos caudatus*) in beef patties. Joint Postgraduate Seminar USM-PSU-UNAND, Penang Island, Malaysia. Oral Presentation.

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3. S. Fatemeh S. Reihani and Azhar Mat Easa (2012). Antioxidant activity and Total phenolic contents in extracts of selected traditional Malay raw salads (*Ulam*), International Conference on Food Science and Nutrition, (ICFSN 2012). Kota Kinabalu, Sabah, Malaysia. Poster Presentation

4. S. Fatemeh S. Reihani & Azhar Mat Easa. (2011). Evaluation of antioxidant activity and phenolic contents in selected traditional Malay raw salads (*Ulam*), Seminar on Functional Foods, UPM, Malaysia. Oral Presentation.

5. Azhar Mat Easa, Reihani, S.F.S. & Tan Thuan Chew. (2014). Gold Award in Biotechnology Asia, Kuala Lumpur Convention centre, Malaysia, 19- 21

6. Reihani, S.F.S. & Easa, A.M. (2013). UREX1: Food grade high ORAC *Ulam Raja* (*Cosmos caudatus*) Extract for Nutracetical Application. *International Conference & Exposition on invention of Institution of Higher Learning*, (*PECIPTA 13*). Silver.

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Publications

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PENILAIAN EKSTRAK ULAM RAJA (*COSMOS CAUDATUS*) SEBAGAI RAMUAN BERFUNGSI DALAM PATTI DAGING LEMBU DAN TEH HIJAU

ABSTRAK

Antioksidan semula jadi dan tiruan mempunyai keupayaan untuk menentang spesies oksigen reaktif, menghalang degradasi pengoksidaan lipid dan dengan itu meningkatkan nilai nutrisi makanan. Kajian ini menilai potensi penggunaan lima salad mentah atau ulam popular kaum Melayu (Daun Kari, Selom, Ulam Raja, Pegaga dan Petai) sebagai bahan berfungsi dalam makanan dan minuman. Pada mulanya, jumlah kandungan fenolik (TPC) dan aktiviti antioksidan telah disiasat dengan menggunakan ujian Folin-Ciocalteu, pemerangkapan radikal DPPH ($TEAC_{DPPH}$) dan penurunan kuasa antioksidan ion ferik ($TEAC_{FRAP}$) masing-masing. Ulam raja menunjukkan aktiviti antioksidan yang paling tinggi, dan telah dipilih untuk pemprosesan dan analisis lanjut. Kesan masa (30, 45 dan 60 min) dan suhu (45, 65 dan 85 °C) pemanasan ke atas hasil pengekstrakan (°Bx) daripada Ulam Raja kemudian dikaji menggunakan reka bentuk komposit berpusatkan muka (FCCD). Yil pengekstrakan yang optimum (4.2 °Bx) telah diperolehi dengan menggunakan pemanasan selama 30 min pada 85 °C. Kromatografi cecair prestasi tinggi (HPLC), digunakan untuk menjelaskan dan menentukan beberapa sebatian penanda, menunjukkan kehadiran beberapa flavonoid iaitu kuersitrin (36.9 mg/g), katekin (25 mg/g) dan rutin (8.2 mg/g) dalam serbuk ekstrak ulam raja terliofil (UREX). Potensi aplikasi UREX (nilai ORAC sebanyak 2833 μmol setara trolox/g) dalam makanan telah disiasat sebagai bahan berfungsi ber ORAC tinggi dalam patti daging lembu.

Penambahan UREX atau ekstrak teh hijau komersial (GTE) pada 500 mg / kg ke patti daging mengurangkan tahap pengoksidaan lipid dan protein dengan signifikan (P<0.05). UREX menunjukkan kesan perencatan pengoksidaan lipid dan protein yang kuat, setanding dengan kesan oleh GTE. Di samping itu, peningkatan yang signifikan (P <0.05) dalam hasil memasak dan sifat tekstur juga direkodkan. UREX juga telah dinilai untuk untuk potensi aplikasinya sebagai nutraseutikal dalam minuman teh. UREX telah ditambah ke dalam teh hijau pada 25 dan 50 mg setiap uncang, dan perubahan dalam warna, aktiviti antioksidan dan ciri-ciri deria sampel (kawalan tanpa UREX, LUREX: 25 mg/uncang, dan HUREX: 50 mg / uncang) telah dinilai. Penerimaan keseluruhan sampel yang ditambah dengan HUREX telah meningkat secara signifikan dibandingkan LUREX dan kawalan. Kuning (b *) dan kroma juga (P <0.05) meningkat secara signifikan. $TEAC_{DPPH}$ dan TPC nyata lebih tinggi (P <0.05) dalam sampel dirawat dengan HUREX berbanding dengan dua yang lain (LUREX dan kawalan). Kesimpulannya , kajian ini menunjukkan nilai potensi penggunaan UREX sebagai bahan berfungsi dalam makanan (patti daging patty) dan minuman (teh hijau).

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ABSTRACT

Natural and synthetic antioxidants have the ability to scavenge reactive oxygen species, hinder oxidative degradation of lipids and thus enhance the nutritional value of food. This thesis aimed to evaluate the potential use of *Cosmos caudatus* as a functional ingredient in food and beverage. In the first phase of this study, the total phenolic contents (TPC) and antioxidant activities of five popular Malaysian raw salads or *Ulam* were investigated by using Folin-Ciocalteu, DPPH radical scavenging ($TEAC_{DPPH}$) and reducing ferric ion antioxidant power ($TEAC_{FRAP}$) assays respectively. Results confirmed that *Ulam raja* possesses the highest antioxidant activities. In the next phase, the effect of heating time (30, 45 and 60 min) and temperature (45, 65 and 85˚C) on the extraction yield (°Bx) of *Ulam raja* was studied by using a face-centered composite design (FCCD). The optimum extraction yield (4.2 °Bx) was obtained by using 85˚C heating temperature for 30 min. To elucidate and quantify some marker compounds in the extract, high performance liquid chromatography (HPLC) was used. The chromatographic data illustrated the presence of several flavonoids *i.e.* quercitrin, catechin and rutin in lyophilized *Ulam raja* extract powder (UREX). Their quantities were reported as 36.9mg, 25mg and 8.2 mg per g dried extract powder (UREX) respectively. Acquiring an ORAC value equivalent to 2833 µmol TE, UREX was utilized as a high-ORAC functional ingredient in beef patties. Incorporation of UREX or a commercial green tea extract (GTE) at 500 mg/kg into beef patties reduced the extent of lipid and protein oxidation significantly ($P<0.05$). UREX showed strong lipid and protein oxidation inhibitory effect, comparable with those of GTE. In addition, significant improvement ($P<0.05$) in cooking yield and textural properties was also recorded. In the last phase of the study, UREX was assessed for its potential application as a nutraceutical in tea beverage. UREX was added into green tea at 25 and 50 mg per sachet, and the changes in color, antioxidant activities and sensory characteristics of the samples (control without UREX, LUREX: 25mg/sachet, and HUREX: 50 mg /sachet) were evaluated. Overall acceptability of samples added with HUREX was significantly improved compared to LUREX and control. Yellowness (b*) and chroma were also significantly ($P<0.05$) increased. $TEAC_{DPPH}$ and TPC were significantly ($P<0.05$) higher in sample treated with HUREX compared to the other two (LUREX and control). In conclusion, this thesis pointed to the potential value of using UREX as a functional ingredient in food (beef patty) and beverage (green tea).

CHAPTER ONE

1. INTRODUCTION

1.1 Background and Rationale

The crucial role of free radicals e.g. reactive oxygen and hydrogen species in various physiological and pathological processes such as aging, tumor and cancer has been proved (Lobo, 2010). Deleterious effects of free radicals cannot be completely controlled and prevented by endogenous antioxidants only which are present in living cells e.g. glutathione (Rao, 2006). Therefore, using exogenous antioxidants seems to be a necessity. Antioxidant phytochemicals in vegetables, fruits, and grains are found to possess prevention abilities against human diseases (Yu et *al*., 2002). In addition to effects on body, free radicals may also have deleterious effects on foods and food products by production of rancid flavors and odors, reducing nutritional quality and the shelf life of food products (Chanwitheesuk et al., 2005). Fat components especially unsaturated fatty acids can be readily oxidized by molecular oxygen and cause adverse effects in foods such as rancidity, off-flavor development and discoloration. To control and reduce the detrimental effects of oxidative stress in foods, synthetic antioxidants such as tertiary Butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been industrially added to a large number of food products. However, due to toxicological safety concerns of synthetic antioxidants much research is being conducted on natural antioxidants derived from plant sources (Devi et al., 2008).

These natural antioxidants can also be formulated as neutraceuticals in order to associate in preventing oxidative deterioration effects in body cellular components.

Natural plant based antioxidants e.g. extracts from rosemary, oregano, sage, thyme, etc in West countries are being widely studied and archived in the USDA data base (USDA, 2010). However, less is known about the antioxidative compounds derived from plants in developing countries, including Malaysia. A large number of freshly eaten local plants, known as *ulam*, is being subjected to intense studies due to its strong history in Malaysian multiracial culture for both preventative and curative medicinal purposes (Huda-Faujan et al., 2007b; Noriham et al., 2004b). These *ulam* are widely popular for being health beneficial as they were claimed to possess anti-aging properties. Most of them are believed to be associated with antioxidant activities (Jayamalar and Suhaila, 1998; Mohd Zin et al., 2002; Noriham et al., 2004a; Zainol et al., 2003). Even though *ulam* are known and widely used by locals in rural areas and also in towns where they are embraced by other races, no much study is available on investigating their antioxidative compounds which may be the main contributors of their health beneficial properties (Andarwulan et al., 2010; Bolling et al., 2010; Halliwell, 1996; Shui et al., 2005; Sulaiman et al., 2011; Zainol et al., 2003).

Extracts taken from *ulam* with high antioxidant activities can be incorporated in food especially those products rich in fats and proteins to enhance their shelf life and maintain their nutritional and sensory quality during storage.

The raising interest toward Western dietary pattern especially in younger generation is of particular concern as globalization of fast foods has had an obvious influence on their eating behavior (Hu, 2008).

Hamburger patty is known as one of the world's most popular processed meat products (Darwish et al., 2011). It is made of ground beef which is considered as a food with high lipid content. Typical ground beef consists of about 18% lipids with 46% saturated, 51% mono-unsaturated and 3% poly-unsaturated fatty acids (Demeyer et al., 2008). The shelf life of meat is highly dependent on the content of unsaturated fatty acids especially those with more than two double bonds; they play an important role in regulating the shelf life of meat (Wood et al., 2003). Natural and synthetic antioxidants have been widely applied in meat industry to enhance the shelf life of meat products and control the development of oxidative reactions in them (Estéves and Cava, 2006).

Incorporation of *ulam* extract into beef patty as an example of food system rich in unsaturated fat and protein, may offer a simple yet effective way to enhance its storage stability by minimizing lipid and protein oxidative spoilage.

In addition to a food system like beef patty, tea as a popular beverage may also be a good target of enrichment by natural extracts from *ulam* in order to increase its health beneficial properties and bio-active compounds intake among tea consumers.

1.2 Objectives

The main objective of this study is to develop a beef burger product enriched with *Ulam Raja (Cosmos caudatus)* extract and to compare the influence of the extract on storage stability of beef patty -as an example of food system rich in fat- with a commercial natural antioxidant as a positive control. The extract product from *Ulam Raja* can be applied in any kind of food and beverage products as a functional and nutraceutical ingredient. The measurable objectives of this study are listed as follows:

1. To rank five popular *ulam* based on their antioxidant activity by using different antioxidant measurement assays.
2. To optimize the extraction process in terms of yield of extraction, and evaluate the effect of optimum conditions on antioxidant activities and total phenolic contents of the extract.
3. To identify and quantify some bio-active (marker) compounds of optimized lyophilized extract powder (UREX).
4. To investigate the frozen storage stability of beef patties incorporated with UREX.
5. To study the potential application of *ulam* extract in tea beverage

1.3 Thesis Outline

The development of a beef patty product enriched with bio-active compounds extracted from selected *ulam (Cosmos caudatus)* as a functional ingredient, and monitoring its storage stability is presented in this dissertation. Besides, the effect of addition of this natural extract in green tea as a popular beverage is depicted and discussed. The present dissertation comprises seven main chapters. CHAPTER ONE is a general introduction on the background of this research in which the current challenges encountered by food industry regarding the oxidative stress. Besides, the rationales and the objectives of this study are briefly discussed.

CHAPTER TWO is based on a general literature review which addresses the free radical damage, antioxidants and their mechanisms of action, and the importance of functional foods.

CHAPTER THREE illustrates assessment of antioxidant activities of five popular *ulam* and confirm the ranking based on antioxidant activity and total phenolic contents by conducting different antioxidant measurement assays. The optimization of the extraction process of the selected *ulam* in terms of yield of extraction by using a face-centered composite design (FCCD) as well as the effect of optimum conditions on antioxidant activities and total phenolic contents of the extract, and at last assessment of selected phytochemicals (marker compounds) are examined and discussed in CHAPTER FOUR. In CHAPTER FIVE, the frozen storage stability of beef patties incorporated with the extract powder as a natural antioxidant is evaluated and a positive reference (commercialized green tea extract) is used to compare the results.

The selected *ulam* extract was assessed for its potential nutraceutical application in tea beverage in CHAPTER SIX. In this phase of study, selected *ulam* extract was added into green tea at 25 and 50 mg per sachet, and the changes in color, antioxidant activities and sensory characteristics of the samples were evaluated.

The last chapter (CHAPTER SEVEN) consists of overall conclusions on the study and recommendations for the future work on this area.

CHAPTER TWO

2. LITERATURE REVIEWS

2.1 Free Radical Damage

The important role of free radicals in biological processes was investigated over a half century ago (Michaelis and Schubert, 1983). In 1983, a good summary of events involved in radical induced cell damage was provided by Willson which led to a clearer understanding of free radical's role in biological redox reaction (Rao et al., 2006). Oxidation reactions are one half of oxidation- reduction (redox) couples and it is important to bear in mind that every oxidation is accompanied by a reduction.

A free radical is a species that possesses one or more unpaired odd or single unpaired electrons. In other words, the term free radical could be attributed to any atom (e.g. oxygen, nitrogen) which possesses at least one unpaired electron in its outermost orbital, while being capable of independent existence. When a covalent bond between two atoms is broken, one electron remains with each newly formed atom and consequently, free radicals are formed (Young et al., 2001). They are highly reactive due to the presence of unpaired electron(s). When free radicals steal an electron from a surrounding compound or molecule a new free radical is formed in its place. The newly formed radical then tends to return to its ground state by stealing electrons with anti parallel spins from cellular structures or molecules. All living cells may contain some odd electron species. Lifetime of most of organic radicals is very short. Without stabilizing features such as steric hindrance at the odd-electron side and extensive delocalization of the odd electron, they decompose rapidly even in the absence of external agents (Rao et al., 2006).

Reactive oxygen species (ROS) is a term attributed to free radicals that involve oxygen. Reactive oxygen and nitrogen species are produced in the human body in both health and disease. In health, they may arise as regulatory mechanisms, intercellular signaling species, or as bactericidal agents (Halliwell, 2004). Table 2-1 shows some of active oxygen and other related species (Yanishlieva-Mashlarova, 2001).

Table 2-1 Active oxygen and related species

Radical	**Non-Radical**
$O_2^{\bullet -}$ Superoxide	H_2O_2 Hydrogen Peroxide
$HO^{\bullet}$ Hydroxyl radical	O_2 Singlet Oxygen
$HO_2^{\bullet}$ Hydroperoxyl radical	O_3 Ozone
$L^{\bullet}$ Lipid radical	LOOH Lipid Hydroperoxide
$LO_2^{\bullet}$ Lipid peroxyl radical	Fe(III) Iron–oxygen complexes
$LO^{\bullet}$ Lipid Alkoxyl radical	HOCl Hypochlorite
$NO_2^{\bullet}$ Nitrogen dioxide	
$^{\bullet}NO$ Nitric oxide	
$RS^{\bullet}$ Thiyl radical	
$P^{\bullet}$ Protein radical	

Although free radicals help the immune system by fighting against invading bacteria and viruses, excess amounts of them are harmful due to their reactivity. Free radicals can damage lipids, proteins, and DNA (Halliwell and Gutteridge, 1999; Pratico and Delanty, 2000). As a consequence, they may alter biochemical compounds, corrode cell membrane and deteriorate cells directly and completely. Rising evidences show that they play a key role in the development of many diseases, such as cancer, heart diseases, cataracts and aging in general (Medicine, 2000). All cells have the capability to fight against oxidative damage by repairing them, however, presence of excess free radicals leads to cell death. The importance of free radicals to cause significant damages is owing to their ability in starting chain reactions. As reported recently, higher levels of oxidative damage to DNA, proteins and lipids have been vastly reported by using a wide range of biomarkers in central nervous system tissue from patients who suffered and died from Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and Huntington's disease (Giasson et al., 2000; Halliwell, 2001).

Intracellular enzymes e.g. glutathione peroxidaze and superoxide dismutase and low molecular-mass compounds such as vitamin E or ascorbic acid are examples of antioxidant defense mechanisms in vivo which can normally control production of free radicals. However, it is impossible to stop some steady-state basal oxidative in all individuals (Halliwell et al., 2004).

In view of the growing body of data on the role of oxidative stress in aging, scientists have initially focused much anti-aging research on attempts to reduce oxidative stress. One of the most widely studied ways to decrease oxidative stress is antioxidant intervention.

2.2 Definition and Importance of Antioxidants

According to a definition by Wills (1980) antioxidants are "substances that in small quantities are able to prevent or greatly retard the oxidation of easily oxidisable materials such as fats". Halliwell (2004) defined the term antioxidant as "any substance that when present in low concentrations compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substance".

This definition covers all oxidizable substrates such as lipids, proteins, DNA and carbohydrates. Nevertheless, antioxidant activity is not confined to any specific group of chemical compounds nor is referred to any particular mechanism of action. It is not easy to classify specific molecules as antioxidants. A recent critical paper outlined the complexity of this classification for the in vivo situation (Azzi et al., 2004). In foods and beverages, antioxidants may be related to the protection of specific oxidation substrates or the formation of specific oxidation products for which threshold values may be defined for different products (Azzi et al., 2004).

Antioxidants can be widely found in the nature and constitute an extremely diversified group of molecules. As stated earlier, antioxidants have many vital functions in cells and many beneficial influences when present in foods. They can significantly help the prevention of degenerative illnesses e.g. different types of cancers, cardiovascular and neurological diseases, cataracts, and oxidative stress dis-functions (Halliwell, 2004; Young et al., 2001). Vitamin E, a natural antioxidant shows anti-carcinogenic properties because it prevents lipid oxidation and scavenges radicals (Rao et al., 2006). The importance of antioxidants in prevention of diseases and as health promoters has been widely investigated and studied. Thus, the demand for functional foods including food products which are supplemented with antioxidants is rising. Each year, more people realize the importance of a diet rich in antioxidants in prevention of diseases. They are now being considered as an important class among nutraceuticals. Their important function in food is increasing shelf-life by preventing lipid oxidation and therefore keeping food fresh for a longer time. Antioxidants (with or without chemical modification) could be incorporated into various food products such as oils, dairy, processed meat and other food products.

Recently the use of antioxidants in the food industry is significantly increased (Huang and Yang, 2006). They have been utilized in food industry not only as dietary supplements but also as shelf life promoters.

2.2.1 Requisite Characteristics for Effective Antioxidants

Antioxidants must have certain characteristics in order to be categorized as effective. These characteristics include a number of structural features:

1. The presence of hydrogen or electron-donating substituents with appropriate reduction potentials, in relation to those of the redox couples of the radicals to be scavenged (Mohammed et al., 2004)
2. The ability to delocalize the resulting radical, whether a phenoxyl radical such as those derived from α-tocopherol or butylated hydroxytoluene, an aryloxyl radical such as those derived from flavonoids, a polyunsaturated hydrocarbon chain radical such as β-caroten, or thiyl radical such as dihydrolipoic acid
3. The transition metal-chelating potential (Yu et al., 2002) dependent on the nature of the functional groups and their arrangement within the molecule.

Another important factor is accessibility of the antioxidant to the site of action which is defined by the lipophilicity or hydrophilicity of the antioxidant or the partition coefficient. For example, α-tocopherol is a much more effective chain breaking antioxidant in scavenging lipid peroxyl radicals than vitamin C Reviewed by Niki (1996).

Interaction of antioxidant radicals with other antioxidant molecules, which cause to spare the original antioxidant from depletion, is also counted as an important factor (Mohammed et al., 2004).

2.3 Antioxidant Defence System

Antioxidants work as radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist, and metal-chelating agents. Both enzymatic and non-enzymatic antioxidants exist in the intracellular and extracellular environment to detoxify reactive species (Rao et al., 2006). Below some functions of antioxidants are given.

2.3.1 Radical termination or inhibition

Antioxidants can terminate oxidation by scavenging free radicals at various steps of oxidation process. In this process, after electron or hydrogen transfer to free radicals an antioxidant becomes an antioxidant-derived radical. The antioxidant-derived radical could become stable, or could decay to a stable state. Similarly, the antioxidant activity of α-tocopherols (AH_2) in the lipid oxidation process is mainly based on the α-tocopherol / α-tocopheryl quinone redox system. α-Tocopherol (AH_2) acts as a radical scavenger during lipid auto-oxidation process, and quenches lipid and peroxyl radicals (L• and LOO•), thus slow down the chain propagation stage (Eq. 2.1). The quenching process may be expressed as below: (Fujisawa et al., 2006; Shahidi and Naczk, 1995)

$AH_2 + L\bullet \rightarrow LH + AH\bullet$ (Eq. 2.1a)

$AH_2 + LOO\bullet \rightarrow LOOH + AH\bullet$ (Eq. 2.1b)

After releasing a hydrogen atom, α-tocopherol radical (AH•) releases another hydrogen atom and produce methyl tocopherol quinine, which is unstable and thus gives rise to tocopheryl quinone (A) as a more stable product. Two tocopherol radicals may form a molecule of α-tocopheryl quinone and a regenerated tocopherol (Eq. 2.2) (Fujisawa et al., 2006).

$AH\bullet + AH\bullet \rightarrow A + AH_2$ (Eq. 2.2)

α-Tocopheryl semiquinone radical (AH•) and α-tocopheryl quinone (A) could also be recovered by using antioxidants such as ascorbate, urate and ubiquinol. Polyphenols are very active in this respect and the radical-scavenging activities of gallates, nordihydroguairetic acid and flavonoids arise from this process. (Yanishlieva-Maslarova, 2001)

2.3.2 Enzymatic Antioxidant Activities

Some enzymes have the ability to catalyze highly reactive free radicals to more stable species. For example, superoxide dismutase (SOD) enzyme catalyzes superoxide radicals to produce hydrogen peroxide and ground-state oxygen. SOD antioxidant mechanisms might be attributed to the complexation of metal ions and SOD. For example, the catalytic ability of Cu-Zn-SOD could be explained by the following reaction (Halliwell and Gutteridge, 1999):

$\text{Enzyme-Cu}^{2+} + O_2\bullet^- \rightarrow \text{Enzyme-Cu}^{+} + O_2$ (Eq. 2.3)

$\text{Enzyme-Cu}^{+} + O_2\bullet^- + 2H^+ \rightarrow \text{Enzyme-Cu}^{2+} + H_2O_2$ (Eq. 2.4)

Net reaction: $O_2\bullet^- + O_2\bullet^- + 2H^+ \rightarrow H_2O_2 + O_2$ (Eq. 2.5)

Hydrogen peroxide is usually removed in aerobes by two types of catalases and peroxidase enzymes.

Catalase directly catalyses the decomposition of H_2O_2 to ground-state oxygen (Eq.2.6), and peroxidase enzymes remove H_2O_2 by using it to oxidize substrate (SH_2) (Eq. 2.7) (Halliwell et al., 2004).

$$2H_2O_2 \xrightarrow{\text{Catalase}} H_2O + O_2 \qquad \text{(Eq. 2.6)}$$

$$H_2O_2 + SH_2 \xrightarrow{\text{Peroxidase}} 2H_2O + S \qquad \text{(Eq. 2.7)}$$

Glutathione peroxidase (GPX) family removes H_2O_2 by coupling its reduction to H_2O with oxidation of reduced glutathione, GSH (Eq.2.8).

$$H_2O_2 + 2\,GSH \xrightarrow{\text{GPX}} GSSG + 2\,H_2O \qquad \text{(Eq. 2.8)}$$

GPX enzymes are specific for GSH as a hydrogen donor. They can also act on peroxides other than H_2O_2 (Halliwell and Gutteridge, 1999). (Eq. 2.9)

$$LOOH + 2\,GSH \xrightarrow{\text{GPX}} GSSG + H_2O + LOH \qquad \text{(Eq. 2.9)}$$

2.3.3 Sequestering Agents

Metals such as iron and copper are known to play an important role in the human health since they synthesize a large number of enzymes and proteins.

Nevertheless, metal ions could be potentially harmful for body health since they can catalyse the auto-oxidation reaction, convert H_2O_2 to HO• and decompose lipid peroxides to reactive peroxyl and alkoxy radicals. Some compounds such as citric acid, amino acids and phosphates exhibit little or no antioxidant activity, but they can chelate metal ions and therefore enhance the activity of other antioxidants (Eriksson and Na, 1993).

2.3.4 Oxygen Scavengers

Compounds that can react with oxygen and remove oxygen in a closed system such as ascorbyl palmatate, sulphites, erythorbic acids and ascorbic acid can be widely used as oxygen scavengers.

2.3.5 Singlet Oxygen Quenchers

Carotenoids such as β-carotene are a good example of strong singlet oxygen quenchers. Their ability of quenching singlet oxygen, their chemical reactivity towards free radicals and instability towards oxidation could be justified basically by their chemical structure which is rich in conjugated double bonds (Britton, 1995; Krinsky, 1994). They can convert singlet oxygen to more stable ground-state oxygen through physical process quenchers (Eq. 2.10 and 2.11).

$^1\beta\text{-carotene} + {}^1O_2^* \longrightarrow {}^3\beta\text{-carotene}^* + {}^3O_2$ (Eq. 2.10)

$^3\beta\text{-carotene}^* \longrightarrow {}^1\beta\text{-carotene} + \text{heat}$ (Eq. 2.11)

Chemical quenching is considered as a very minor side reaction against $^1O_2$ and thus, carotenoids cannot contribute in protection against $^1O_2$ significantly (Edge et al., 1997).

2.4 Different Class of Antioxidants

Based on the mechanism of actions, antioxidants are classified into primary, secondary and synergistic antioxidants.

2.4.1 Primary/Chain Breaking Antioxidants

Chain breaking or primary antioxidants are able to scavenge radicals, inhibit chain initiation, and break chain propagations (Niki, 1996). They can donate an electron or a hydrogen atom to free radicals and react directly with them. They can effectively prevent oxidation even when present in low concentrations. Natural phenolic compounds such as eugenol, vanillin, rosemary, and some vitamins such as vitamin C and E are categorized under this class of antioxidants (Niki, 1996; Rajalakshmi and Narasimhan, 1996). These antioxidants are able to react with ROS either by single electron transfer or hydrogen atom transfer (Ou et al., 2002).

Some anti-oxidative compounds with chain breaking properties are naturally available. In spite of being in the same category, they have different mechanism of reaction with radicals and thus, different oxidation products. Some of the important natural antioxidants, their reaction mechanisms and their oxidation products are discussed below.

Vitamin E (α-TOH), present in phospholipid bilayers of cells play an efficient role as an antioxidant. It scavenges lipid peroxy radicals (LOO•) by hydrogen atom transfer (Equation 2.12) (Chaudiere and Ferrari-iliou, 1999).

α-TOH + LOO• $\longrightarrow$ α-TO• + LOOH (Equation 2.12)

The oxidation of α-TOH leads to the formation of tocopheryl radical (α-TO•**)** which can be stabilized by aromatic delocalization. After further oxidation α-TO• produces α-tocopheryl quinone as shown in Figure 2.1 (Chaudiere and Ferrari-iliou, 1999).

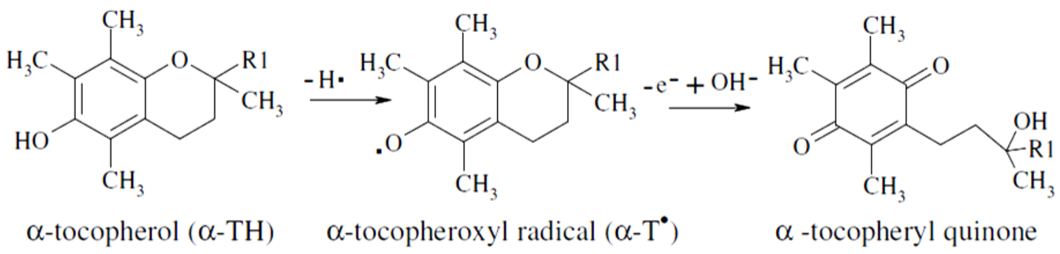

Fig 2.1 Chemical structures of α-tocopherol and its oxidation products

Phenolic compounds such as catechol act as antioxidants by donating an electron to radical cation, forming semi-quinone which can further donate an electron to form quinine (Pannala *et al*., 2001) (Figure 2.2).

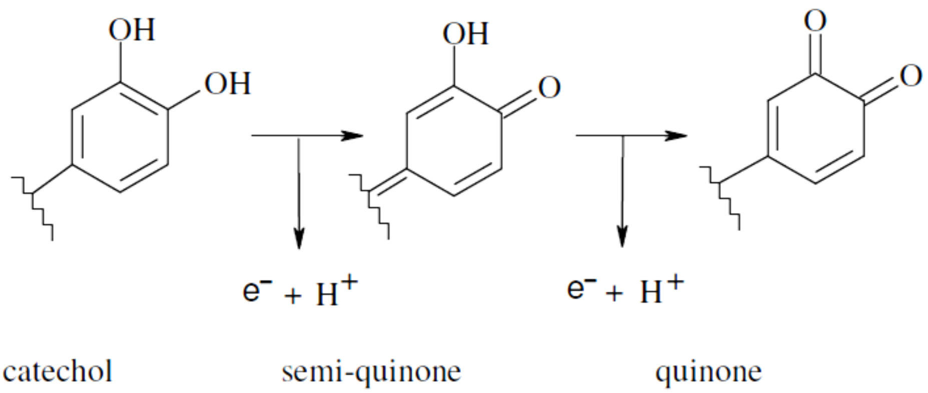

Figure 2.2 Major oxidation products of catechols

β-carotene can act as an antioxidant by following two pathways. In the first pathway it donates an electron to a radical to form a cation radical (β-carotene$\bullet^{+}$) and in the second it involves in direct free radical addition to it to form an adduct [β-carotene (OOR)] (Figure 2.3) (Everett et al., 1996).

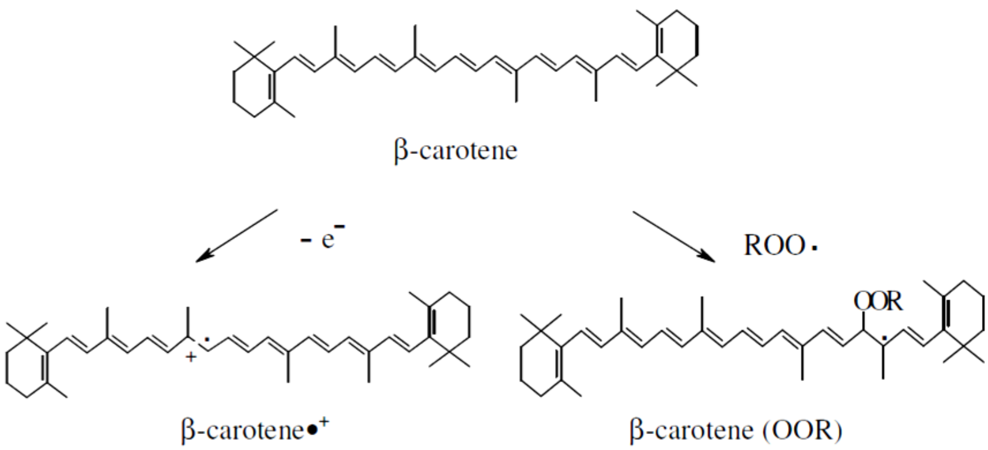

Figure 2.3 Structures of β-carotene, its cation radical and lipid peroxy adduct

2.4.2 Secondary/Preventive Antioxidants

Secondary or preventive antioxidants suppress the generation of free radicals (Niki, 1996). They can react with lipid peroxides through reduction or hydrogen donation and convert them into stable end products such as alcohols. Sulfur, thiols, sulfides, and disulphides act as preventive antioxidants by inhibiting autoxidation. Thiols (RSH) such as cysteine and gluthathione, sulphides (R-S-R) such as methionine and 3,3′-thiodipropionic acid and free amine groups of proteins ($R\text{-}NH_2$) react with lipid peroxides (LOOH) and form stable products as given by Equations 2.13 to Equation 2.15 (Yanishlieva-Mashlarova, 2001).

$RSH + LOOH \rightarrow R\text{-}S\text{-}S\text{-}R + LOH + H_2O$ (Eq. 2.13)

$R\text{-}S\text{-}R + LOOH \rightarrow R\text{-}SO\text{-}R + LOH$ (Eq.2.14)

$R\text{-}NH_2 + LOOH \rightarrow R\text{-}N\,(OH)\,L + H_2O$ (Eq. 2.15)

Carotenoids such as β-carotene, lycopene, zeaxanthin, lutein, and canthaxanthin can quench singlet oxygen (Halliwell, 2004).

The process involves energy transfer from singlet oxygen ($^1O_2$) to carotenoid molecule (Car) resulting in the formation of triplet state carotenoid ($^3Car^*$) which will revert to its original state as it can transfer excess energy to the solvent (Eq. 2.16 and Eq. 2.17) (Fujisawa et al., 2006).

$^1O_2 + Car \rightarrow {}^3O_2 + {}^3Car^*$ (Eq. 2.16)

$^3Car^* \rightarrow Car + heat$ (Eq. 2.17)

Preventive antioxidants (class 2) are different from chain-breaking antioxidants (class 1) in which they can form stable products by reacting with lipid hydroperoxides and thus inhibiting lipid hydroperoxides from further decomposing into peroxy or alkoxy or hydroxy radicals. However, chain-breaking antioxidants react with radicals by donating an electron or hydrogen atom to reduce them. In other words, opposite to chain breaking class, preventive antioxidants are not involved in reaction with radicals or donation of electrons.

2.4.3 Synergistic Antioxidants

Synergistic antioxidants are those compounds that can associate with the reactivation of primary antioxidants, or may inhibit lipid peroxidations, and thus, maintain primary antioxidants active and prevent them from depletion.

For instance, ascorbic acid can donate a proton and regenerate tocopherols. The metal ions initiate the formation of radicals that are responsible for the chain reactions in lipids. The metal chelators like citric and phytic acids (inositol hexaphosphate) form a stable complex with metal ions. The chelated metal ions no longer can exhibit pro-oxidant properties. Therefore, metal chelators can control the homolytic cleavage of hydroperoxides that produce radicals (Yanishlieva-Mashlarova, 2001).

Free radicals oxidize vitamin to vitamin E semiquinone, while ascorbic acid can reduce it back to vitamin E (May et al., 1998; May et al., 1997).

2.5 Classification of Antioxidants Based on their Sources

Antioxidants can generally be categorized into synthetic or natural antioxidants. Synthetic antioxidants are those which can be produced synthetically in the laboratory. Natural antioxidants are extracted from plant and animal sources.

2.5.1 Synthetic Antioxidants

Synthetic antioxidants are mainly phenolic compounds and can be prepared synthetically in the laboratory. Therefore, their mechanism of reaction with radicals is similar with that of phenolic antioxidant compounds. For example, they can act as chain breaking antioxidants. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) can delocalize the electrons and stabilize after they donate a hydrogen atom. By donating electros and protons, they can form stable quinones.

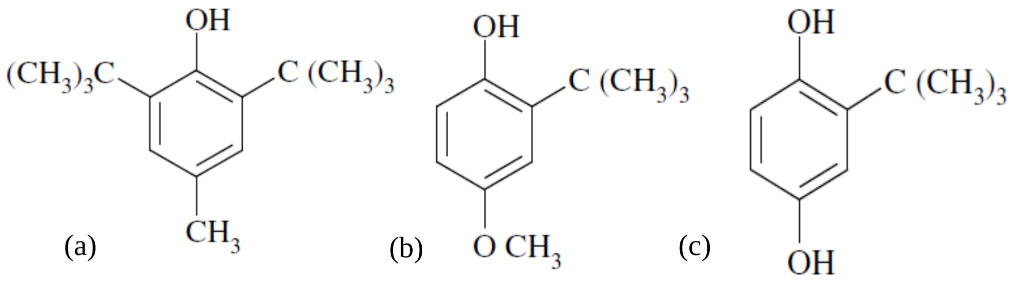

Fig 2.4 Structure of (a) BHT and (b) BHA and (c) tertiary butyl hydroquinone

Figure 2.4 shows some of the common synthetic antioxidants i.e. butylated hydroxyanisole, butylated hydroxytoluene, and tert-butyl hydroquinone (Devi et al., 2008; Rajalakshmi and Narasimhan, 1996).

Synthetic antioxidants are being used routinely along with natural food antioxidants in foods and medicine especially those containing oils and fats to protect the food against oxidation. Although synthetic phenolic antioxidants (such as BHT and BHA) have been widely used as antioxidants in food, cosmetics, and therapeutic industries, due to some undesirable physical properties such as their high volatility and instability at high temperature, carcinogenic risks, and consumer preferences, they seem no longer popular and the attention of consumers turned from synthetic to natural antioxidants (Papas, 1999). This has been caused an increasing trend towards the utilization of natural compounds present in plants as antioxidants.

2.5.2 Natural antioxidants

The term natural antioxidants is attributed to substances which occur in the nature and can be extracted from plant or animal tissues. This definition includes also those substances which may be formed as a consequence of cooking or processing plant or animal components (Pokorny et al., 2001). The antioxidants present in cells such as superoxide dismutase, enzymes that metabolize reactive oxygen species, superoxide reductase that catalyzes direct reduction of superoxide, catalases that catalyze dismutation of hydrogen peroxide to water and molecular oxygen, glutathione-related systems, selenium compounds, lipoic acid, and ubiquinones are other examples of naturally occurring antioxidants.

Organic acids, such as citric acid and phytic acid act as chelating agents by binding metal atoms and prevent them from initiating radicals. Table 2.2 shows some naturally occurring antioxidants and their sources.

Table 2.2 Some natural antioxidants and their sources (Pokorny 2007)

Natural Antioxidants	**Sources**
Tocopherols, tocotrienols, sesamol, phospholipids, olive oil resins	Oils and oils seeds
Several lignin-derived compounds	Oats and rice bran
Ascorbic acid, hydroxycarboxylic acids, flavonoids, carotenoids	Fruits and vegetables
Phenolic compounds	Spices, herbs, tea, cocoa
Amino acids, dihydropyridines, Maillard reaction products	Proteins and protein hydrolysates
Catechin, Epicatechin, Myricetin, Quercetin, Kaempferol	Teas

Natural antioxidants can be found in almost all plants, microorganisms, fungi, and even in animal tissues (Pokorny, 2001). Some examples of natural antioxidants taken from plants include rosemary (ORAC value 1,652 per g db), oregano (ORAC value 1,752 per g db), sage (ORAC value: 1199 per g db), thyme (ORAC value 1,573 per g db), bilberries and etc (Akhtar et al., 1998) (Figure 2.5).

The antioxidative compounds and properties of natural extracts high in antioxidants are being widely studied in West countries and archived in the USDA data base (USDA, 2010).

Fig. 2.5 (a) Oregano, (b) Rosemary, (c) Thyme, (d) Sage

The most important groups of natural antioxidants are believed to be tocopherols, flavonoids and phenolic acids (Potterat, 1997).

Phenolic compounds are deemed to be the most dominant contributor of antioxidant activity in plant extracts owing to their high concentration compared to other phytochemicals (Hodzic et al., 2009), their redox property and interaction of an individual or combination of their diverse chemical structures with assays used (Teixeira et al., 2005) and their synergistic characteristic as hydrogen donors, reducing agents and free radical scavengers (Vattem et al., 2005; Zhou et al., 2009). Phenolic antioxidant's intake is generally from plants and vegetables, fruits and drinks such as tea.

2.5.2.1 Phenolic Compounds

Phenolic compounds possess an aromatic ring with one or more hydroxyl substituent. The phenolic hydroxyl group is known to be the main structure which is responsible for the antioxidant and radical-scavenging activities of the phenolic derivatives (Lazarus et al., 2001).

Phenolics are able to donate the hydrogen atom of phenolic hydroxyl to the free radicals easily and consequently halt the propagation chain reactions during the oxidation process. The resonance delocalization in phenoxyl radicals make them act as stable phenoxyl radicals and this stability is the main reason of phenolics being effective antioxidants.

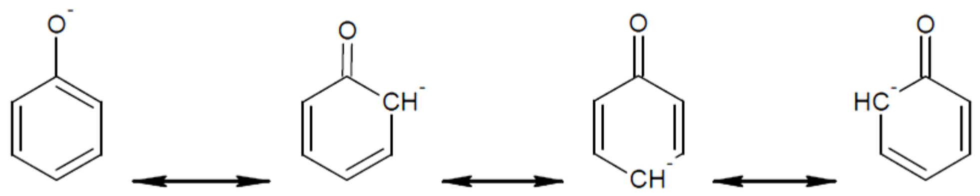

Fig. 2.6 Resonance stabilization of phenoxyl radical

Various substituents may affect the rate of free-radical scavenging and the phenolic capacity since it is directly related to the stability of the phenoxyl radical. A second hydroxyl group at the ortho-position of a catechol ring can reduce the O–H bond dissociation enthalpy and consequently increases the rate of H-atom transfer to radicals. Likewise, a third hydroxyl group in the phenolic ring can further improve the antioxidant capacity.

Another effective factor in antioxidative activity is the steric effect of substituent which may prevent phenoxyl radicals from coupling.