Chapter 6
Antioxidants

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6.1 Introduction

Antioxidants are the molecules that prevent cellular damage caused by oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from one molecule to an oxidizing agent. Oxidation reactions are known to produce free radicals. These free radicals are highly reactive species which contains one or more unpaired electrons in their outermost shell. Once they are formed, the chain reaction starts. Antioxidant reacts with these free radicals and terminates this chain reaction by removing free radical intermediates and inhibits other oxidation reactions by oxidizing themselves.

Though oxidation reactions are crucial for life, they can also be damaging. Plants and animals have a complex system of multiple types of antioxidants, such as vitamin C and vitamin E, as well as enzymes, such as catalase (CAT), superoxide dismutase (SOD), and various peroxidases (Hamid et al. 2010). Oxidative stress plays a key role in causing various human diseases, such as cellular necrosis, cardiovascular disease, cancer, neurological disorder, Parkinson's dementia, Alzheimer's disease, inflammatory disease, muscular dystrophy, liver disorder, and even aging (Amit and Priyadarsini

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2011). Besides, there are some antioxidants in the form of micronutrients which cannot be manufactured by the body itself such as vitamin E, β-carotene, and vitamin C, and hence these must be supplemented in the normal diet (Teresa et al. 2011).

Antioxidants can also act as prooxidants when these are not present at the right place at the right concentration at the right time (Touriño et al. 2008). The relative importance of the antioxidant and prooxidant activities is not yet explored fully and needs further research.

In this chapter, authors have tried to discuss the various types, sources, synthesis, uses, and protective efficacy of antioxidant with examples.

6.2 Classification of Antioxidants

Antioxidants can be classified into two major types based on their source, i.e., natural and synthetic antioxidants (schematic representation of the classification of antioxidants is shown in Fig. 6.1).

6.2.1 Natural Antioxidants

Natural antioxidants either are synthesized in human body through metabolic process or are supplemented from other natural sources, and their activity very much depends upon their physical and chemical properties and mechanism of action. This can be further divided into two categories, i.e., enzymatic antioxidants and nonenzymatic antioxidants.

6.2.1.1 Enzymatic Antioxidants

Enzymatic antioxidants are uniquely produced in the human body and can be subdivided into primary and secondary antioxidant.

Primary Antioxidants

Primary antioxidants mainly include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) as described below.

Superoxide Dismutase Superoxide dismutase (SOD) enzyme is found in both the dermis and the epidermis. It removes the superoxide radical ($O_2^{\cdot-}$) and repairs the body cells damaged by free radical. SOD catalyzes the reduction of superoxide anions to hydrogen peroxide (6.1). SOD is also known to compete with nitric oxide (NO) for superoxide anion, which inactivates NO to form peroxynitrite. Therefore, by scavenging superoxide anions, it promotes the activity of NO (Chakraborty et al. 2009).

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \tag{6.1}$$

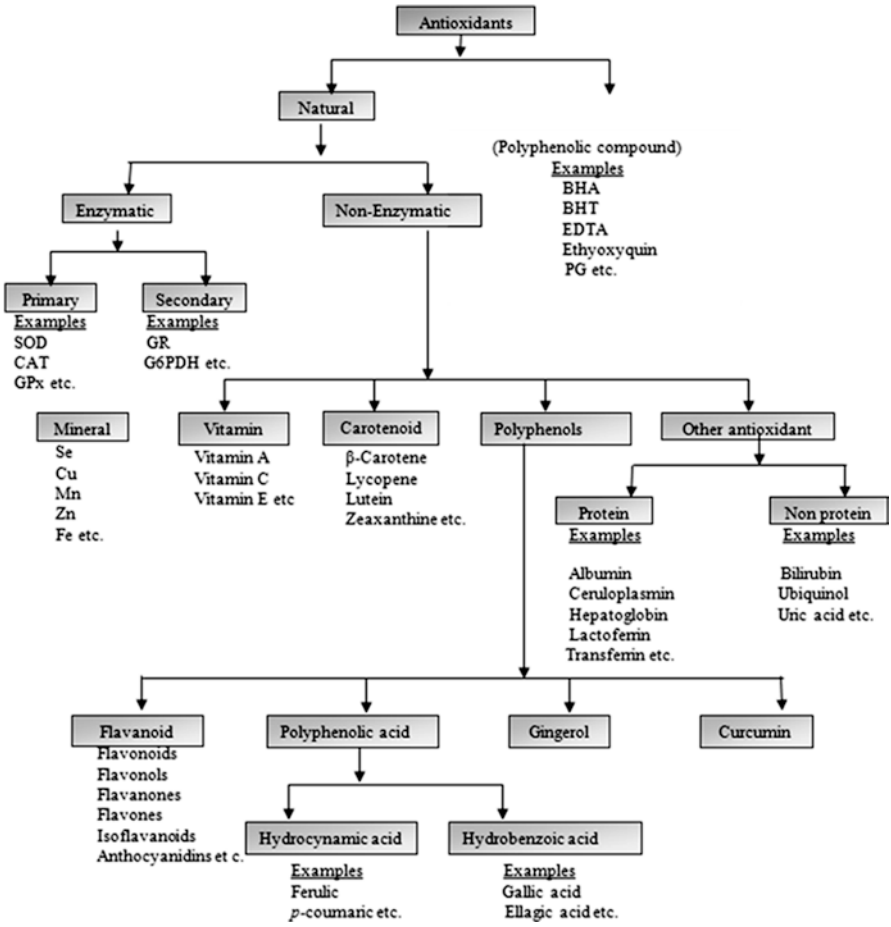

Fig. 6.1 Schematic representation of classification of antioxidants

Catalase Catalase enzyme (CAT) is found in the blood and most of the living cells and decomposes H_2O_2 into water and oxygen (6.2). Catalase with glucose peroxidase is also used commercially for the preservation of the fruit juices, cream consisting of egg yolk, and salad by removing the oxygen (Chakraborty et al. 2009).

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2 \tag{6.2}$$

Glutathione Peroxidase Glutathione peroxidase (GPx) is a group of selenium-dependent enzymes, and it consists of cytosolic, plasma, phospholipid hydroperoxide, and gastrointestinal glutathione peroxidase (Chakraborty et al. 2009). GPx (cellular and plasma) catalyzes the reaction of H_2O_2 by reduced glutathione (GSH); as a

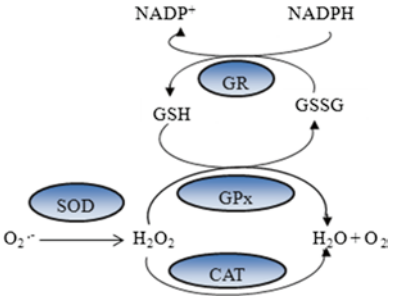

Fig. 6.2 Outline of the mechanism of enzymatic antioxidants in the removal of free radical

result, oxidized glutathione (GSSG) is produced (6.3) and it is again recycled to its reduced form by glutathione reductase (GR) and reduced nicotinamide adenine dinucleotide phosphate (NADPH).

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O \tag{6.3}$$

Secondary Antioxidant

Secondary antioxidant includes glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH). G6PDH generates NADPH. GR is required to recycle the reduced glutathione (GSH) using secondary enzyme GR and NADPH (6.4).

$$GSSG + NADPH \xrightarrow{GR} NADP + 2GSH \tag{6.4}$$

Glutathione is a cysteine containing peptide-type antioxidant and is synthesized in the body cells. The thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized and reduced. A high level of glutathione is found in the cells (~3,100 μg/g of tissue) (Hissin and Hilf 1976), maintained in the reduced form (GSH) by the enzyme GR, and in turn reduces other metabolites and enzyme systems, such as ascorbate. Due to its high concentration and its role in maintaining redox state in the cells, it is considered one of the most important cellular antioxidants. (Outline of the mechanism of enzymatic antioxidants in the removal of free radical is shown in Fig. 6.2.)

6.2.1.2 Nonenzymatic Antioxidants

They are a class of the antioxidants which are not found in the body naturally but are required to be supplemented for the proper metabolism (Raygani et al. 2007). Some of the known nonenzymatic antioxidants are minerals, vitamins, carotenoids, polyphenols, and other antioxidants as listed below.

Minerals

Minerals are required in the body cells for the proper functioning of the enzymes. Their absence is known to affect the metabolism of many macromolecules. They include selenium, copper, iron, zinc, and manganese. They act as cofactors for the enzymatic antioxidants.

Iron (Fe) Iron is the most abundant trace metal found to bound with protein in the biological system. Normally the concentration of free iron is very low and the low concentrations of iron-binding proteins promote ROS production, lipid peroxidation, and oxidative stress (Dabbagh et al. 1984). Hence iron supplementation helps in reducing the oxidative stress.

Magnesium (Mg) Magnesium is a cofactor for glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) involved in pentose cycle which catalyzes the production of NADPH from NADP during the glucose metabolism and hence maintains the normal ratio of GSH to GSSG and a normal redox state in cells. Deficiency of magnesium reduces GR activity and GSSG does not reduce to GSH, hence causing oxidative damage to the cells (Fang et al. 2002).

Selenium (Se) Selenium is also a very important component of enzymatic antioxidant. In the presence of selenium (Se), glutathione peroxidase (GPx) plays a protective role against oxidation of lipid and protects the cell membrane and takes part in H_2O_2 and lipids' hydroxyperoxide metabolism. Hence, Se behaves like vitamin E and can be substituted in place of vitamin E and is used to prevent the risk of cancer and cardiovascular diseases (Sikora et al. 2008).

Copper (Cu), Zinc (Zn), and Manganese (Mn) SOD is a class of enzyme that consists of different types of SODs, depending upon their metal cofactor such as Cu–Zn and Mn. Cu–Zn SOD is found in the cytosol having Cu and Zn at their active sites which helps in proton conduction, whereas Mn-SOD is found in mitochondria and has Mn at its active site. These metals are responsible for SOD's antioxidant activities.

Vitamins

Vitamins form the class of micronutrients required for the proper functioning of the body's antioxidant enzyme system, such as vitamin A, vitamin C, vitamin E, and vitamin B. They cannot be synthesized in our body and hence need to be supplemented in the diet.

Vitamin A Vitamin A is helpful in night vision and in maintenance of epithelial cells in mucus membranes and skin. Because of its antioxidant properties, it assists immune system also and is found in three main forms: retinol, 3,4-didehydroretinol, and 3-hydroxyretinol. The main sources of this include sweet potatoes, carrots, milk, egg yolks, and mozzarella cheese.

Vitamin C Vitamin C is water soluble and is also called as ascorbic acid. It is found in fruits (mainly citrus), vegetables, cereals, beef, poultry, fish, etc. It is helpful in

preventing some of the DNA damage caused by free radicals, which may contribute to the aging process and the development of diseases, such as cancer, heart disease, and arthritis.

Vitamin E Vitamin E is a lipid-soluble vitamin. This consists of eight different forms such as α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol. Most abundantly found in almonds, safflower oil, soybean oils, oil of wheat germs, nuts, broccoli, fish oil, etc., α-tocopherol possesses highest bioavailability and is the most important lipid-soluble antioxidant which reacts with the lipid radical and protects the membranes from lipid peroxidation; as a result, oxidized α-tocopheroxyl radicals are produced that can be recycled to the reduced form through reduction by other antioxidants, such as ascorbate and retinol.

Carotenoid

Carotenoid consists of β-carotene, lycopene, lutein, and zeaxanthin. They are fat-soluble colored compounds found in fruits and vegetables. β-Carotene is found mostly in radish-orange-green color food items including carrots, sweet potatoes, apricots, pumpkin, mangoes, and cantaloupe along with some green and leafy vegetables, including collard greens, spinach, and kale. Lutein is abundant in green leafy vegetables such as collard greens, spinach, and kale (Hamid et al. 2010). Lutein is best known for its role in protection of retina against harmful action of free radicals and also prevents atherosclerosis (Sikora et al. 2008).

Although lycopene, lutein, canthaxanthin, and zeaxanthin do not possess provitamin A activity, β-carotene is known as a precursor for vitamin A (Fang et al. 2002). Tomato is a good source of lycopene and spinach is a good source of zeaxanthin. It has been shown that lycopene is a potent antioxidant and is the most effective compound in removing singlet oxygen found in tomatoes, watermelon, guava, papaya, apricots, pink grapefruit, and other foods.

Polyphenols

Polyphenols is a class of the phytochemicals that possess marked antioxidant activities. Their antioxidant activities depend on their chemical and physical properties which in turn regulates the metabolism depending on their molecular structures (Ajila et al. 2011). These consist of phenolic acids, flavonoids, gingerol, curcumin, etc. (Amit and Priyadarsini 2011).

Flavonoid is a major class of polyphenolic compound and is mostly found in vegetables, fruits, grains, seeds, leaves, flower, bark, etc. Some of the spices, such as ginger and turmeric, are also good sources of polyphenolic compound, e.g., gingerol is obtained from the rhizomes of ginger, whereas curcumin (diferuloylmethane) is the main bioactive component of turmeric and is known to possess good antioxidant activity. Curcumin is an excellent scavenger of ROS, such as $O_{2^{\cdot}}^{-}$ radicals, lipid peroxyl radicals ($LO_{2^{\cdot}}$), OH radicals, and nitrogen dioxide ($NO_{2^{\cdot}}$)

radicals, which induced oxidative stress. Curcumin has been shown to inhibit lipid peroxidation and has been shown to increase GSH levels also in epithelial cells which lead to lower ROS production (Biswas et al. 2005).

Other Antioxidants

Transition Metal-Binding Proteins Albumin, ceruloplasmin, hepatoglobin, and transferrin are the transition metal-binding proteins found in human plasma, bind with transition metals, and control the production of metal catalyzed free radicals. Albumin and ceruloplasmin are the copper ion sequesters, hepatoglobin is hemoglobin sequester, and transferrin acts as free iron sequester.

Nonprotein Antioxidants Bilirubin, uric acids, and ubiquinol are nonprotein antioxidants which inhibit the oxidation processes by scavenging free radicals (Papas 1998).

Bilirubin Bilirubin is an end product of heme catabolism. It is a lipid-soluble cytotoxic product that needs to be excreted. However, bilirubin efficiently scavenges peroxyl radical at micromolar concentrations in in vitro model (Stocker et al. 1987) and is regarded as the best antioxidant against lipid peroxidation.

Uric Acid Uric acid is a powerful antioxidant and is a scavenger of singlet oxygen and radicals. Urate reduces the oxo-heme oxidant formed by peroxide reaction with hemoglobin and protects erythrocytes from peroxidative damage. The plasma-urate levels in humans are about 300 μM, making it one of the major antioxidants in humans (Ames et al. 1981).

Coenzyme Q Coenzyme Q is also known as ubiquinol (Co Q) and is an oil-soluble antioxidant. This is produced in the body through monovalent pathway, in heart, liver, kidney, pancreas, etc. The mechanism of the action may occur in two ways:

In the first mechanism, reduced form of ubiquinol (CoQH) acts as chain-breaking antioxidant and reduces peroxyl ($ROO^{\cdot}$) and alcoxyl radicals ($LO^{\cdot}$) (Papas 1998) (6.5 and 6.6).

$$CoQH + ROO^{\cdot} \rightarrow Q^{\cdot} + ROOH \tag{6.5}$$

In the second mechanism, it reacts with vitamin E radical ($TO^{\cdot}$) and regenerating vitamin E.

$$CoQH + TO^{\cdot} \rightarrow Q^{\cdot} + ROOH \tag{6.6}$$

6.2.2 Synthetic Antioxidants

Synthetic antioxidants are artificially produced or synthesized using various techniques. Generally, they are polyphenolic compounds mainly that capture the free

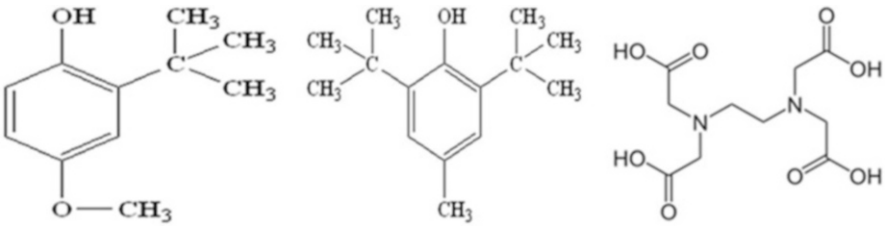

Fig. 6.3 (1) BHA, (2) BHT, (3) EDTA, (4) Ethoxyquin, (5) PG, (6) TBHQ

radicals and stop the chain reactions. Polyphenolic derivatives usually contain more than one hydroxyl or methoxy group. Ethoxy quinine is the only heterocyclic, N-containing compound reported to be used as antioxidant in the food, especially animal feed. Mostly reported synthetic phenolic antioxidants are *p*-substituted, whereas the natural phenolic compounds are mostly *o*-substituted. The *p-substituted* substances are preferred because of their lower toxicity. Synthetic phenolic antioxidants are always substituted with alkyl groups to improve their solubility in fats and oils and to reduce their toxicity. These synthetic compounds possessing antioxidant activity are commonly used in pharmaceuticals, as preservatives for cosmetics and to stabilize the fat, oil, and lipid in food (Gupta and Sharma 2006).

These new findings about the synthetic antioxidants have led the researches to develop new synthetic antioxidants in terms of their water solubility, stability, and non-toxicity. Characteristics of some of the known synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethylenediaminetetraacetic acid (EDTA), 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (ethoxyquin), propyl gallate (PG), and tertiary butylhydroquinone (TBHQ), are given below (Hamid et al. 2010) (structures of these antioxidants are shown in Fig. 6.3).

6.2.2.1 BHA

It is a monophenolic, lipid-soluble antioxidant, better used for the lipid oxidation in animal fat compared to vegetable oil.

6.2.2.2 BHT

It is also a monophenolic fat-soluble antioxidant but is more stable than BHA at high temperature, and both act synergistically. Many commercially available antioxidant formulations contain both of these antioxidants. BHA interacts with peroxy radicals to produce a BHA phenoxy radical which in turn may remove a hydrogen atom from the hydroxyl group of BHT. BHA is regenerated by the hydrogen radical provided by BHT. The BHT radicals so formed can react with a peroxy radical and act as a chain terminator.

6.2.2.3 EDTA

EDTA is a common sequestrant, water-soluble antioxidant added to foods, body care, and household products. It binds with trace minerals, such as copper, iron, and nickel, that may be present in the food product. If not inactivated, these minerals may lead to discoloration, rancidity, and textural breakdown. When added as an antioxidant, EDTA prevents oxygen from causing color changes and rancidity.

6.2.2.4 Ethoxyquin

It is as an antioxidant primarily used to protect carotenoid oxidation in animal feeds, vegetables, and fruits during storage.

6.2.2.5 PG

It is an ester formed by the condensation of gallic acid and propanol. It acts as an antioxidant which is used as a food additive to protect mainly oils and fat in the food products.

6.2.2.6 TBHQ

TBHQ is a highly effective diphenolic antioxidant. In foods, it is used as a preservative for unsaturated vegetable oils and many edible animal fats. It does not cause discoloration even in the presence of iron and does not even change flavor or odor of the material to which it is added. It is used industrially as a stabilizer to inhibit auto-polymerization of organic peroxides. It is also used as a corrosion inhibitor in biodiesel. In perfumery, it is used as a fixative to lower the evaporation rate and improve stability. It is also added to varnishes, lacquers, resins, and oil field additives. It can be used alone or in combination with BHA or BHT (Said et al. 2002).

6.3 Sources of Antioxidants

Antioxidants can be derived from two main sources: natural source such as fruits, vegetables, cereals, legumes, beverages, spices, and animals (Table 6.1) and from agro-industry, e.g., waste processing industry (Table 6.2).

Table 6.1 List of natural sources of antioxidants

Source	Name	Type of antioxidants	References
Fruits	Blackcurrant	Vitamin C, carotene (lutein and β-carotene), phenolic compound (anthocyanins), and phenolic acids (hydroxycinnamic acid)	Hägg et al. (1995a), Benvenuti et al. (2004a), Zadernowski et al. (2005a)
	Strawberry	Vitamin C and phenolic compound (anthocyanins and ellagic acid with its derivatives)	Hägg et al. (1995b), Anttonen and Karjalainen (2005)
	Grapes	*Seed*: gallic acid, catechins, and epicatechins *Peel*: ellagic acid, myricetin, quercetin, kaempferol, *trans-resveratrol*, anthocyanins, flavonols, etc.	Pastrana-Bonilla et al. (2003)
	Bilberries	Anthocyanin, vitamin C, carotenoids, etc.	Kähkönen et al. (1999)
	Cranberries	Anthocyanins (peonidin and cyanidin), flavanones, procyanidin, flavonols (quercetin and myricetin), derivatives of hydroxycinnamic acid, etc.	Kähkönen et al. (1999, 2001), Taruscio et al. (2004), and Määttä-Riihinen et al. (2004)
	Crowberry fruits	Vitamin C, carotenoids (lutein, β-carotene), and phenolic compounds	Kähkönen et al. (1999, 2001), Halvorsen et al. (2002), and Olsson et al. (2004)
	Blackberry	*Pulp*: anthocyanins, flavonols, and ellagic acid *Seed*: procyanidins and epicatechins	Benvenuti et al. (2004b), Siriwoharn et al. (2004), Reyes-Carmona et al. (2005), and Zadernowski et al. (2005b)
	Citrus food (lemons, oranges, grapefruits, etc.)	Vitamin C, polyphenolic compounds such as ferulic acid, *p*-coumaric acid, and caffeic acid	Gorinstein et al. (2001)
	Apple (peel contains seven times higher polyphenols than in pulp)	*Polyphenols*: epicatechin and its dimmer procyanidin B2 *Phenolic acid*: chlorogenic acid *Dihydrochalcones*: phlorizin, phloretin-2-xyloglucoside, etc.	Lu and Foo (1997, 2000)
	Cherries	Hydroxycinnamic acid and anthocyanins	Chaovanalikit and Wrolstad (2004)
	Plums, prunes, pears, kiwi	Hydroxycinnamic acid, catechins	Belitz and Grosch (1999), Yanishlieva-Maslarova and Heinonen (2001), and Mannach et al. (2004)
Vegetables	Tomatoes	Lycopene, quercetin, etc.	Stewart et al. (2000) and Knoblich et al. (2005)
	Onion	Flavonoids	Lachman et al. (2003)
	Parsley roots, carrot, and pumpkin	Vitamin C and β-carotene	Sikora et al. (2008)
	Parsley	Flavones	Beecher (2003)
	Brassica vegetables (white cabbage, kale, broccoli sprouts, or cauliflower)	Vitamin C, carotenoids, derivatives of hydrocinnamic acids such as chlorogenic acid, ferulic acid, and flavonols	Kurilich et al. (1999), Kopsell et al. (2004), and Vallejo et al. (2003)
	Spinach	Flavonoids, *p*-coumaric acid, etc.	Lomnitski et al. (2003)

Cereals and legumes	Wheat and rye	Phenolic acid: ferulic, vanillic, and *p*-coumaric acids	Yanishlieva-Maslarova and Heinonen (2001), and Mannach et al. (2004)
	Oats	Avertramidin (polyphenols of oats), catechins, etc.	Nie et al. (2006) and Peterson et al. (2001)
	Buckwheat	Polyphenols: rutin, catechins, etc.	Holasova et al. (2002)
	Rice	Anthocyanins, gallic acid, ferulic acids, etc.	Laokuldilok et al. (2011)
	Beans	Seed: flavanols	Mannach et al. (2004)
	Soybeans	Isoflavones and phenolic acid (*p*-hydroxybenzoic acid, salicylic acid, *p*-coumaric acid, and ferulic acid) along with tocopherol, sterols, phospholipids, etc.	Kim et al. (2006)
	Cocoa seeds	Procyanidins, quercetin and its glycosides, caffeic acid, etc.	Sikora et al. (2008) and Jolić et al. (2011)
Beverages: alcoholic drink	Red wine	Polyphenols: flavan-3-ols, flavanols, anthocyanins, etc.	Mannach et al. (2004) and Beecher (2003)
	Cider	Hydrocinnamic acid	Mannach et al. (2004)
	Beer	*Phenolic acid*: cinnamic, chlorogenic, vanillic, ferulic, and gallic acids *Flavan-3-ol*: catechin, epicatechin, procyanidin, prodelphinidin, etc.	Sikora et al. (2008) and Walters et al. (1997)
Other drinks	Orange juice	Flavanols etc.	Mannach et al. (2004)
	Tea	Catechins, theaflavins, etc.	Sikora et al. (2008) and Friedman et al. (2006)
	Coffee	Hydrocinnamic acid and chlorogenic acid	Sikora et al. (2008) and Mannach et al. (2004)
	Chocolate	Flavanols etc.	Mannach et al. (2004); Beecher (2003)
Spices	Ginger	Gingerol	Wang et al. (2003)
	Rosemary	Carnosol, carnosic acid, rosmanul, etc.	Senorans et al. (2000)
	Sage	Carnosol, carnosic acid, luteolin, rosmanul, rosmarinic acid, etc.	Yanishlieva-Maslarova and Heinonen (2001)
	Thyme	Thymol, flavonoids, luteolin, etc.	Zheng and Wang (2001) and Exarchou et al. (2002)
	Summer savory	Flavonoids, carnosol, carvacrol, etc.	Dimitrios (2006)
	Black pepper	Piperine	Srinivasan (2007)
	Red pepper	Vitamin C, luteolin, apigenin, etc.	Sikora et al. (2008) and Materska and Perucka (2005)
	Clove	Eugenol, gallic acid, etc.	Pathak et al. (2004)
Animal derived food	Red crabs	Carotenoid, astaxanthin, etc.	Pokorny et al. (2001)
	Blue crab	Canthaxanthin, 4-hydroxyechinenone, 3-hydroxycanthaxanthin, echinenone, isocryptoxanthin, β-carotene, and astaxanthin	Pokorny et al. (2001)
	Crustacea	Carotenoids etc.	Pokorny et al. (2001)

Table 6.2 List of source of antioxidants from agro industry (waste and processing industry)

Source	Antioxidant compounds	References
Liquid state culture of *Phanerochaete chrysosporium* ATCC 24275 with apple pomace sludge and synthetic medium	Polyphenolic compounds	Gassara et al. (2012)
Aspergillus niger NRRL 567 cultivated on apple pomace as a solid substrate	Citric acid	Dhillon et al. (2013)
Olive mill wastewater (OMW)	*Derivative of benzoic acid*: 4-hydroxybenzoic, protocatechuic, vanillic acids *Derivative hydroxycinnamic acid*: ferulic acid caffeic acids *Tyrosol*: 4-hydroxyphenethyl alcohol, homovanillyl alcohol: 4-hydroxy-3-methoxyphenethyl alcohol *Hydroxytyrosol*: 3,4-dihydroxyphenethyl alcohol	Federici et al. (2009)
Grape pomace	*Flavonol glycosides*: quercetin 3-*O*-glucoside and quercetin 3-*O*-glucuronide *Anthocyanin*: malvidin 3-*O*-glucoside *Methanolic extract*: flavonols, flavonols glucosides, flavanols and their gallate esters, anthocyanins, and low molecular weight proanthocyanins *Ethanolic extract*: triterpenes lupeol, oleanolic acid, flavonol quercetin, and daucosterol	Spatafora and Tringali (2012)
Tomato peel and seed by-products	*Peel byproduct as carotenoids*: lycopene, lutein, β-carotene, and *cis*-β-carotene *Seed byproduct as carotenoid*: lycopene and other carotenoids	Knoblich et al. (2005)

6.3.1 Natural Sources

6.3.2 Agro-industry

In recent past, agro industry is also found to be one of the major sources for the production of antioxidants. They can be derived either from the waste produced from the agro industries or as a by-product during the processing of the food material.

Food processing processes generally produce large amount of waste as well as by-products along with reasonable quantity of effluent. These food processing by-products, agro-industrial waste, and effluents typically consist of high amounts of proteins, sugars, and lipids along with specific organic compounds as well. Therefore, this could be used as a cheap and abundant source of fine chemicals and secondary metabolites. Valuable natural antioxidants, antimicrobial agents,

vitamins, etc., along with macromolecules, can be produced by pretreating the waste by physical and biological agents followed by tailored recovery procedures. Efficient pretreatment is necessary for the optimal recovery of the main classes of products. Here we have discussed a case study related to the agro-industrial waste management for the recovery of the natural antioxidants. Some of the known sources of the agro-industrial waste and the recovery vis-à-vis by-production of antioxidants are summarized in Table 6.2

6.3.2.1 Some Examples

There are very few scientific groups involved in this kind of studies worldwide. Some of the examples for such studies are briefly described below.

The use of liquid state culture of *Phanerochaete chrysosporium* ATCC 24275 for the production of polyphenolic compounds by employing apple pomace sludge and synthetic medium has been studied. Increased polyphenol content was observed by acetone extraction (383–720 mg Gallic Acid Equivalent/l) (GAE/l) during the fermentation of apple pomace and it is further increased by ~1.5-fold until 67 h of fermentation by ethanolic extraction (Gassara et al. 2012).

The bioproduction of citric acid and optimization of extraction from *Aspergillus niger* NRRL 567 cultivated on apple pomace as a solid substrate with inducers, ethanol and methanol, by rotating drum-type solid-state bioreactor has been carried out. Results showed that optimum conditions achieved for higher citric acid bioproduction (220.6 ± 13.9 g/kg dry solids, DS) were 3 % (v/v) methanol, intermittent agitation of 1 h after every 12 h at 2 rpm and 1 vvm (volume per volume per minute) of aeration rate and 120 h incubation time. Highest production of citric acid was of 294.19 g/kg DS (dry substrate) (Dhillon et al. 2013). In this study, scientists have reviewed the results of various studies carried out for the recovery of value-added products from the Olive mill wastewater (OMW). OMW is supposed to be a rich source of polyphenolic compounds, such as benzoic acid derivatives (4-hydroxybenzoic, protocatechuic, vanillic acids), hydroxycinnamic acid derivatives (ferulic acid, caffeic acids), tyrosol (4-hydroxyphenethyl alcohol), homovanillyl alcohol (4-hydroxy-3-methoxyphenethyl alcohol), and hydroxytyrosol (3,4-dihydroxyphenethyl alcohol). Methods have been optimized for the maximum yield of hydroxytyrosol (a polyphenols). The process for the recovery of these antioxidants involved filtration to eliminate the suspended solids followed by physicochemical processes, such as ultrafiltration, nanofiltration, and reverse osmosis. However, hydroxytyrosol is one of the main polyphenol recovered from OMW (Federici et al. 2009).

The antioxidant phenolic compounds were isolated and identified by HPLC, LC-MS, and flash chromatography in the fractions of methanolic and ethanolic extracts of destemmed grape pomace. The analytical studies showed the presence of the main flavonols, flavonol glucosides and their gallate esters, anthocyanins, and low molecular weight proanthocyanins. Five pyranoanthocyanins were also identified for the first time in grape pomace. Quercetin 3-*O*-glucoside and quercetin

3-*O*-glucuronide resulted the most abundant flavonol glycosides, and malvidin 3-*O*-glucoside is the main anthocyanin. Triterpenes lupeol, oleanolic acid, flavonol quercetin, and daucosterol were the main constituents identified for the ethanolic extract (Spatafora and Tringali 2012).

In the study, carried out by Knoblich et al. (2005), tomato peel and seed by-products were used for the isolation of antioxidants (carotenoids). The lycopene content of peel byproduct was found to be 734 μg/g of dry material. Significant amount of lutein, β-carotene, and *cis*-β-carotene were also identified. Seed by-product mainly contained lycopene while the other carotenoids were approximately half of that present in the peels (Knoblich et al. 2005).

6.4 Mechanism of Antioxidant Activity

There are mainly three types of mechanism known for the antioxidant activity, viz., chain breaking, preventive, and synergetic. Schematic representation of these mechanisms is given in Fig. 6.4a–c.

6.5 Techniques for Measurement of Antioxidant Activity

There are three major techniques mostly used for the measurement of antioxidant activity in various samples.

6.5.1 Chemical Assays for Antioxidant Activity

There are many chemical assays used for the assessment of antioxidant activity in the products (herbal, nutraceuticals, and food items). Some of the well-documented and most practiced methods are described below.

6.5.1.1 Oxygen Radical Absorption Capacity

Oxygen radical absorption capacity (ORAC) method uses dichlorofluorescein as the fluorescent probe and an azo-compounds, such as 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) as the radical generator. It measures the inhibition of the peroxyl radical induced oxidation initiated by thermal decomposition of AAPH. Over time, the free radical generated from the thermal decomposition of AAPH quenches the signal from the fluorescent probe fluorescein. The subsequent addition of an antioxidant produces a more stable fluorescence signal due to the inhibition of fluorescein decay by single antioxidant and/or complex mixture. Rate of decay of fluorescence measures the antioxidant's capacity (Číž et al. 2010).

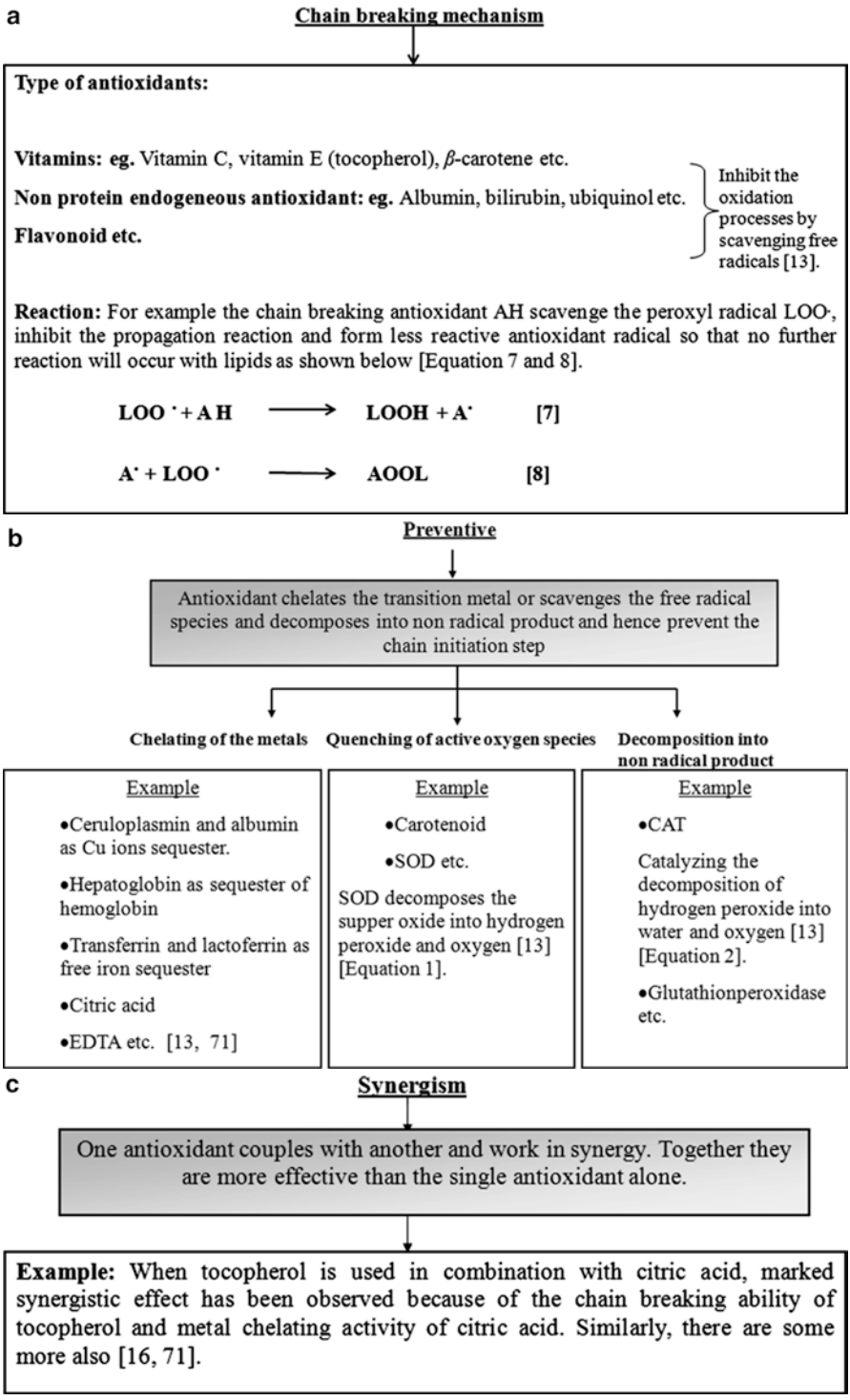

Fig. 6.4 (**a–c**) showing schematic representation of mechanism of chain breaking, preventive and synergetic action of antioxidants respectively

6.5.1.2 Determination of Total Phenolic Content (TPC)

Total phenolic content of the extracts are determined using Folin–Ciocalteu (FC) reagent using spectrophotometer, measured at 725 nm. This method is based on reduction ability of phenolic functional group. Oxidation and reduction reaction of phenolate ion takes place at base condition. The reduction of phosphotungstate–phosphomolybdenum complex (Folin–Ciocalteu reagent) by phenolat ion will change its color to blue. The reduction of complex will increase when the extract contains more phenolic compounds. Thus the color will be darker and the absorbance will be higher, showing higher antioxidant activity (Prior et al. 2005).

6.5.1.3 1,1′-Diphenyl-2-Picrylhydrazyl

DPPH (1,1′-diphenyl-2-picrylhydrazyl) assay is carried out as per the reported method of Brand-Williams et al. (1995). $DPPH^-$ free radical is obtained by dissolving DPPH in methanol and is stable when placed under the dark at −20 °C until used. As $DPPH^-$ reacts with antioxidants present in the sample, color changes from violet to yellow and absorbance of the solution so obtained is measured spectrophotometrically at 515 nm (Brand-Williams et al. 1995).

6.5.1.4 Trolox Equivalent Antioxidant Capacity

In this assay, ABTS {2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)} is used to measure the antioxidant capacity of the substance (food stuffs). Trolox equivalent antioxidant capacity (TEAC) is also known as ABTS assay and the procedure is based on the reported method of Arnao et al. (2001). When ABTS reacts with potassium persulfate, it becomes a free radical ($ABTS^+$) which gives blue color to the solution. The phenolics, thiols, or vitamin C present in the food stuffs scavenge this $ABTS^+$ free radical and convert it into its neutral colorless form which is measured spectrophotometrically. $ABTS^+$ absorbs light at 734 nm (Arnao et al. 2001).

6.5.1.5 Ferric Reducing Antioxidant Power

Ferric reducing antioxidant power (FRAP) assay is carried out using the earlier reported method as described by Benzie and Strain (1996). When ferric chloride reacts with 2,4,6-tripyridyl-*s*-triazine (TPTZ) at low pH, ferric is converted into ferrous causing formation of ferrous tripyridyl triazine complex. FRAP values are obtained by comparing the absorbance change at 593 nm in reaction mixture with those containing ferrous ions in known concentration (Benzie and Strain 1996).

6.5.1.6 Determination of Total Reducing Power (TRP)

TRP is determined following the method of Negi et al. (2005). It is measured spectrophotometrically in terms of their capacity to reduce the potassium ferricyanide (Fe^{3+}) to the potassium ferrocyanide (Fe^{2+}), depending upon the concentration of the antioxidant compounds present in the sample, which in turn reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm (Negi et al. 2005).

6.5.2 Biochemical Assays for Antioxidant Activity Assessment

Antioxidant activity may also be measured in biological system, i.e., in vivo and in vitro models. These include measurement of oxidative stress marker of the adduct or end product of ROS with the molecules, such as lipid, protein, DNA, and other molecules. These methods include thiobarbituric acid reactive substances (TBARS), SOD, CAT, GPx, GSH, and ferrous oxidation-xylenol orange (FOX) assay. These assays may be carried out in blood, urine, breath and tissues. Some of the examples are described below:

6.5.2.1 TBARS

TBARS method determines the extent of lipid peroxydation in sample. TBARS is the reaction product of thiobarbituric acid (TBA) and malondialdehyde (MDA) which results from the decomposition of lipid hydroperoxide in the sample which is read spectrophotometrically at 532 nm (Ohkawa et al. 1979).

6.5.2.2 Protein Carbonyl

Protein carbonyl content results from the oxidative cleavage of protein. In this case, 2,4-dinitrophenylhydrazine (DNPH) reacts with protein carbonyl and forms a Schiff base to produce corresponding hydrazone. The amount of protein hydrazone produced is quantified spectrophotometrically at an absorbance between 360 and 380 nm (Levine et al. 1990).

6.5.2.3 FOX

Hydroperoxide content of the lipid can be determined from its ability to oxidize ferric (Fe^{2+}) to ferrous (Fe^{3+}). Ferrous (Fe^{3+}) formed a complex with xylenol orange reagent (bluish-purple color) which is measured at 560 nm (Nourooz-Zadeh et al. 1994).

6.5.2.4 CAT

Catalase activity can be measured by using H_2O_2 as a substrate according to the method of Aebi (1984).

6.5.2.5 SOD

SOD is measured using the method of Kakkar et al. (1984) where nicotinamide adenine dinucleotide (NADH) is used as a substrate. The color intensity of the chromogen (purple color) in butanol layer is measured against butanol (blank) on spectrophotometer at 560 nm (Kakkar et al. 1984).

6.5.2.6 ROS

In this assay, 2′,7′-dichlorofluorescein diacetate (DCFDA) is used to measure ROS level. It undergoes cellular oxidation by ROS and gets converted into fluorescent dichlorofluorescein (DCF) which is highly fluorescent at 530 nm (Moein and Moein 2012).

6.5.3 Instrumental Technique (Antioxidant Analyzer)

Recently, an instrument named PHOTOCHEM Antioxidant Analyzer developed by Analytik Jena UK is being used for the measurement of antioxidant property of different products. It is capable of measuring both water-soluble and lipid-soluble antioxidants in a single system. It is based on the principle of photochemiluminescence with luminometric detection. It can measure the antioxidant capacity of lyophilized vegetables, fruits juices, beer, and water; lipid-soluble antioxidative capacity in baker's yeast, cheese, tea, and coffee; and lipid-soluble antioxidative capacity in edible oil and salami extracts (http://www.selectscience.net/product-news/rapid-and-accurate-antioxidant-measurement-in-foods).

6.6 Conclusions

Antioxidant defense system is always maintained in the body to counter the adverse effect of oxidative stress developed in the biological system due to the formation of reactive oxygen species. Oxidative stress is a key factor which plays an important role in the progression of various pathological diseases. There are many reactive species produced in the body as a result of metabolic functions. These active oxygen species are crucial for life because they are also responsible for various

physiological activities, such as production of energy, synthesis of essential compounds, and in signal transduction. They may also attack the macromolecules including protein, DNA, and lipid, causing cell damage. Each body type maintains a particular ratio of antioxidants and ROS and the imbalance causes oxidative stress. Therefore, antioxidants are widely supplemented in diets for the maintenance of proper health and prevention of various pathological diseases. Besides, they also have many industrial uses, such as for the preservation of food and cosmetics as well as in preventing the degradation of rubber and gasoline.

It is further concluded that production of antioxidant compounds from the agro-industrial waste should be highly encouraged and practiced more and more in order to minimize the waste production and reduce the adverse effect on the environment.

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