Imbalance in antioxidant defence and human diseases: Multiple approach of natural antioxidants therapy

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The origin of diseases of multifactorial nature is being understood now due to vitiation in the basic homeostatic balance phenomenon in the body. A majority of disease conditions like atherosclerosis, hypertension, ischaemic diseases, Alzheimer's disease, Parkinsonism, cancer and inflammatory conditions are being considered caused primarily due to the imbalance between pro-oxidant and antioxidant homeostasis. Antioxidant principles from natural resources possess multifacetedness in their multitude and magnitude of activities and provide enormous scope in correcting the imbalance. Therefore, much attention is being directed to harness and harvest the antioxidant principles from natural resources. In the light of present understanding about the role of free radicals in pathogenesis of multiple diseases, this article provides an account of multifaceted activities of antioxidants and discusses the multiple approach due to which these phytochemicals deserve proper position in therapeutic armamentarium.

THERE is an explosion of global awareness concerning increasing imbalances in natural ecosystem. Therefore, various measures are being taken up to correct the *root cause* of the imbalance. Human beings constantly struggle against the changing environmental conditions to maintain optimum health and vigour throughout their life, during all the seasons. The human body depends on the continuous holistic interaction between internal and external factors. When this interaction is in a state of equilibrium, man enjoys health and when it fails, either due to internal deficiency or hostile environmental factors, the balance is disturbed and leads to disharmony and disease.

Recently, Hellstrom[1] has proposed the *altered homeostatic theory* which asserts that multiple acquired and genetic factors move the basic homeostatic balance of the body in such a direction that it inappropriately activates defence mechanisms and favours multiple diseases. Therefore, it is important to understand the homeostatic process and find out the *causes*, which create inappropriate imbalance. These *causes* may be the *real factor*, lack of which may be important in ensuing the disease process. The ideal curative approach would then be the restoration of these factors, which will improve the imbalance and shift the physiological process in the direction of homeostatic balance. The altered homeostatic theory is regarded as a holistic approach to the disorders of multifactorial nature, because bodily homeostasis epitomizes the idea of a functioning whole.

As one of the aspects of the body's natural ecosystem, it is increasingly being realized now that a majority of the diseases/disorders are mainly due to the imbalance between pro-oxidant and anti-oxidant homeostatic phenomenon in the body. Pro-oxidant conditions dominate either due to increased generation of free radicals and/or their poor quenching/scavenging into the body[2–4].

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and our metabolism. They are continuously produced by the body's normal use of oxygen such as respiration and some cell-mediated immune functions. They are also found or generated through environmental pollutants, cigarette smoke, automobile exhaust fumes, radiation, air-pollutants, pesticides, etc.[5–8]. Naturally, there is a dynamic balance between the amount of free-radicals generated in the body and antioxidants to quench and/or scavenge them and protect the body against their deleterious effects[9,10]. However, the amount of these protective antioxidant principles present under the normal physiological conditions, are sufficient only to cope with the physiological rate of free-radical generation. It is obvious, therefore, that any additional burden of free-radicals either from environment or produced within the body, can tip the free-radical (pro-oxidant) and anti-free-radical (anti-oxidant) balance leading to oxidative stress, which may result in tissue injury and subsequent diseases[4,11–14] (Figure 1). Thus the antioxidants status in human reflects the dynamic balance between the antioxidant defence and pro-oxidant conditions and has been suggested as a useful tool in estimating the risk of oxidative damage[9,15,16].

In this context, research in the recent past has accumulated growing body of evidences to show that enrichment of body systems with natural antioxidant principles may correct the vitiated homeostasis[2,17–21].

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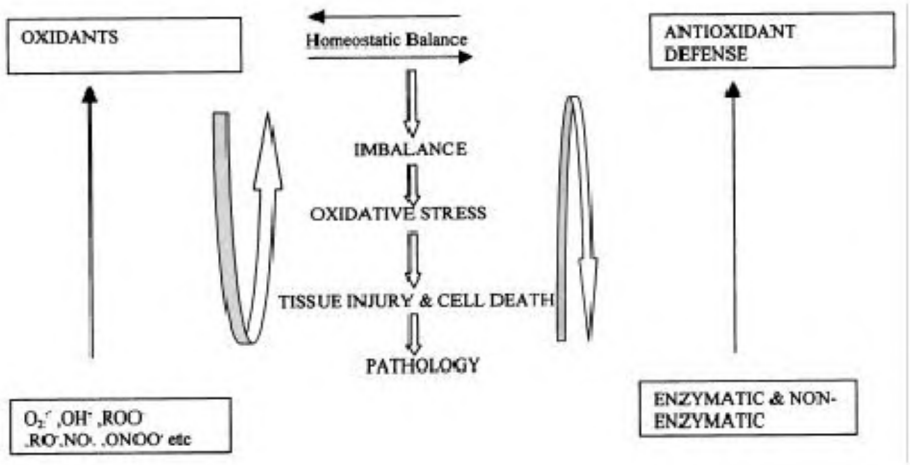


Figure 1. Balance of reactive oxygen species (ROS) generation and antioxidant defence. Under normal physiological conditions the body copes with the flux of ROS. Oxidative stress describes a condition in which antioxidant defence is insufficient to keep the level of ROS below a toxic threshold. This may be due to either the excessive generation of ROS or loss of antioxidant defence or both.

Further, these efforts can prevent the onset as well as treat the disease caused/or fostered due to free-radicals and related oxidative stress. These evidences accelerated the search for antioxidant principles, which led to the identification of natural resources and isolation of active antioxidant molecules. Collectively, these molecules are called polyphenolics, and flavonoids have contributed enough to the understanding in this area.

Based on recent understanding of the multifarious, multifaceted aspects of natural antioxidants, success and failures of antioxidant therapy in clinical studies, this article aims to review the present knowledge, limitations and future prospects of this high-yielding therapeutic avenue. Since flavonoids have been studied in detail as antioxidants, favourable as well as critical evaluation of different aspects of these phytochemicals relating to their antioxidant properties have been analysed in particular. Nonetheless, descriptions provided herein, may be applicable to other polyphenolic compounds also.

Flavonoids

Flavonoids present a great variety of structural specificities. As potent antioxidants, flavonoids are especially important for protection against human disease. The multiple properties of these phytochemicals have made them more attractive, as they can modulate various aspects of disease like lipid peroxidation involved in atherogenesis, thrombosis, carcinogenesis, hepatotoxicity and a variety of disease conditions. The basic structure of a flavonoid contains a flavon nucleus (2-phenyl-benzo-ϒ-pyran) consisting of benzene rings (A) and (B) combined by an oxygen-containing pyran ring (C). The differences in substitution on ring (C) distinguish the different classes of flavonoids (Figure 2).

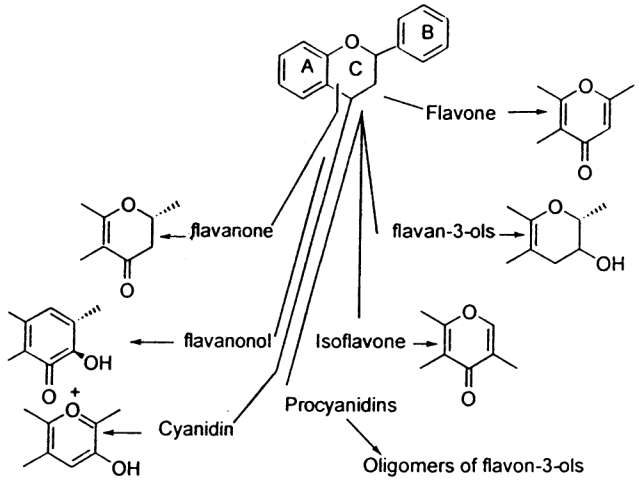


Figure 2. Skeletal structure of flavonoid and related structures.

Antioxidant properties are related to structural features

It is the free-radical entity, which is primarily responsible to initiate damage to the biological targets leading to different disorders. However, as the understanding is progressing, it is being realized that merely radical scavenging activity of a compound is not directly related to the protection in biological system rather, structure matters much and the *in vivo* radical-generating conditions[22]. According to the mode of action, antioxidants may be classified as free-radical scavenger/terminator, chelater of metal ions, capable of catalysing lipid peroxidation or as oxygen scavengers that react with superoxides and so on[23–27]. Similarly, according to the environment they are exposed to and the situation they find, polyphenolic antioxidants act either by trapping the initiating radical, propagating lipid peroxyl radicals,

recycling α-tocopherol and or deactivating the excited photosensitizer, etc.[28].

Structural determinants for radical scavenging property and antioxidant potential

With the increasing knowledge about various free-radicals and free-radical scavengers, several structural determinants have been proposed. Bors *et al.*[23] proposed the following structural determinants for effective radical scavenging properties by flavonoids (Figure 3). (1) The *O*-dihydroxy (catechol) structure in ring (B), which is the obvious radical target site for all flavonoids, with a saturated 2,3-double bond. (2) The 2,3-double bond in conjugation with a 4-oxo function which is responsible for electron delocalization from the (B) ring. (3) The additional presence of both 3 and 5 hydroxyl groups for maximum radical scavenging potential and strong radical absorption.

Frankel[24] further elaborated the explanation for these structural criteria and added that flavonoids are recognized for both their ability to donate electrons and stop chain reactions. This activity is attributed to the phenolic hydroxyls, particularly in the 3′,4′-OH of the (B) ring and the 2,3-double bond in the (C) ring. Activity increases with the number of –OH groups in rings (A) and (B). These phytochemicals impart their antioxidant activity by scavenging diverse groups of radicals like hydroxyl radicals ($^{\bullet}OH$), superoxide anion radicals ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), alkoxyl radicals ($RO^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$), peroxynitrite ($ONOO^{\bullet}$) and mechanisms like complexing proteins (enzymes and their metal-binding sites), inducing synergistic effects by reducing oxidized antioxidants (mixture of vitamins E and C). Jovanovic *et al.*[29] specified that organic $ROO^{\bullet}$ selectively attacks the (B) ring of any 3′,4′ or 2′,5′-dihydroxyl flavonoid. Pannala *et al.*[30] suggest that flavonoids scavenge $ONOO^{\bullet}$ by two possible mechanisms: (i) preferential nitration for the monohydroxycinnamates, and (ii) electron donation for catecholes (Figure 4). The metal chelating activity of these phytochemicals is due to their binding metals at two points of their molecules: (i) the orthodiphenol (3′,4′-di OH) grouping in ring (B) and (ii) the ketal structure in ring (C) (Figure 5).

Concurrently, Georgi and colleagues[31] suggested that the free-radical scavenging properties of antioxidant compounds are often associated with their ability to form stable radicals after their reaction with free-radicals. Flavonoids that can scavenge radicals effectively usually give rise to semiquinone free-radical in alkaline solution. This special feature may also give rise to their active sites. The semiquinone free-radical or aroxyl radical may react with a second radical, acquiring a stable quinone structure[18] (Figure 4). However, aroxyl radical can also interact with oxygen, generating quinones and $O_2^{\bullet-}$ which may be responsible for the undesired pro-oxidant effects of flavonoids. Therefore the possible side reactions with aroxyl radical also play an important role. The structural criteria discussed above and below meet the requirement in scavenging highly reactive $^{\bullet}OH$ as well as $O_2^{\bullet-}$ radicals[18,31]. As illustrated in Figure 4, it is proposed that *O*-dihydroxyl structure in ring (A) makes better antioxidant than others[31].

Therefore, the activities of antioxidants are related to the stability of the free-radicals formed after they react with active radicals. Flavonoids with *O*-tri or *O*-dihydroxyls in the (B) ring and/or in the ring (A) form stable free-radicals. This is an important feature with flavonoid compounds, due to which many are better antioxidants than antioxidant nutrient vitamins C and E

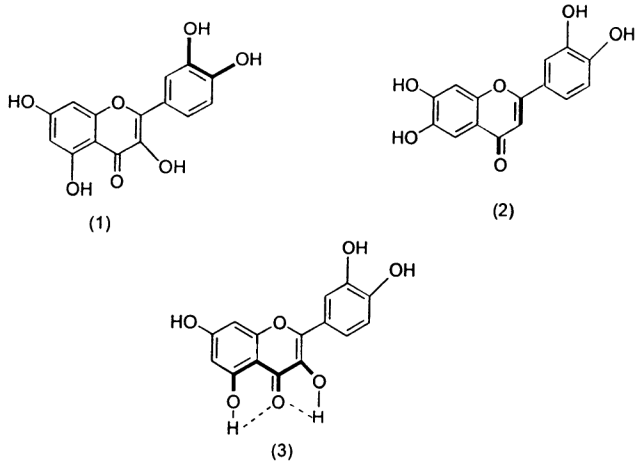


Figure 3. Structural determinants of radical scavenging property.

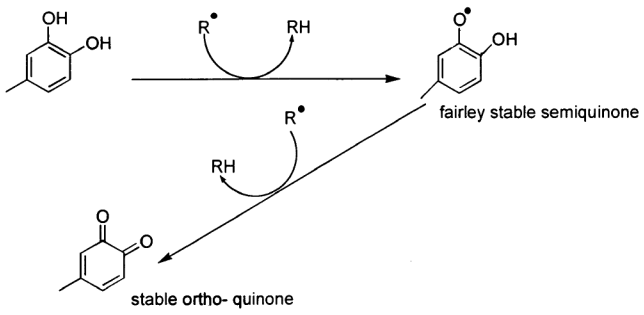


Figure 4. Scavenging of ROS (R•) by flavonoids and formation of stable structure.

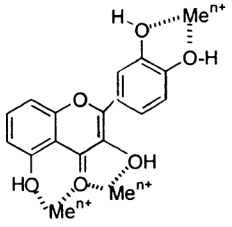


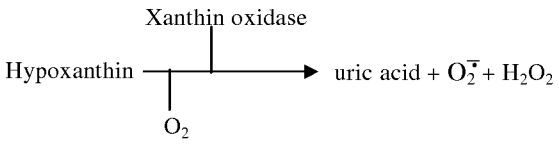
Figure 5. Binding sites in flavonoids for transition metals.

and β-carotene, on a mole-for-mole basis. These antioxidant nutrients do not form stable radicals and are dependent for their scavenging/transport on other systems[2,32,33].

Apart from the above criteria, other features also play an important role in considering antioxidant properties: (i) Rate constant with different types of radicals. The lower the reactivity of a free-radical to be scavenged, more important are the structural requirements as described for a flavonoid to act as effective as an scavenger. However, de Groot[12] argues that rate constant for the reaction of a flavonoid with a free-radical or any other reactive oxygen species (ROS), is just one factor which determines its potency to act as an antioxidant. (ii) Stoichiometry of the radical scavenging process. (iii) Effective concentration to be reached at the site where the reactive species is being formed. (iv) Stability and decay kinetics of the resulting product such as flavonoid aroxyl radical, is also an important aspect.

Structural determinants for scavenging superoxides ($O_2^{\cdot-}$) and their sources

$O_2^{\cdot-}$ radical has been of intense interest owing to its increased dominance *in vivo* in different disease conditions. In a majority of the cases, this radical is generated enzymatically. Xanthin oxidase (XOD)-mediated generation of $O_2^{\cdot-}$ is extensively studied. Oxidation of hypoxanthin to uric acid with simultaneous generation of $O_2^{\cdot-}$ and H_2O_2 has been observed to play a crucial role during myocardial ischaemia, reperfusion injury, gout, rheumatoid arthritis and many other inflammatory conditions. Molecular oxygen, which is easily available *in vivo*, acts as an electron acceptor during reoxidation of XOD and generates $O_2^{\cdot-}$ and H_2O_2 (refs 34 and 35).



XOD is considered an important biological source of $O_2^{\cdot-}$ radical. These and other ROS contribute to oxidative stress in an organism and are involved in many pathological conditions such as inflammation, atherosclerosis, cancer, ageing, etc. Decades ago, Robac and Gryglewski[36] had reported that antioxidative property of several flavonoids was a result of scavenging of $O_2^{\cdot-}$.

Detailed study by Cos and colleagues[37] provides very important insight to categorize flavonoids into different classes based on their structure and biological activity related to XOD inhibition and/or $O_2^{\cdot-}$ scavenging: (i) Flavonoids which can scavenge only $O_2^{\cdot-}$ without inhibitory activity on XOD such as (+) taxifolin, (–) epicatechin, (–) epigallocatechin; (ii) Flavonoids which can effectively inhibit XOD activity, but cannot scavenge $O_2^{\cdot-}$ radicals, such as kaempferol, morin, isorhamntin; (iii) Compounds which possess both the $O_2^{\cdot-}$ scavenging activity as well as XOD inhibitory capacity such as quercetin, 7-neohespiridosylluteolin,4′,7-dimethylquercetin, 3-rutinosylkaempferol; (iv) Compounds which possess XOD inhibitory activity, but may become pro-oxidant and increase the generation of $O_2^{\cdot-}$ such as luteolin, galangin, apigenin; (v) Compounds with marginal effect on XOD inhibition along with pro-oxidant properties such as 7-hydroxyflavone; (vi) Flavonoids with neither XOD inhibitory nor $O_2^{\cdot-}$ scavenging capacity such as 4′-hydroxyflavanone, 3-hydroxylflavone, cirsimarin, 6-glucosyl-8-xylosylapigenin.

Based on the above categorization, at least two structural criteria for a flavonoid have been proposed: (a) to possess strong XOD inhibitory activity, flavonoids should have hydroxyl groups at C-5 and C-7 with a double bond between C-2 and C-3, and (b) to scavenge $O_2^{\cdot-}$ effectively, on the other hand, a hydroxyl group at C-3′ in ring (B) and at C-3 position is essential.

This study acknowledges that the knowledge of structural criteria provides a better tool to identify compounds with desired biological activity and also an insight into looking for better therapeutic agents. For example, allopurinol, an anti-rheumatic, anti-gout drug, possesses only XOD inhibitory activity and has no check on the $O_2^{\cdot-}$ produced already at the target site. It becomes obvious, therefore, that compounds bearing both the $O_2^{\cdot-}$ scavenging as well as XOD inhibitory activity may offer better therapeutic potential than allopurinol. Flavonoids with both these properties possess in common OH-groups, either at C-5, C-3 or C-3′ and C-4′. However, for a single compound to meet every criterion is not encouraging as the presence of –OH group at C-3 slightly decreases XOD inhibitory activity, which suggests that a planar flavonoid structure is a necessary requirement for this particular activity. Although these observations and interpretations provide important understanding in structure–activity relationships, it is important to note that *in vitro* observations may have limitations while interpreting *in vivo* effects, as in the case of luteolin. According to Cos *et al.*[37], luteolin apart from possessing XOD inhibitory activity may accelerate generation of $O_2^{\cdot-}$. However, recent *in vivo* studies report its important therapeutic contribution in inflammatory, chronic obstructive pulmonary disease, and bronchitis, as antitusive expectorant without any adverse effect on cardiovascular damage[38,39]. It is also important to note that flavonoids can be oxidized and may exert pro-oxidant effects *in vitro* under some assay conditions; however, most reported studies have emphasized their antioxidant effects as they may not meet similar conditions *in vivo*.

It seems important, therefore that a combination of antioxidant molecules is better suited to satisfy diverse biological activity, where compounds impart their biological activity working synergistically for better therapeutic value.

Transition metals, lipid peroxidation and antioxidant

Metals play an important role in biological processes. There are several important biochemical and physiological mechanisms where involvement of metals plays a crucial role. Although they are trapped and transported into the body via highly sequestered and controlled processes, there are several circumstances where any deviation from their controlled mechanism leads to their leakage into the surrounding environment, where they contribute to overt generation of free-radicals and consequently, lipid peroxidation, tissue injury and oxidative stress[40–44].

Most of the biological studies of lipid peroxidation involve transition metal ions, added to or contaminating reaction mixtures. Copper ions, Cu^{2+}, are classically used to stimulate low-density lipoprotein (LDL) peroxidation *in vitro* and Fe^{2+} for lipid peroxidation models[45,46]. The presence of Cu^{2+}/Fe^{2+} has been frequently observed in atherosclerotic lesions. When Fe^{2+}, Cu^{2+} or certain chelates of these ions (Fe^{2+} + ADP) are added to liposomes, lipoproteins or isolated biological membranes, peroxidation of lipids ensues[41,47,48]. Oxidized forms of these transition metals can also accelerate peroxidation, if a reducing agent is added to or present ($FeCl_2$/ascorbate) in the experimental set-up.

Brown *et al.*[49] studied LDL oxidation mediated by Cu^{2+} and observed structural requirements for flavonoids. They noticed that the ortho 3′,4′-dihydroxy substitution in the ring (B) is shown to be important for Cu^{2+}-chelate formation, thereby influencing antioxidant activity (Figures 4 and 5). Presence of a 3-hydroxy group in a flavonoid structure enhances the oxidation of quercetin and kaempferol, whereas luteolin and rutin, each lacking the 3-hydroxyl group, do not oxidize readily in the presence of Cu^{2+} ions. They demonstrated that the reactivities of flavonoids in protecting LDL against Cu^{2+} ion-induced oxidation are dependent on their structural properties in terms of the response of a particular flavonoid to Cu^{2+} ions. Whether chelation or oxidation, their partitioning abilities between the aqueous compartment and the lipophilic environment within LDL particles and their hydrogen-donating antioxidant properties are important aspects. A number of flavonoids effectively chelate trace metals, which play an important role in oxygen metabolism. Free iron and copper are potential enhancers of ROS formation, as exemplified by the reduction of H_2O_2 with the generation of highly aggressive hydrogen radicals,

$$H_2O_2 + Fe^{2+}\ (Cu^{+}) \rightarrow {}^{\bullet}OH + OH^{-} + Fe^{3+}\ (Cu^{2+})$$

Or, by copper-mediated LDL (LH) oxidation,

$$LH + L^{\bullet} \rightarrow LOO^{\bullet}.$$

Pietta[18] proposed that binding sites for trace metals to flavonoids are the catechol moiety in the (B) ring, the 3-hydroxyl, 4-oxo groups in the heterocyclic ring, and the 4-oxo, 5-hydroxyl groups between the heterocyclic ring and the ring (A) (Figure 5).

Generation of hydrogen peroxide and antioxidant activities of flavonoids

Miura *et al.*[50] suggest that flavonoids which possess pyrogallol (adjacent trihydroxyl) and/or catechol (adjacent dihydroxyl) moieties in their structure show strong H_2O_2 generating activity via an $O_2^{\bullet-}$ anion radical and also possess inhibitory activities in rat liver microsomal lipid peroxidation. Flavonoids which generate H_2O_2 can scavenge free-radicals. Flavonoids act as antioxidants not only by free-radical scavenging, but also by the metal chelation and by inhibition of enzymes like NADPH oxidase in human neutrophils, mitochondrial succinoxidase and NADH oxidase and inhibit microsomal cytochrome P-450 (Fe^{2+})-dependent enzymatic reactions by their metal-chelating activity[50–52].

The electron and H^+ donating capacity of flavonoids seem to contribute to the termination of lipid peroxidation chain reaction based on their reducing power[29,53]. Due to their reducing power these phytochemicals act as both antioxidant as well as pro-oxidant depending upon the environment they are exposed to. They act as pro-oxidant in the absence of radicals. The classical antioxidants, α-tocopherol and vitamin C, are also reported to behave in a similar fashion.

Flavonoids generate H_2O_2 by donating a hydrogen atom from their pyrogallol or chatechol structure to oxygen, through a superoxide anion radical[50] (Figure 6). The pyrogallol-type flavonoids generate more H_2O_2 than catechol. More the H_2O_2 generation, more potent is the radical trapping.

The increased generation of H_2O_2 by flavonoids has been a matter of considerable debate, where most of the studies come from *in vitro* cell culture. H_2O_2 has many cellular effects depending on the cell types examined and H_2O_2 concentration. The response of cells to H_2O_2 varies according to the cell studied, its level of enzymatic

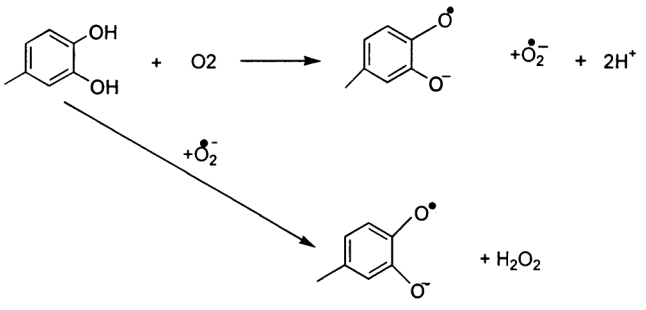


Figure 6. Scheme representing H_2O_2 generation by flavonoids.

antioxidants like catalase and other H_2O_2 removing enzymes. H_2O_2 has been reported to raise intracellular Ca^{2+}, activate transcription factors, repress expression of certain genes, promote or inhibit cell proliferation, be cytotoxic, activate or suppress certain signal transduction pathways, promote or suppress apoptosis. However, the observations that flavonoids increase the generation of H_2O_2 appears ambiguous. Long *et al.*[54] very recently cautioned that increased generation of H_2O_2 by flavonoids may be due to artifacts in the cell-culture media and therefore, it must be precautiously interpreted as such relating *in vivo* effects. It appears important that most of the reported effects of polyphenolic compounds on cells may be due to their oxidation in the culture media, leading to H_2O_2 generation and again it may not be the real situation *in vivo*. Therefore, more studies are required to differentiate direct cellular effects of phenolic compounds from effects caused by H_2O_2 generation in the culture media and factual conditions *in vivo*.

Anticytotoxicity properties and structural requirement for antioxidants

A variety of synthetic and dietary polyphenols protect mammalian and bacterial cells from cytotoxicity induced by hydroperoxides, especially H_2O_2. The polyphenols bearing *O*-dihydroxyl or its equivalent structures in flavonoids are essential for protection against H_2O_2 mediated cytotoxicity[22]. It seems difficult for polyphenols possessing free carboxyl groups to be incorporated into the cells or the cell membranes, because of electrostatic repulsion occurring between the negative charge of the carboxyl group and the membrane phospholipids. This may be the reason that neither α-tocopheral nor ascorbate offers cytoprotection due to H_2O_2-mediated cytotoxicity.

Several hindered alkyl phenols such as BHT (2,6-ditertbutyl-4-methyl phenol) are widely used as antioxidants and free-radical scavengers[55]. Apart from their beneficial antioxidant effects, these alkyl phenols also produce detrimental effects in animals. Liver microsomes and hepatocytes from rats metabolize BHT extensively through two main routes: (i) hydroxylation of alkyl substituents and (ii) oxidation of the pi-electron system. The latter pathway generates initially a phenoxyl radical that partitions between two reactive products: combination with molecular oxygen and followed by reduction yields hydroperoxides (BHT-OOH) and one electron oxidation yields the quinone methide (BHT-QM). Both these metabolic products are more toxic than the parent compound. These observations further suggest the importance of structural requirement in order to achieve desired beneficial properties.

It is important to note that cases with flavonoids are also not fair throughout in this context. Griffiths[56] observed that flavonoids are metabolized *in vivo* as phenolic acids. Limasset *et al.*[57] analysed several phenolic acids which are attributed to be the metabolites of flavonoids and are known to present antioxidant properties through their ability to reduce H_2O_2 produced by stimulated polymorphonuclear cells (PMNCs). They propose the following structural features based on biological activities: (i) Monophenolic compounds have a little, if any, inhibitory effect; (ii) Ortho-diphenolic acids have strong inhibitory effect with a little difference depending on acidic chain; (iii) metaorthomethylation of diphenolic compounds has a little effect on the inhibitory action. On the other hand, *O*-methylation on parahydroxyl reduces the inhibitory effect; (iv) Methylation, both in para and meta, suppresses completely the inhibitory effect; (v) unsaturation in α and β position of propionic chain leads to the most effective inhibitory compounds.

In a recent study in this regard, Silva *et al.*[58] observed structure–activity relationship of caffeic acid and derivatives. They note that esterification of the carboxyl group of dihydrocaffeic acid dramatically enhanced the radical scavenging potency of the compound. However, similar effects were not observed with caffeic acid. The authors suggest that the *n*-alkyl esters of both phenolic series had similar potencies, and their antiradical activities are independent of the alkyl chain length.

Knowledge of the driving forces related with antiradical and/or antioxidant behaviour of these compounds is worthy research therefore, because it could be a very important basis to explain some of their biological properties, especially those related with deleterious oxidative processes[59].

Present status and future prospects

The molecular components of free-radical biology and biological inter-relationships of these components in mediating various disease processes are beautifully being unravelled for better understanding and exploitation in biomedical/clinical sciences[52,60,61]. Parallel identification and isolation of anti-free-radical/antioxidant principles from natural resources are simultaneously presenting enormous scope for their better therapeutic application. Sustained interest in the use of antioxidants for treatment of human disease[62–66] and the role of dietary antioxidant in prevention of disease development offer better understanding for the development of newer and better therapeutic entities[42,67–69]. These developments in both the therapeutic and nutritional fields have not only been punctuated by some successes, but by some spectacular failures as well[70–73]. In this concern, major contribution relates to the trials with antioxidant compounds, vitamins E and C and β-carotene, either in combination or alone. Several authors have reviewed the positive and negative aspects associated with these widely studied molecules[2,32,74]. Halliwell[75] has explained the antioxidant

paradox. The protective effect of diet may not be equivalent to the protective effect of antioxidant in the diet, rather a combination in association with antioxidant principles may be responsible for beneficial effects. For example, β-carotene in the diet may protect against cancer development, but β-carotene may not be the only true protective agent. Similarly, it is important to note that most of these studies emerge from *in-vitro* observations and their *in vivo* interpretation has got certain limitations. This may be the reason that individual isolated compounds number in thousands; however, *in vivo* observations of only a few are reported. Pietta[18] also supports this view and suggests that the basis of epidemiological observations are fruits, vegetables, medicinal plants and beverages. Preventive role of these materials is due to a variety of constituents, including vitamins, minerals, fibre and numerous phytochemicals, including flavonoids. Thus, it is possible that flavonoids also contribute to the protective effect. A possible protective role against coronary heart disease (CHD) of flavonoid intake has been reported in four out of six epidemiological studies[76]. Therefore, foundation studies based on epidemiologic observations that antioxidants impart beneficial effects are presenting a strong basis for the exploitation of their therapeutic potential.

Very recently, Polidori *et al.*[16] have reviewed the human plasma profiles of antioxidant molecules in different disease conditions. The authors observed the imbalance/deficiencies of different antioxidants in different disease states. This study suggests that there is an urgent need to have fingerprints of antioxidant profiles in different disease conditions. This information can pave the way for selecting a particular type of preparation with desired permutations for the treatment of a particular disease. Though population studies are emerging to show the reduced antioxidant status in CHD, cancer and neurological disorders[16,77–82], global impact is yet to be substantiated.

The holistic therapeutic effects of dietary antioxidants may be observed, as they can display their first antioxidant defence in the digestive tract by limiting ROS formation[15,83–85] and scavenging them and may impart antiulcer activity[86]. Further, once absorbed, either as aglycons or glycosides[87–89] or to a larger extent, as phenolic acids, they continue to exert antioxidant effect along with other systemic activities.

One of the main purposes of antioxidant therapy is to reduce arterial wall and other target site's inflammation, induced by oxidative stress. Increased oxidative stress and induction of redox-sensitive genes by activating NF-kβ (ref. 90) is a common final pathway of action of classical risk factors: hypercholesterolemia, hypertension, smoking and diabetes. Abundance of NF-kβ is the marker of imbalance between pro-oxidant and antioxidant. Pharmacological manipulation by antioxidants ameliorates the abundance of NF-kβ (ref. 91).

The use of a combination of antioxidants with varied modes of action has better effects and resembles much more an antioxidant-rich diet supplementation, on which the positive data from epidemiological studies are based. The arguments proposed by Fryer[92] are very pertinent in this respect. He argues that although a diet rich in β-carotene protects against cancer development, β-carotene may not be the only protective agent. When we are looking for natural products, the approach should also be natural. Analysis of plant material always reveals the presence of six carotenoids, of which β-carotene is usually the most abundant. The remaining five are present at much lower concentrations. However, they are by no means less important. Two of these dietary components (zeaxanthin and lutein) are thought to be essential for well-being of the retina[93]. A diet rich in high β-carotene will also contain much lower concentrations of these rare carotenoids and mere supplementation of pure β-carotene alone cannot be expected to produce the same effects as a balanced intake provided by a diet rich in fruit and vegetables. Also, isolation and administration of β-carotene from carotinoids may lead to the loss of certain biological activities and synergistic actions observed with holistic approach.

Similarly, van Acker *et al.*[94] have suggested that vitamin E is a generic term used to collectively describe a range of tocopherol and tocotrienol molecules. *In vivo*, α-tocopherol is most abundant and therefore, the most important vitaminer. It terminates the chain reaction of lipid peroxidation by scavenging lipid peroxyl radicals. In this reaction, α-tocopherol itself becomes a radical. Glutathion seems to be able to regenerate α-tocopherol via a so-called free-radical reductase. The authors observed that introduction of antioxidant flavonoids such as 7-monohydroxyethyl rutoside, fisetin or naringenin restored GSH-dependent protection in case of α-tocopherol deficiency. This suggests that flavonoids can take over the role of α-tocopherol as an antioxidant. Concurrently, Jiang and colleagues[95] reported that in contrast to α-tocopherol, it is the γ-tocopherol and its metabolites which possess anti-inflammatory activities (particularly COX-2 inhibition), predominantly in macrophages and epithelial cells. This activity is independent of their antioxidant property. Apart from free-radicals, local expression of COX-2 and mediated inflammatory activity deteriorate pathological conditions in atherosclerotic plaques and cancer. Therefore, apart from antioxidant activity of tocopherol, its other aspects are also important. The finding that flavonoids can take over the role of tocopherols appears hence, an important development.

Antioxidants are ubiquitous in natural products. The putative therapeutic impression of many traditional medicines appears to be attributed to the presence of antioxidant principles. The great variation in their magnitude as well as multitude of activities may even become more important for protective effects in situations

where free-radical species are not directly involved in the disease process but may participate and/or foster the secondary events[2,69,96]. Better understanding, therefore, of the structure–activity relationship of these phytochemicals, synergistic mode of action and their relative importance in different mechanisms may provide deeper insights in finding out better and safer therapeutics.

The concept of synergy is central to the holistic approach. The trend of the modern concept to isolate pure compounds may not achieve the desired results, as observed in the natural version. Once an active principle is isolated from the natural product without its synergical colleagues to support and/or balance its action, it may lose its character as present in its natural form. The isolation and study of a problem from its environment is the beauty of modern science. However, the natural/holistic approach attempts to solve problems by taking these in their entirety, with all their interlinkages and their complexity. This may be the reason why Ayurvedic preparations have different permutations according to the disease conditions.

It has been observed by our own experience that a majority of Ayurvedic preparations prescribed in disease conditions now being explained to be mediated through oxidative stress, possess strong antiradical properties[97]. Kumar[98] observed that Ayurveda, the science of longevity is not just a collection of therapeutic recipes, but a framework which defines the conditions of sickness and connotes them with healing practices. As rightly and timely perceived by Kochhar[99] that it is in the framework of cultural Copernicanism, where concerted efforts are being made in the world of medicine to identify traditional knowledge systems and accord them the recognition. It is high time for us therefore, to explore the possibility of identifying our traditional therapeutic knowledge and interpret it according to the recent advancements, in order to give it a deserving place.

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