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Research Paper

Structure-Activity Relationship of Antioxidative Property of Hesperidin

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Present study was designed to investigate the structure-activity relationship of antioxidative property of hesperidin *via* a simple free radical scavenging system including, reducing power, chelating activity on Fe^{2+}, free radical-scavenging, total antioxidant, superoxide radical scavenging, hydrogen peroxide scavenging and hydroxyl radical scavenging activities in an attempt to understand its mechanism of antioxidant activity, which may pave the way for possible therapeutic applications. Different antioxidant tests were employed, namely, reducing power, chelating activity on Fe^{2+}, free radical-scavenging, total antioxidant, superoxide radical scavenging, hydrogen peroxide scavenging and hydroxyl radical scavenging activities. In addition, the results were compared with natural and synthetic antioxidants, such as α- tocopherol, ascorbic acid, butylated hydroxytoluene(BHT), butylated hydroxyanisole (BHA) and trolox. Hesperidin exhibited a strong reducing power, chelating activity on Fe^{2+}, free radical-scavenging, hydrogen peroxide scavenging and hydroxyl radical scavenging activities. Antioxidant activity of hesperidin increased with increased concentrations, in the range of 20-140 µg/ml. Hesperidin also exhibited a strong superoxide radical scavenging activity. Total antioxidant activity of hesperidin and both standards decreased in the order of hesperidin > α-tocopherol > trolox > BHA > BHT. This study showed that hesperidin exhibited antioxidant activity in all tests and could be considered as a source of natural antioxidants.

Key word: Hesperidin, Structure Activity Relationship, antioxidant activity, α- tocopherol, ascorbic acid and butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA).

INTRODUCTION

Antioxidants are vital substances that protect the body from damage caused by free radical-induced oxidative stress. Free radicals can be generated from metabolic pathways within body tissues and can also be introduced from external sources such as drugs, food, UV radiation and environmental pollution[1]. Free radicals have been implicated in the cause of several diseases such as liver cirrhosis, atherosclerosis, cancer and diabetes and play an important role in ageing. Oxidative stress can also contribute to the development of neuro-degenerative disorders, such as Alzheimer's and Parkinson's as well as other diseases. These free radicals attack unsaturated fatty acids of biomembranes, resulting in lipid peroxidation and desaturation of proteins and DNA, causing a series of deteriorative changes in the biological systems leading to cell inactivation[2]. Thus, antioxidants are important inhibitors of lipid peroxidation, not only for food protection but also in

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defending living cells against oxidative damage. Lipid peroxidation is an important deteriorating reaction in food during storage and processing that causes a loss in nutritional quality. The addition of antioxidants is required to preserve food quality. Antioxidant supplements or antioxidant-rich food are used to help the human body reduce oxidative damage from free radicals and active oxygen species[3]. Anther synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and trolox are widely used as antioxidants in the pharmaceutical and food industry. However, they have been shown to have toxic and/or mutagenic effects[2]. Because of their toxicity, the development and isolation of natural antioxidants from plant species, especially edible plants, such as silymarin, polyphenols and flavonoids are in progress[4].

In vitro investigations have demonstrated that the antioxidant properties of flavonoids are linked with their capability for scavenging free radicals, chelating metals and inhibiting the activity of oxidases[5-8]. Among these herbal resource, flavonoids. Flavonoids are a family of polyphenolic compounds found in fruits and vegetables. Flavonoids have wide biological properties including antibacterial, antiviral, anticancer, immunostimulant and antioxidant effects[9] Flavonoids' activity as antioxidants refers to their ability to transfer a hydrogen atom or an electron and to the possibility of their interactions with other antioxidants[10]. Hesperidin (HES) is a flavonone glycoside, belonging to the flavonoid family. This natural product is found in citrus species. Hesperidin was reported to have many biological effects including anti-inflammatory, antimicrobial, anticarcinogenic and antioxidant effects, and decreasing capillary fragility[11]. The flavonoid hesperidin may serve as a hydrogen donor for α-tocopherol radical, thus regenerating α-tocopherol, a key element of redox balance in biosystems. Hesperidin, in combination with a flavon called diosmin is used as Daflon®(Servier, France) to treat chronic venous insufficiency in Europe[12]. Other biological effects of hesperidin are unknown. However, the structural activity relationship of hesperidin on their antioxidative activity has not been fully reported. In the present study, the structural activity effects of hesperidin on the antioxidative activities were investigated using a simple free radical scavenging system including, reducing power, chelating activity on Fe^{2+}, free radical-scavenging, total antioxidant, superoxide radical scavenging, hydrogen

peroxide scavenging and hydroxyl radical scavenging activities in an attempt to understand its mechanism of action, which may pave the way for possible therapeutic applications.

MATERIALS AND METHODS:

Chemicals : -Hesperidin was from Aldrich (USA).

- α- tocopherol, ascorbic acid, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and trolox were from Sigma, USA.

Determination of reducing power

The reducing power of hesperidin was measured according to the method of Oyaizu [13]. Various concentrations of hesperidin (20-140µg) in 1 ml of distilled water were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 7.6) and 2.5 ml potassium ferricyanide [K_3 $Fe(CN)_6$] (1%, w/v), and then the mixture was incubated at 50°C for 30 min. Afterwards, 2.5 ml of trichloroacetic acid (10%, w/v) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of upper-layer solution was mixed with 2.5 ml distilled water and 0.5 ml $FeCl_3$ (0.1%, w/v), and the absorbance was measured at 700 nm. α- tocopherol, BHA and BHT were used as standard antioxidants. Higher absorbance of the reaction mixture indicated greater reducing power.

Determination of chelating activity on Fe^{2+}

The chelating activity of hesperidin on ferrous ions (Fe^{2+}) was measured according to the method of Decker and Welch[14]. Aliquots of 1 ml of different concentrations (0.25, 0.50, 0.75 and 1.0 mg/ml) of the samples were mixed with 3.7 ml of deionized water. The mixture was incubated with $FeCl_2$ (2 mM, 0.1 ml) for 5, 10, 30 and 60 min. After incubation the reaction was initiated by addition of ferrozine (5 mM and 0.2 ml) for 10 min at room temperature, and then the absorbance was measured at 562 nm in a spectrophotometer. A lower absorbance indicates a higher chelating power. The chelating activity of hesperidin on Fe^{2+} was compared with that of EDTA at a level of 0.037 mg/ml. Chelating activity was calculated using the following formula:

Chelating activity (%) = [1–(AbS/AbC)] x 100

AbS = Absorbance of sample
AbC = Absorbance of control

Control test was performed without addition of hesperidin.

Determination of free radical-scavenging activity

The free radical scavenging activity of hesperidin was measured with 1,1-diphenyl-2-picrylhydrazil (DPPH·) using the slightly modified methods of Brand William et al.[15] and Takashira and

Ohtake[16]. Briefly, $6x10^{-5}$ mol/l DPPH$^{\cdot}$ solution in ethanol was prepared and 3.9 ml of this solution was added to 0.1 ml of the hesperidin (2- 6 mg/ml) and trolox solution (0.02- 0.06 mg/ml). The mixture was shaken vigorously and the decrease in absorbance at 517 nm was measured at 5, 10, 30 and 60 min. Water (0.1 ml) in place of hesperidin was used as control. The percent inhibition activity was calculated using the following equation:

Inhibition activity(%)= $[(A_0 - A_1)/A_0 \times 100]$

Where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of hesperidin sample.

Determination of total antioxidant activity determination

The antioxidant activity was determined according to the thiocyanate method with slight modifications (Osawa and Namiki[17]). For the stock solution, 10 mg of hesperidin was dissolved in 10 ml water. Then the solution of hesperidin or standards samples (tocopherol, trolox, BHA and BHT) [100mg/l] in 2.5 ml of potassium phosphate buffer (0.04 M, pH 7.6) was added to 2.5 ml of linoleic acid emulsion. Fifty ml linoleic acid emulsion contained Tween-20 (175 µg), linoleic acid (155 µl) and potassium phosphate buffer (0.04 M, pH 7.6). On the other hand, 5.0 ml of control contained 2.5 ml of linoleic acid emulsion and 2.5 ml of potassium phosphate buffer (0.04 M, pH 7.6). Each solution was then incubated at 37°C in a glass flask in the dark. At 24 h intervals during incubation, 0.1 ml of this incubation solution was added to 4.7 ml of 75% (v/v) ethanol and 0.1 ml of 30% (w/v) ammonium thiocyanate. Precisely 3 min after 0.1 ml of 0.02 M $FeCl_2$ in 3.5% (w/v) HCl was added to the reaction mixture, the absorbance of the red colour was measured at 500 nm in a spectrophotometer. The solutions without added hesperidin or standards were used as the control. The inhibition of lipid peroxidation in percentage was calculated by the following equation:

Inhibition % = $[(A_0 - A_1)/A_0 \times 100]$

Where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of hesperidin or standards.

Determination of superoxide radical scavenging activity

Measurement of superoxide anion scavenging activity of hesperidin was based on the method described by Liu et al.[18]. Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system by oxidation of NADH and assayed by reduction of nitroblue tetrazolium (NBT). In this experiment, the

superoxide anion was generated in 3 ml of Tris-HCl buffer (16 mM, pH 8.0) containing 1 ml of NBT (50 µM) solution, 1 ml of NADH (78 µM) solution and different concentrations (0.1-1.25mg/ml) of sample solution. The reaction was started by adding 1 ml of PMS solution (10 µM) to the mixture. The reaction mixture was incubated at 25°C for 5 min and absorbance at 560 nm was recorded against blank samples in a spectrophotometer. Trolox, ascorbic acid and BHA were used as standard samples (0.1-1.25 mg/ml).

Determination of hydrogen peroxide scavenging activity

Hesperidin (100µg/ml) was dissolved in 3.4 ml of 0.1 M phosphate buffer (pH 7.6) and mixed with 0.6 ml of 43 mM hydrogen peroxide solution. The absorbance value (at 230 nm) of the reaction mixture was recorded after 40 min. For each concentration, a separate blank sample was used for background subtraction (Ruch et al.)[19]. α- tocopherol, BHT and BHA (100µg/ml) were used as standard antioxidants. The solutions without added hesperidin or standards were used as the control. The percentage of scavenged hydrogen peroxide of hesperidin and standard compounds was calculated using the following equation:

Scavenged H_2O_2 % = $[(A_0-A_1)/A_0 \times 100]$

Where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of hesperidin and standards.

Determination of hydroxyl radical scavenging activity

The effect of hydroxyl radical was assayed by using the 2-deoxyribose oxidation method (Chung et al.,)[20]. 2-Deoxyribose is oxidized by the hydroxyl radical that is formed by the Fenton reaction and degraded to malondialdehyde. The reaction mixture contained 0.45 ml of 0.2 M sodium phosphate (pH 7.6), 0.15 ml of 10 mM 2-deoxyribose, 0.15 ml of 10 mM $FeSO_4$-EDTA, 0.15 ml of 10 mM hydrogen peroxide, 0.525 ml of distilled water and 0.075 ml (20-120µg/ml) of sample solution in a tube. The reaction was started by the addition of hydrogen peroxide. After incubation at 37°C for 4 h, the reaction was stopped by adding 0.75 ml of 2.8% (w/v) trichloroacetic acid and 0.75 ml of 1.0% (w/v) of thiobarbituric acid. The mixture was boiled for 10 min, cooled in ice and then measured at 520 nm. The reaction mixture not containing test sample was used as the control. Trolox, ascorbic acid, BHT and BHA (20-120µg/ml) were used as standard antioxidants The scavenging activity on hydroxyl radicals was expressed as:

The scavenging activity on hydroxyl radicals = $[(A_0 - A_1)/A_0 \times 100]$

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Where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of hesperidin sample.

Results

Figure 1 shows the reducing power of hesperidin. The reducing power of hesperidin increased with increasing concentration. Based on a comparison of the absorbance at 700 nm, the reducing power of hesperidin at a concentration of 20µg/ml was similar to that of BHA at 20µg/ml. This indicates that hesperidin was electron donors and could also react with free radicals, converting them to more stable products and terminate the radical chain reaction. Also, 120µg/ml hesperidin is the best concentration which exhibits the most reducing power.

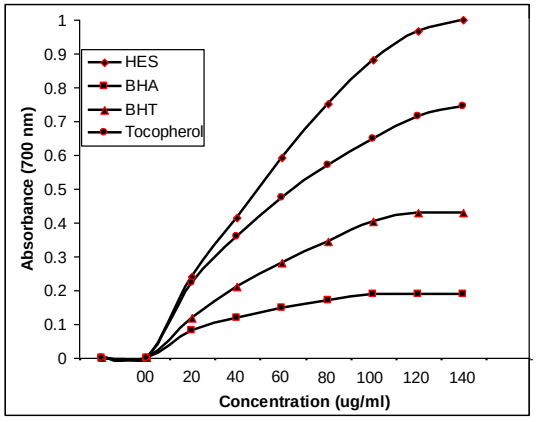

Fig. 1: Reducing power of hesperidin (HES), BHA, BHT and α-tocopherol.

The reducing power of hesperidin and both standards decreased in the order of hesperidin > BHA > BHT> α-tocopherol. Fig. 2. Shows the chelating effect of hesperidin. All samples at 1.0, 1.25 and 1.50 mg/ml concentration showed more than 45% chelating effect on ferrous ions at an incubation time of 120 min.

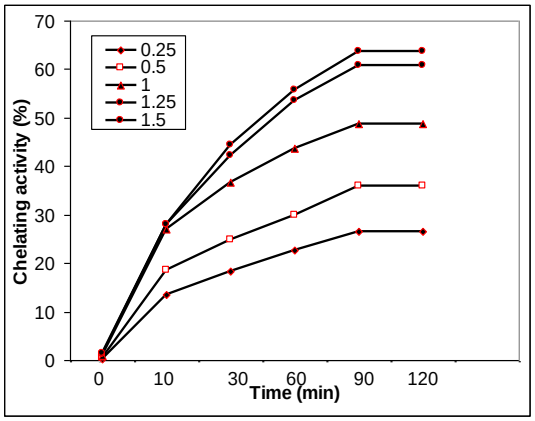

Fig. 2: Chelating effects of different concentrations of hesperidin on Fe^{2+} ions at different incubation times with $FeCl_2$

Also, the chelating activity of hesperidin at a concentration of 1.0, 1.25 and 1.50 mg/ml are the same at the first 10 min. In addition, no change in chelating activity of hesperidin at a concentration more than 1.25 mg/ml. The chelating activity of samples increased with increasing incubation times with $FeCl_2$. However, the chelating activity of hesperidin of 1.0 mg/ml was nearly equal to EDTA at 0.037 mg/ml (43.67%) for an incubation time of 30 min. This indicates that the chelation property of the samples on Fe^{2+} ions may afford protection against oxidative damage.

The DPPH$^{\cdot}$ radical scavenging effects of hesperidin are presented in Fig. 3. and showed appreciable free radical scavenging activities. The free radical scavenging activity of hesperidin was

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compared to trolox, as a synthetic antioxidant. Hesperidin of 6 mg/ml had the highest radical scavenging activity when compared with 0.06 mg/ml trolox.

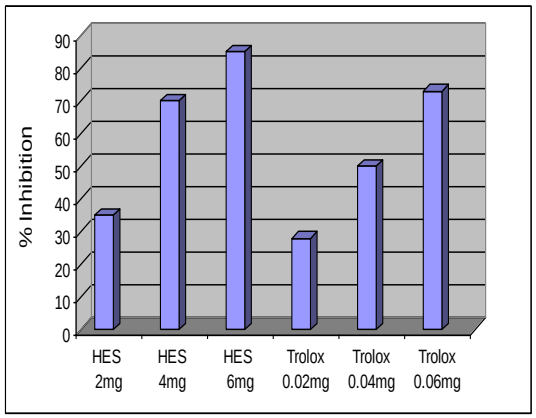

Fig. 3: Scavenging activities of different concentrations of hesperidin (HES) and trolox against the 1,1-diphenyl-2-picryl-hydrazil (DPPH˙) radical.

The effects of 100 mg/l of hesperidin on peroxidation of linoleic acid emulsion are shown in Fig. 4. Hesperidin showed higher antioxidant activity when compared to α-tocopherol, trolox, BHA, and BHT.

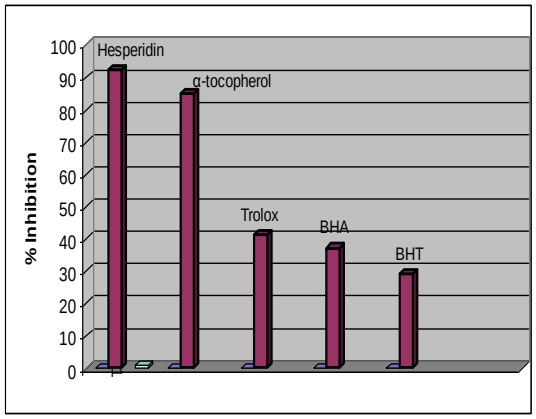

Fig. 4: Total antioxidant activities of hesperidin, α-tocopherol, trolox and BHA, BHT (100 mg/l concentration) on peroxidation of linoleic acid emulsion

Total antioxidant activity of hesperidin and both standards decreased in the order of hesperidin > α-tocopherol > trolox > BHA >BHT.

Fig. 5 shows the superoxide radical scavenging activity by 0.1-1.25 mg/ml of hesperidin in comparison to the same amount of BHA, trolox and ascorbic acid. At 0.1-1.25 mg/ml concentrations, hesperidin showed higher superoxide radical scavenging activity than trolox, BHA, and ascorbic acid.

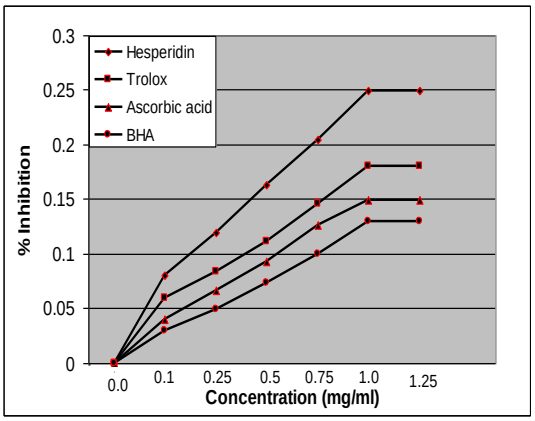

Fig. 5: Superoxide radical scavenging activity of hesperidin (HES), trolox, ascorbic acid and BHA at different concentrations

The superoxide radical scavenging activity of hesperidin and both standards decreased in the order of hesperidin > trolox > ascorbic acid > BHA.

Figure 6 presents the scavenging activity of samples on H_2O_2. The results are compared with BHA, BHT, and α-tocopherol as standards. Hesperidin was capable of scavenging activity in a concentration–dependent manner. At 100µg/ml, hesperidin exhibited 83.5%. On the other hand, BHA, BHT, and α-

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tocopherol exhibited 51.2%, 65.4%, and 77.6%, respectively, of H_2O_2 scavenging activity at the same concentration.

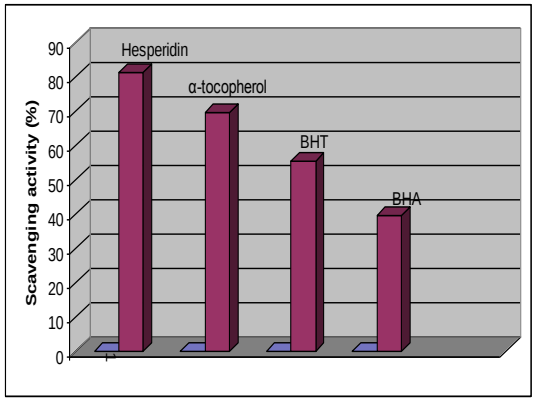

Fig. 6: Hydrogen peroxide scavenging activity of hesperidin, α-tocopherol, BHT and BHA at 100µg/ml concentration

Fig. 7 shows the hydroxyl radical scavenging effects of hesperidin. The scavenging effect of hesperidin on hydroxyl radical was concentration dependent. At 20-120µg/ml concentrations, hesperidin exhibited higher hydroxyl radical scavenging activity than ascorbic acid. Among the oxygen radicals, hydroxyl radical is the most reactive chemical species known.

The hydroxyl radical induces some oxidative damage to biomolecules such as all proteins, DNA, nucleic acid. The hydroxyl radical scavenging activity of hesperidin and both standards decreased in the order of hesperidin > trolox > ascorbic acid > BHT> BHA.

Discussion

Damages of biological systems caused by reactive oxygen species belong to

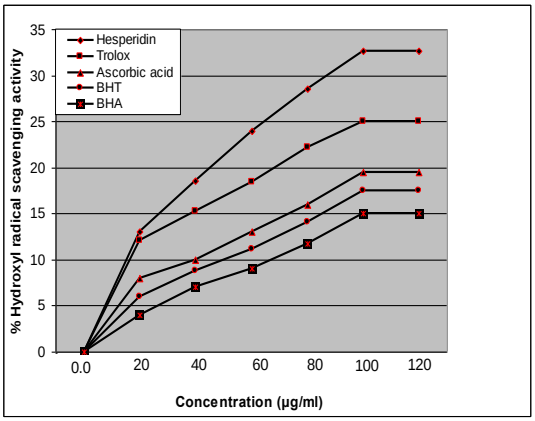

Fig. 7: Hydroxyl radical scavenging activities of hesperidin, trolox, ascorbic acid, BHT and BHA at different concentrations.

processes directly linked with development of cardiovascular and malignant diseases. Human organism possesses systems controlling oxidation processes posing a threat to structures and functions of cells. Three defense mechanisms has been developed[21], including: prevention of reactions of reactive oxygen species with biologically-significant compounds, breaking free-radical chain reactions and undesirable non-radical oxidation reactions, scavenging the products of free radicals reactions with biological substances and repair of damages.

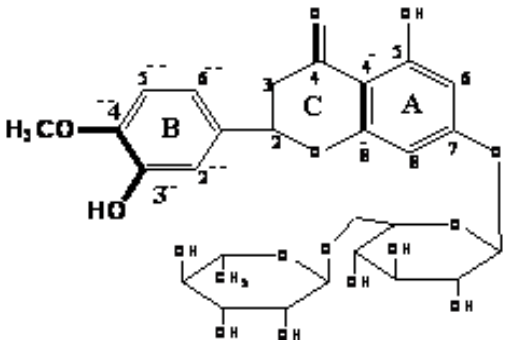

Fig. 8: Hesperidin structure

Flavonoids' activity as antioxidants refers to their ability to transfer a hydrogen atom or an electron and to the possibility of their interactions with other antioxidants[10]. The reducing power has been used as one of the antioxidant capability indicators of plants[22]. In the reducing power assay, the presence of reductants (antioxidants) in the tested samples resulted in the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}) fig. 1. The amount of Fe^{2+} complex can therefore be monitored by measuring the formation of Perl's Prussian Blue at 700 nm[2].

Hesperidin may serve as a hydrogen donor for α-tocopherol radical, thus regenerating α -tocopherol – a key element of redox balance in biosystems. The hesperidin radical formed may be reduced by vitamin C which is converted into ascorbyl radical[5]. The results in fig. 2 indicated that A significant property of hesperidin is its capability for blocking the oxidative activity of systems with transition metal ion (Fe^{2+}/Fe^{3+}) that play an essential role in the formation of reactive oxygen species in Fenton's reactions.

Flavonoids are naturally occurring phenolic phytochemicals, which have been reported to possess several biological properties in vitro. Despite the similarity between flavonoid structures, the biological properties vary considerably with only minor modifications in their structure. The number and specific positions of hydroxyl groups and the nature of the substitutions determine whether flavonoids function as strong antioxidative[23], anti-inflammatory, antiproliferative[24] or enzyme modulating agents. Hydroxylation of the B-ring, where a catechol group is the key to the flavonoid activity, coupled with a 2, 3 double bond in conjugation with a 4-oxo function (carbonyl group) in the C-ring[23].

The antioxidant properties of hesperidin result from their chemical structure: 3"–o-hydroxy,4"-o-methoxy system in the B ring, reciprocal configuration of the double bond C4'-C8' and the 4-carbonyl group of the C ring, and configuration of the 5-hydroxyl group and the double bond C5-C6 of the A ring[25](Figure 8). All the mentioned structural conditions may be found in a hesperidin molecule which, in the *in vitro* systems efficiently scavenges hydroxyl radical ($OH^{\bullet}$), superoxide radical ($LOO^{\bullet}$), superoxide anion radical ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), and nitrogen oxide ($NO^{\bullet}$).

Free radicals are known to be a major factor in biological damages, and $DPPH^{\bullet}$ has been used to evaluate the free radical-scavenging activity of natural antioxidants[26]. $DPPH^{\bullet}$, which is a molecule containing a stable free radical

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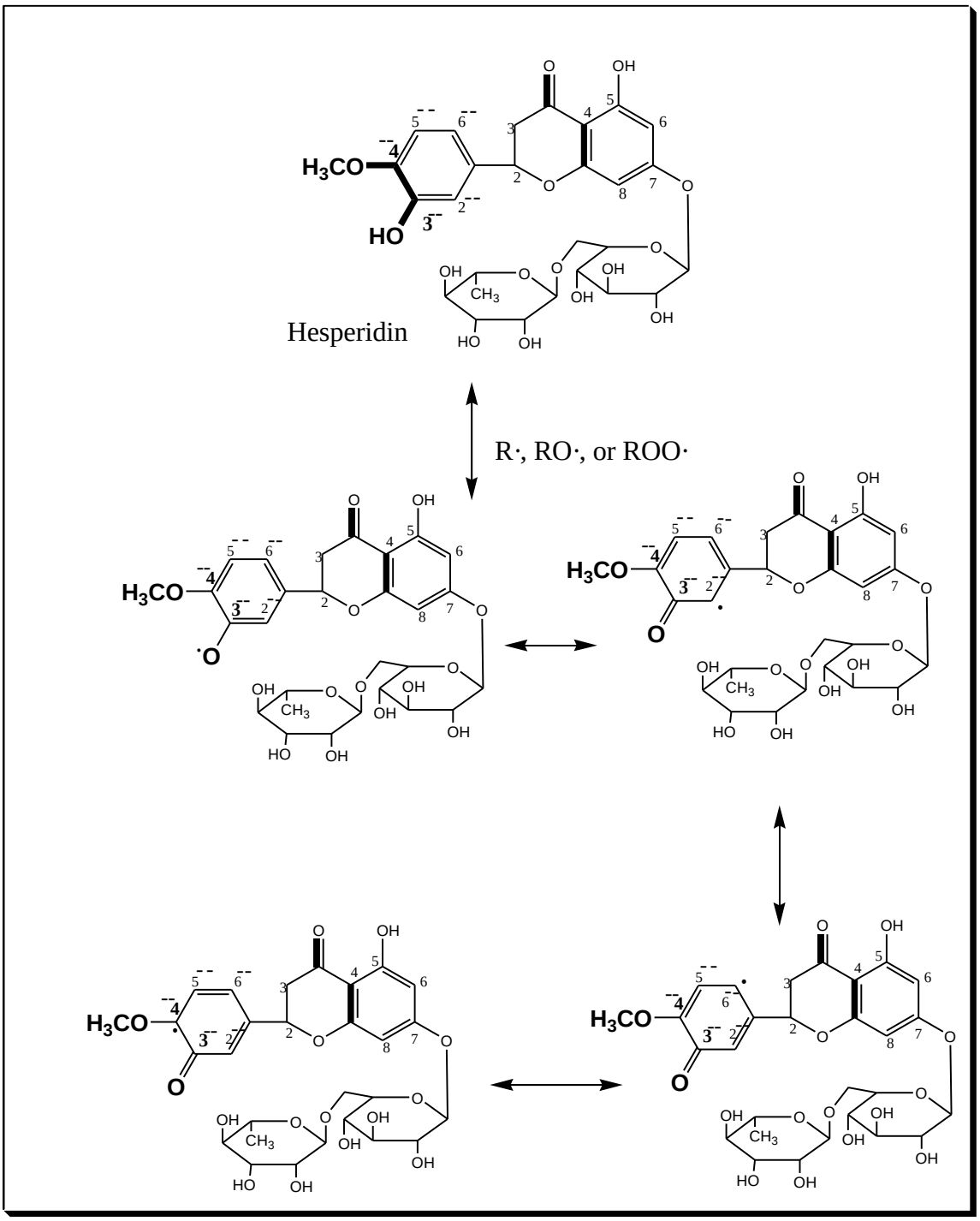

Scheme 1: Proposal mechanism of hesperidin antioxidant activity

with a purple color, changes into a stable compound with a yellow color by reacting with an antioxidant which can donate an electron to DPPH•. In such case, the purple color typical of the free DPPH• radical decays, a change which can be followed either spectrophotometrically (517 nm). The proton radical scavenging action is known as an important mechanism of antioxidation. 1,1-Diphenyl-2-picrylhydrazil (DPPH•) is used as a free radical to evaluate the antioxidative

activity of some natural sources[2]. The DPPH radical scavenging effects of hesperidin was presented in Fig. 3. From these results, it can be stated that hesperidin have the ability to scavenge free radicals and could serve as a strong free radical inhibitor or scavenger according to trolox. On the other hand, such dietary antioxidant, hesperidin may be particularly important in protecting cellular DNA, lipids and proteins from free radical damage. Many attempts at explaining the structure-activity relationships of some natural antioxidant compounds have been reported in the literature. It has been reported that the antioxidant activity of phenolic compounds may result from the neutralization of free radicals initiating oxidation processes, or from the termination of radical chain reactions, due to their hydrogen donating ability[27]. It is also known that the antioxidant activity of polyphenolic compounds is closely associated with their structures, such as substitutions on the aromatic ring and side chain structure. Their accessibility to the radical centre of DPPH$^{\bullet}$ could also influence the order of the antioxidant power. Free radical scavenging activity of polyphenolic compounds is believed to be influenced by the number and position of phenolic hydrogen in their molecules[23]. It is also proposed that the higher antioxidant activity is related to the greater number of hydroxyl groups on the flavonoid nucleus[28].

In overall agreement with structure-activity relationship studies of the free radical scavenging capacity of flavonoids [23&29], several structural features were shown to be important for the protective effect of flavonoids against glutamate-mediated programmed cell death[30] including the presence of a hydroxyl group on C-3" and a 4'-8' double bond in conjugation with a C-4 ketone function The formation of hydrogen bonds between the ketonic oxygen and the hydroxyls at C-3" and C-5 may have some influence on the scavenging power as well [29].

The total antioxidant activity of hesperidin was determined by the thiocyanate method in linoleic acid emulsion. The antioxidative activities of hesperidin were compared with commercial antioxidants such as α-tocopherol (Toc), BHT, BHA and trolox. Total antioxidant activity of hesperidin and both standards decreased in the order of hesperidin > α-tocopherol > trolox > BHA > BHT fig. 4.

Superoxide radical, known to be very harmful to cellular components as a precursor of the more reactive oxygen species, contributes to tissue damage and various diseases. In a biological system, its

toxic role can be eliminated by superoxide dismutase[2]. The radicals may also play an important role during the peroxidation of unsaturated fatty acids and other potential susceptible substances[31]. The superoxide radical scavenging activity of hesperidin and both standards decreased in the order of hesperidin > trolox > ascorbic acid > BHA fig. 5. Hydrogen peroxide is an intermediate during endogenous oxidative metabolism and mediates radical oxygen formation such as OH, which may be used to predict the scavenging capability of antioxidants in biological systems[32]. H_2O_2 has only a weak activity to initiate lipid peroxidation, but its activity as an active oxygen species comes from its potential to produce the highly reactive hydroxyl radical through the Fenton reaction. These results in fig. 6 showed that hesperidin have a strong H_2O_2 scavenging activity. At 100µg/ml concentration, H_2O_2 scavenging activity of hesperidin and both standards decreased in the order of hesperidin > α-tocopherol > BHT > BHA. The mechanism of hesperidin antioxidant activity was designed by hydrogen donation to free radicals and formation of a complex between the lipid radical and the antioxidant radical (hesperidin, free radical acceptor). The following scheme explains the proposal mechanism of hesperidin antioxidant activity.

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