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Short Communication

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A Convenient Mechanism for the Free Radical Scavenging Activity of Resveratrol

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A B S T R A C T

Resveratrol(3,5,4'-trihydroxy-trans-stilbene) is a stilbenoid, a type of natural phenol, and a phytoalexin produced naturally by several plants when under attack by pathogens such as bacteria or fungi was evaluated for possible antioxidant and free radical scavenging activities. 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 ⍺-tocopherol, ascorbic acid, butylated hydroxytoluene(BHT), butylated hydroxyanisole (BHA) and trolox. Resveratrol exhibited a strong reducing power, chelating activity on Fe^{2+}, free radical-scavenging, hydrogen peroxide scavenging and hydroxyl radical scavenging activities. Antioxidant activity of resveratrol increased with increased concentrations.Total antioxidant activity of resveratrol and both standards decreased in the order of ⍺-tocopherol > resveratrol > trolox > BHA > BHT. This study showed that resveratrol exhibited antioxidant activity in all tests and could be considered as a source of natural antioxidants.

Keyword: Resveratrol, antioxidant activity, ⍺- tocopherol, ascorbic acid and butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA).

Introduction

Oxygen, an element indispensable for life, can under certain circumstances, adversely affect the human body. It is produced by plants during photosynthesis, and is necessary for aerobic respiration in animals. The oxygen consumption inherent in cell growth leads to the generation of a series of reactive oxygen species (ROS). They are continuously produced by the body's normal use of oxygen such as respiration and some cell mediated immune functions. ROS include free radicals such as superoxide anion radicals ($O_2 \cdot^-$), hydroxyl radicals ($OH^{\cdot}$) and non free-radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$)[1–3]. ROS are continuously produced during normal physiologic events and can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. 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]. Resveratrol (3, 4', 5-trihydroxystilbene) is a phytoalexin found in a wide variety of dietary sources including grapes, plums and peanuts. It is also present in wines, especially red wines and to a much lesser extent in white wines. Its stilbene structure is related to the synthetic oestrogen diethylstilbestrol. Resveratrol exists as cis- and trans-isomers. In in vitro and in vivo experiments, resveratrol

displays diverse pharmacological effects including modulation of lipoprotein metabolism and cardiovascular protection[5], anti-inflammation[6], platelet antiaggregatory activity[5], antimicrobial activity[7], antiallergic activity[8], anticancer properties[9,10], and most notably, antioxidant properties[11]. In the present study, the iron chelating and antioxidant activities of resveratrol was 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

-Resveratrol was from Aldrich (USA).

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

Methods

Determination of reducing power

The reducing power of resveratrol was measured according to the method of Oyaizu [12]. Various concentrations of resveratrol (120-720μg) 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. BHT was used as standard antioxidant. Higher absorbance of the reaction mixture indicated greater reducing power.

Determination of chelating activity on Fe^{2+}

The chelating activity of resveratrol on ferrous ions (Fe^{2+}) was measured according to the method of Decker and Welch[13]. 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 resveratrol 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–(Absorbance of sample/Absorbance of control)]x100]

Control test was performed without addition of resveratrol.

Determination of free radical-scavenging activity

The free radical scavenging activity of resveratrol was measured with 1,1-diphenyl-2-picrylhydrazil (DPPH•) using the slightly modified methods of Brand William et al.[14] and Takashira and Ohtake[15]. Briefly, $6x10^{-5}$ mol/l DPPH• solution in ethanol was prepared and 3.9 ml of this solution was added to 0.1 ml of the resveratrol (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 resveratrol 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 resveratrol sample.

Determination of total antioxidant activity determination

The antioxidant activity was determined according to the thiocyanate method with slight modifications (Osawa and Namiki[16]). For the stock solution, 10 mg of resveratrol was dissolved in 10 ml water. Then the solution of resveratrol 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 resveratrol 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 resveratrol or standards.

Determination of superoxide radical scavenging activity

Measurement of superoxide anion scavenging activity of resveratrol was based on the method described by Liu et al.[17]. Superoxide anions were generated in a non-enzymatic phenazine methoxy sulfate-nicotinamide adenine dinucleotide (PMS-NADH) system by oxidation of NADH and assayed by reduction of nitro blue 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 100 mg/l of resveratrol solution. The reaction was started by adding 1 ml of PMS-NADH 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 and BHT were used as standard samples (100 mg/l).

Determination of hydrogen peroxide scavenging activity

Resveratrol (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.,)[18]. α- tocopherol, BHT and BHA (100μg/ml) were used as standard antioxidants. The solutions without added resveratrol or standards were used as the control. The percentage of scavenged hydrogen peroxide of resveratrol 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 resveratrol 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.,)[19]. 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 (20-100μg/ml) of resveratrol 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 (20-100μg/ml) were used as standard antioxidants. The scavenging activity on hydroxyl radicals was expressed as:

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

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

Results

Figure 1 shows the reducing power of resveratrol. The reducing power of resveratrol increased with increasing concentration. Based on a comparison of the absorbance at 700 nm, the reducing power of resveratrol at a concentration of 120μg/ml was similar to that of BHT at 480μg/ml. This indicates that resveratrol was electron donors and could also react with free radicals, converting them to more stable products and terminate the radical chain reaction. Also, 480μg/ml resveratrol is the best concentration which exhibits the most reducing power. The reducing power of resveratrol and BHT decreased in the order of resveratrol > BHT.

Fig. 2.shows the chelating effect of resveratrol. 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. Also, the chelating activity of resveratrol at a concentration of 1.0, 1.25 and 1.50 mg/ml are the same at the zero time. In addition, the maximum chelating activity of resveratrol at a concentration at 1.50 mg/ml. The chelating activity of samples increased with increasing incubation times with $FeCl_2$. However, the chelating activity of resvsratol of 1.50 mg/ml was nearly equal to EDTA at 0.037 mg/ml (43.67%) for an incubation time of 60 min. This indicates that the chelation property of the samples on Fe^{2+} ions may afford protection against oxidative damage.

The DPPH· radical scavenging effects of resveratrol are presented in Fig. 3. and showed appreciable free radical scavenging activities. The free radical scavenging activity of resveratrol was compared to trolox, as a synthetic antioxidant. Resveratrol of 150 ug/ml had the highest radical scavenging activity when compared with 150 ug/ml trolox.

The effects of 100 mg/l of resveratrol on peroxidation of linoleic acid emulsion are shown in Fig. 4. Resveratrol showed higher antioxidant activity when compared to α-tocopherol, trolox, BHA, and BHT. Total antioxidant activity of resveratrol and both standards decreased in the order of α-tocopherol > resveratrol > trolox > BHA > BHT.

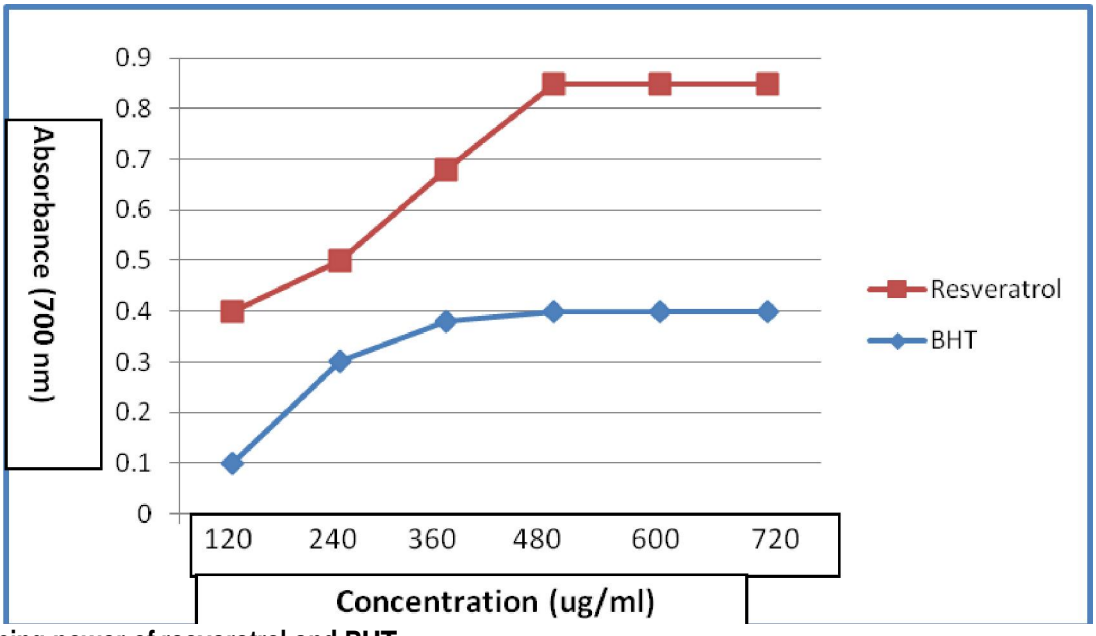


Fig. 1: Reducing power of resveratrol and BHT.

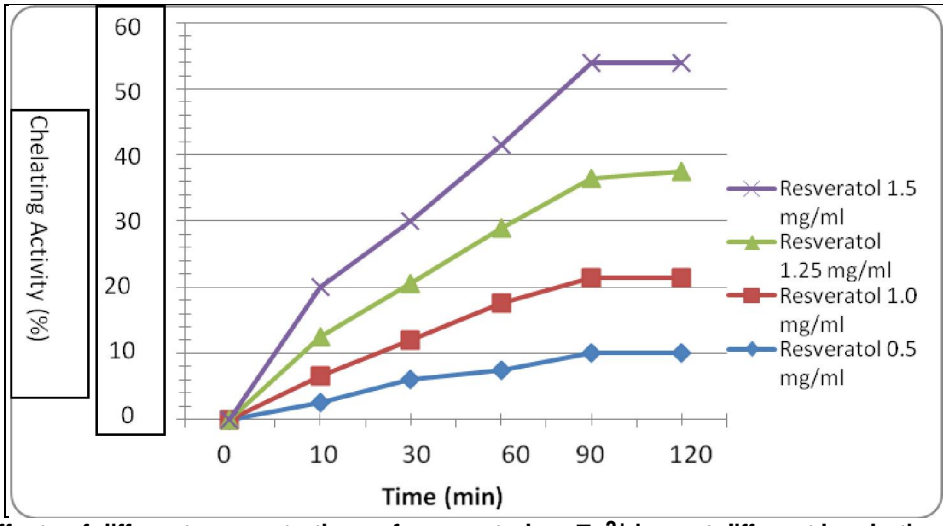


Fig. 2: Chelating effects of different concentrations of resveratrol on Fe^{2+} ions at different incubation times with $FeCl_2$.

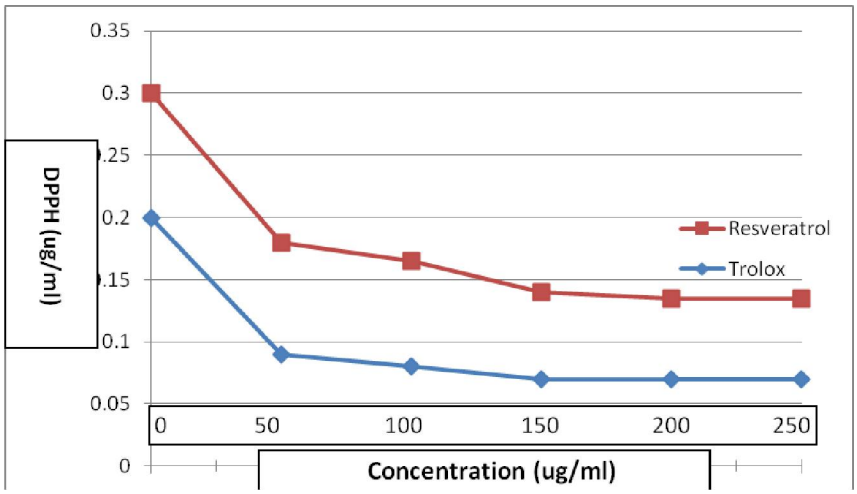


Fig. 3: Scavenging activities of different concentrations of resveratrol and trolox against the 1,1-diphenyl-2-picryl-hydrazil ($DPPH^{\cdot}$) radical.

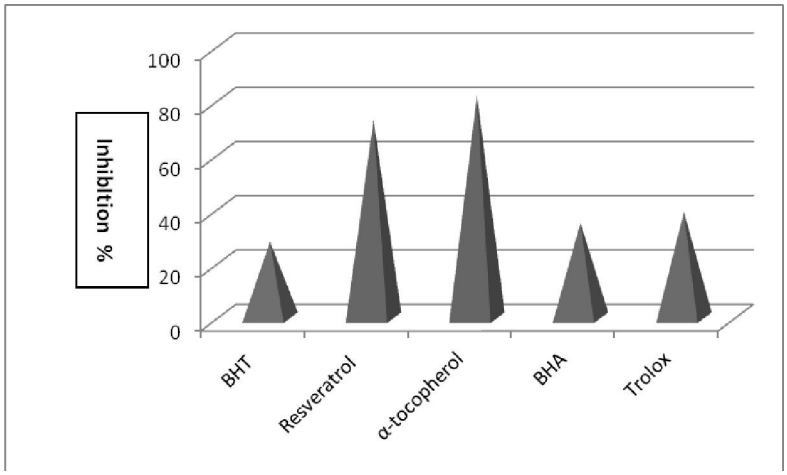


Fig. 4: Total antioxidant activities of resveratrol, α-tocopherol, trolox and BHA, BHT (100 mg/l concentration) on peroxidation of linoleic acid emulsion.

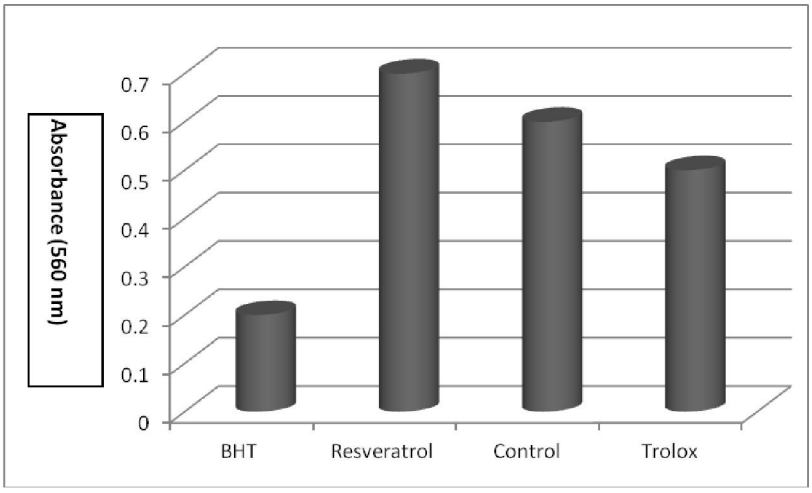


Fig. 5: Superoxide radical scavenging activity of resveratrol, trolox and BHT at 100 mg/l concentration.

Fig. 5 shows the superoxide radical scavenging activity at 100 mg/l of resveratrol in comparison to the same amount of BHT, trolox and ascorbic acid. At 100 mg/l concentrations, resveratrol showed higher superoxide radical scavenging activity than trolox, BHA, and ascorbic acid. The superoxide radical scavenging activity of resveratrol and both standards decreased in the order of resveratrol > trolox > BHT.

Figure 6 presents the scavenging activity of samples on H_2O_2. The results are compared with BHA, BHT, and α-tocopherol as standards. Resveratrol was capable of scavenging activity in a concentration–dependent manner. At 100μg/ml, resveratrol exhibited 59.5%. On the other hand, BHA, BHT, and α-tocopherol exhibited 34.6%, 48.3%, and 73.6%, respectively, of H_2O_2 scavenging activity at the same concentration.

Fig. 7 shows the hydroxyl radical scavenging effects determined by the 2-deoxyribose oxidation method. The scavenging effect of resveratrol on hydroxyl radical was concentration dependent. At 20-100μg/ml concentrations, resveratrol 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 resveratrol and both standards decreased in the order of resveratrol > trolox > ascorbic acid > BHT> BHA.

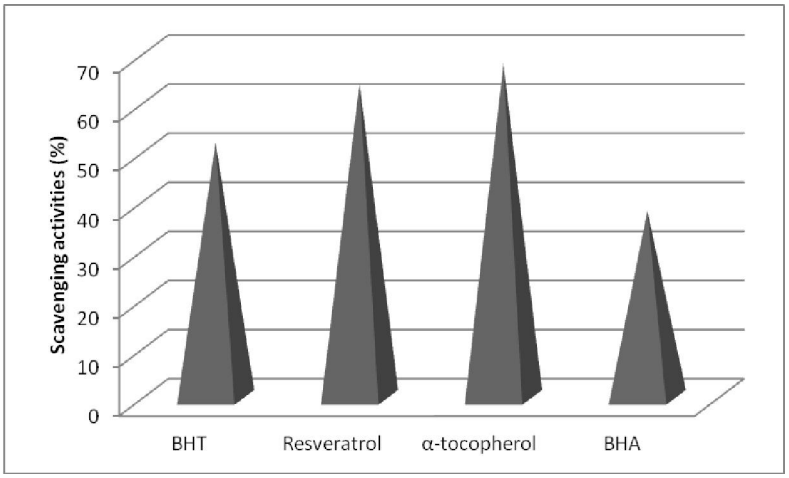


Fig. 6: Hydrogen peroxide scavenging activity of resveratrol, α-tocopherol, BHT and BHA at 100μg/ml concentration.

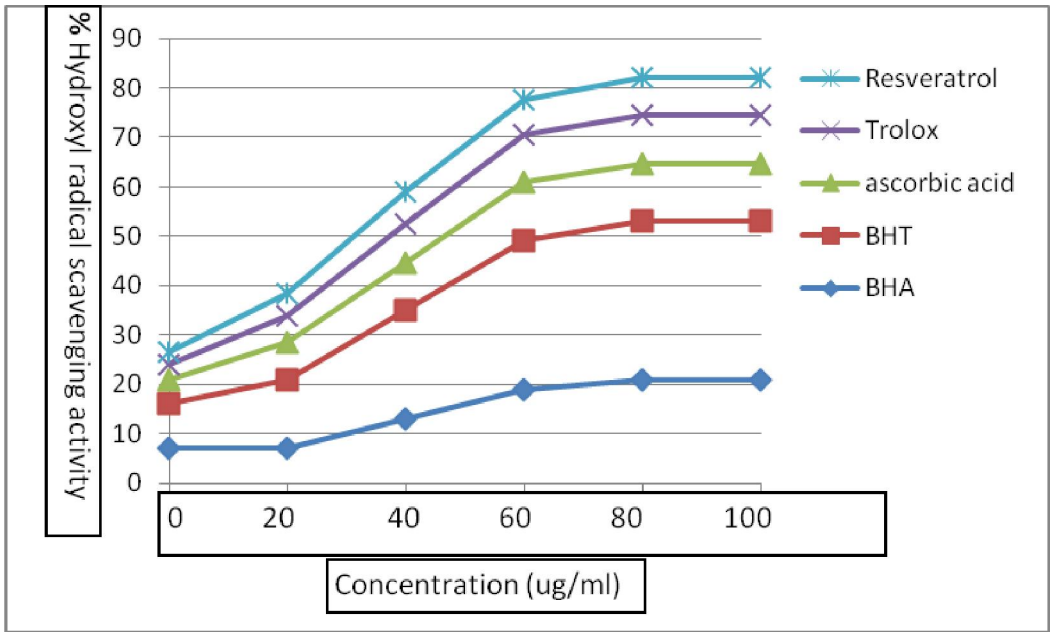


Fig. 7: Hydroxyl radical scavenging activities of resveratrol, trolox, ascorbic acid, BHT and BHA at different concentrations.

Discussion

Damages of biological systems caused by reactive oxygen species belong to 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[20], 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. Resvsratrol's 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[4]. The reducing power has been used as one of the antioxidant capability indicators of plants[21]. 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].

Resveratrol may serve as a hydrogen donor for ⍺-tocopherol radical, thus regenerating ⍺ -tocopherol – a key element of redox balance in biosystems. The resveratrol radical formed may be reduced by vitamin C which is converted into ascorbyl radical[22]. The results in fig. 2 indicated that A significant property of resvsratrol 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.

Resveratrol 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.

The antioxidant properties of resveratrol result from their chemical structure: P-hydroxyl group in ring A and conjugated double bond system. All the mentioned

structural conditions may be found in a resveratrol 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 ($NO^{\bullet}$).

Resveratrol, non flavonoid was found to exhibit antiradical activity was stronger than BHT Fig. 1. The strong antiradical activity of resveratrol could be due to the presence of conjugated double bond, which makes the electrons more delocalized. 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[25]. $DPPH^{\bullet}$, which is a molecule containing a stable free radical with a purple color, changes into a stable compound with a yellow color by reacting with an antioxidant which can donate an electron to $DPPH^{\bullet}$. In such case, the purple color typical of the free $DPPH^{\bullet}$ 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 ($DPPH^{\bullet}$) is used as a free radical to evaluate the antioxidative activity of some natural sources[26]. The $DPPH^{\bullet}$ radical scavenging effects of resveratrol are presented in Fig. 3. From these results, it can be stated that resveratrol 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, resveratrol 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 phenolic 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 phenolic 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 of resveratrol is related to the greater number of hydroxyl groups.[28]

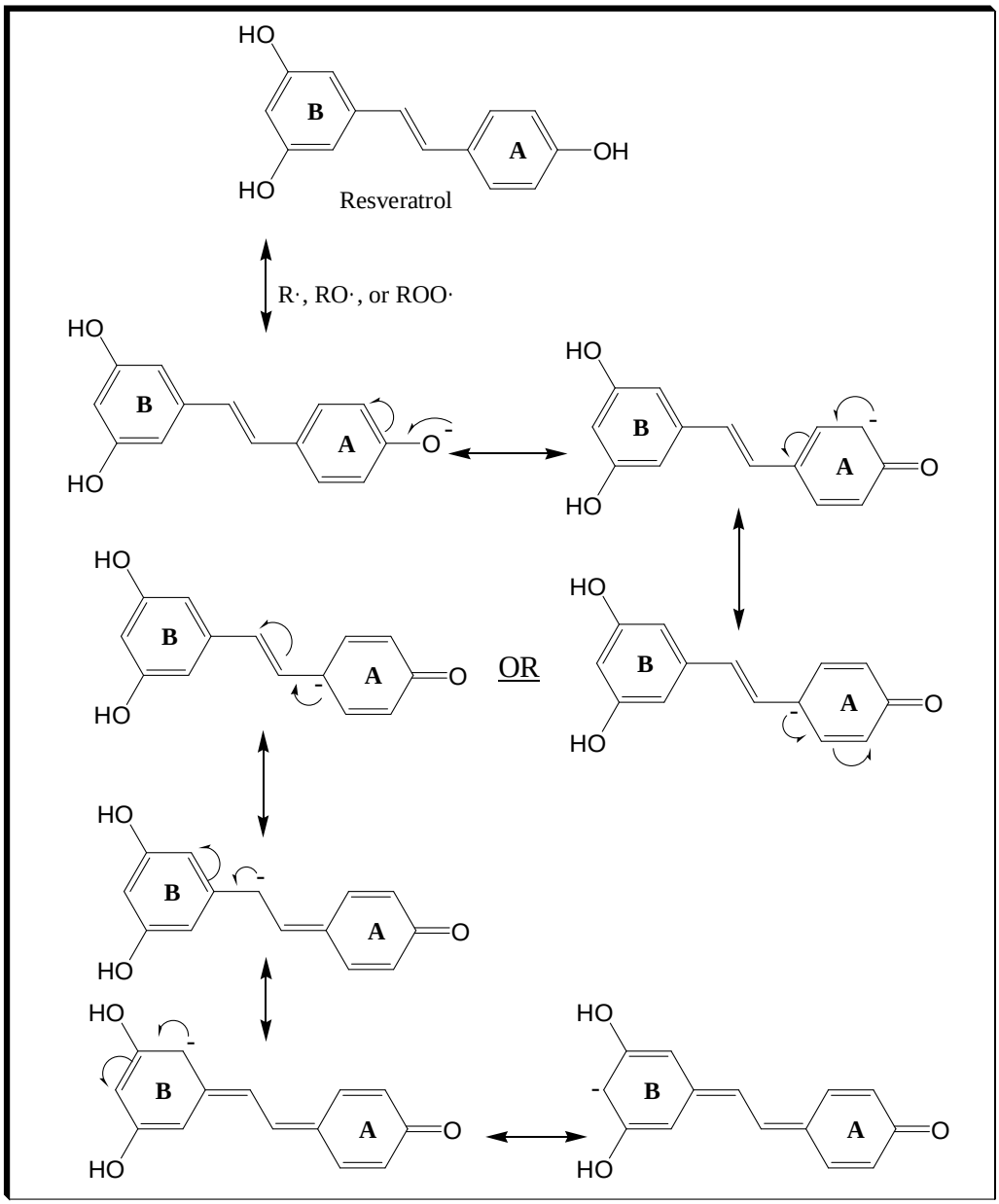
The total antioxidant activity of resveratrol was determined by the thiocyanate method in linoleic acid emulsion. The antioxidative activities of resveratrol were compared with commercial antioxidants such as α-tocopherol (Toc), BHT, BHA and trolox. Total antioxidant activity of resveratrol and both standards decreased in the order of α-tocopherol > resveratrol > trolox > BHA > BHT fig. 4. In the terms of molecules of DPPH, resveratrol was equivalent to α-tocopherol. The reaction mechanism of DPPH with α-tocopherol can be explained by two-steps reaction: in the first step, one molecule of DPPH reacts with one molecule of α-tocopherol produce α-tocopheroxy radical and then, one α-tocopheroxy radical reacts with another molecule of DPPH to form α-tocopherolquinone. Hence, two molecules of DPPH are reduced by one molecule of α-tocopherol.

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[26]. The radicals may also play an important role during the peroxidation of unsaturated fatty acids and other potential susceptible substances[29]. The superoxide radical scavenging activity of resveratrol and both standards decreased in the order of resveratrol > trolox > 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[30]. 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

resveratrol have a strong H_2O_2 scavenging activity. At 100μg/ml concentration, H_2O_2 scavenging activity of resveratrol and both standards decreased in the order of α-tocopherol > resveratrol > BHT > BHA. The mechanism of resveratrol antioxidant activity was designed by hydrogen donation to free radicals and formation of a complex between the lipid radical and the antioxidant radical (resveratrol, free radical acceptor). The following scheme explains the proposal mechanism of resveratrol antioxidant activity.

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, and almost any biological molecule it touches, as food additives in order to increase the shelf life of foods by preventing lipid peroxidation. However, the mechanism of antioxidative activities of resveratrol is still unknown. The hydroxyl radical scavenging activity of resveratrol and both standards decreased in the order of resveratrol > trolox > ascorbic acid > BHT> BHA.



Scheme 1: Proposal mechanism of resveratrol antioxidant activity.

The structural requirement considered essential for effective radical scavenging by resveratrol is the presence of P-hydroxyl group in ring A and conjugated double bond. The presence of double bond between the two rings A and B makes the electrons more delocalized. P-hydroxy system in the A ring, which possesses electron donating properties and is a radical target. Also, the double bond between the two phenolic rings conjugated with a phenolic double bond, which is responsible for electron delocalization from the A ring to ring B to give the resveratrol-quinone structure (scheme 1).

Structure-activity relationship effect of resveratrol on their antioxidant property has not been reported earlier to our knowledge, and this study is perhaps the first observation of its kind.

In conclusion, the present study showed that the effects of antioxidative activity of resveratrol depend on the number and order of OH groups and the presence of double bond conjugated make the electrons more delocalized.

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