Stable free radical scavenging and antiperoxidative properties of resveratrol compared in vitro with some other bioflavonoids

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Stable free radical scavenging and antiperoxidative activities of resveratrol, a component of grapes and red wine, were evaluated and compared with some other known bioflavonoids (quercetin, catechin, kaempferol, myricetin, fisetin, ellagic acid and naringenin) widely present in the plant kingdom. Free radical scavenging activity was measured in an in vitro chemical system (DPPH assay), while for antiperoxidative activity, biological system comprising of hepatic and pulmonary homogenates was employed. Antiradical activity assay showed quercetin and myricetin to be stronger antiradical agents than resveratrol. Structure-activity study revealed that O-dihydroxy group on ring B of flavonoid plays a crucial role. A double bond at 2-3 position conjugated with a 4-oxo function and hydroxy groups at positions 3 and 5 also contribute towards antiradical activity of flavonoids. Resveratrol exhibited stronger antiradical activity than kaempferol and naringenin and was also more efficient than α-tocopherol, a known strong endogenous non-flavonoid antioxidant, used for comparison. In vitro antiperoxidative assay showed fisetin as the strongest and kaempferol as the weakest antioxidant. Resveratrol was found to be stronger antioxidant than catechin, myricetin, kaempferol and naringenin, but was weaker than quercetin, fisetin and α-tocopherol. Antiradical and antiperoxidative activities of resveratrol may explain its beneficial effects in disease states.

Assays exhibited no direct correlation between antiradical and antiperoxidative activities of the phenolics.

Keywords: antiradical, antioxidant, DPPH, resveratrol, lipid peroxidation, flavonoids, polyphenols, α-tocopherol

Reactive oxygen species play an important role in pathogenesis of several oxidative stress related diseases like carcinogenesis, cardiovascular diseases, rheumatoid arthritis, ulcerative colitis and neurological degenerative diseases. The micronutrients and non-nutrient components of fruits and vegetables play a preventive role in the development of these oxidative stress-induced disease conditions. Particular interest has been focused on polyphenolic compounds, comprising of flavonoids and non-flavonoids. Polyphenols are synthesized by the plants in response to injury or attack. Many of them are scavengers of free radicals, antioxidants, chelating agents, phytoestrogens and modifiers of various enzymatic and biological functions. Plant extracts that possess antioxidant activities, include tea, olives, grapes, grains, apple, etc. The phenolic antioxidants in plants belong to various classes, such as flavonols, flavonones, flavones, isoflavones, catechins and anthocyanidins, etc. The phenolic antioxidants like myricetin and kaempferol, catechin, quercetin and ellagic acid and naringenin also show anticarcinogenic activity in numerous animal model cell lines and in various organs such as colon, oesophagus, lung, liver, mammary gland and skin. Out of phenolics, stilbene compounds like resveratrol are receiving tremendous attention due to their chemopreventive activity.

Resveratrol (3, 5, 4′-trihydroxy-trans-stilbene), a naturally occurring stilbene found in grapes and in certain medicinal plants is believed to provide protection from fungal infection and other stress. Appreciable quantity of resveratrol is present in red wine, which in recent years has stimulated considerable interest on the effects of this compound on human health. Indeed, it has been proposed that resveratrol may be linked to the apparent inverse relationship between red wine consumption and the incidence of heart disease, the so-called 'French paradox'. Resveratrol is reported to have a diverse range of pharmacological properties, including anti-inflammatory, antiplatelet aggregation and anticarcinogenic activities, but its mechanism of action is not clear. One of its modes of action could

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be due to its antioxidative property as reported by Acquaviva et al.\textsuperscript{19}, however, no structure-activity relationship is reported. Hence, we evaluated the antiradical and antiperoxidative activities of resveratrol and compared them with some other known phenolics having anticarcinogenic activity like quercetin, catechin, kaempferol, myricetin, fisetin, naringenin and \( \alpha \)-tocopherol (Fig. 1). Antiradical activity of all the tested compounds was assessed in a chemical model, i.e., DPPH system and antiperoxidative activity was evaluated in a biological system. The novelty of the present work is the kinetic studies of DPPH-scavenging, finding number of molecules of DPPH scavenged by each molecule of resveratrol and other test compounds, and finally comparing chemical system with biological system of free radical scavenging.

Materials and Methods

1,1-Diphenyl-2-picrylhydrazyl (DPPH), \( \alpha \)-tocopherol, catechin, myricetin, fisetin, quercetin, kaempferol, naringenin and resveratrol were procured from Sigma Chemical Company, MO, St. Louis, USA. All other chemicals of analytical grade were purchased locally.

Radical scavenging assay

Antiradical activity or hydrogen donating ability of different compounds was measured by spectrophotometric method, using DPPH, as described\textsuperscript{20}. The assay is based on the reduction of

Fig. 1—Chemical structures of the tested compounds
DPPH. Because of its odd electron, DPPH gives strong absorption maxima at 517 nm (purple color) by visible spectroscopy. As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, i.e., a free radical scavenging antioxidant, the absorption intensity is decreased, and the resulting decolourization is stochiometric with respect to the number of the electrons captured. For DPPH assay, an aliquot of antioxidant solution (200 µl) was mixed with 800 µl of 100 mM tris-HCl buffer (pH 7.4) and then added to 1 ml of 500 µM DPPH in ethanol (final concentration of 250 µM). The mixture was shaken thoroughly and the absorbance was measured at 517 nm at different time intervals. The reaction was performed in dark. There was a remnant optical density of DPPH at 517 nm (16.3% in average) even after its complete scavenging and thus a computer program in basic language was used for the correction factor (C.F), calculated by using the following formula:

\[
\text{C.F} = \frac{\text{Remnant optical density}}{(\text{Maximum O.D.}-\text{Remnant O.D.)}}
\]

By using the same computer program, µmoles of DPPH scavenged per µmoles of test compound was calculated. Percentage of DPPH scavenged by various phenolics at different concentrations, viz. 12.5, 25, 50, 100 and 200 µM at 10 min was calculated. From these concentration response curves, half maximal inhibitory concentration (IC$_{50}$) (average ± SD of four assays) for the individual phenolic was calculated.

**In vitro antioxidant activity assay**

Male Balb/c mice were sacrificed under light ether anaesthesia. Lungs and liver were perfused in situ through heart and portal vein, respectively. Both organs were excised and homogenates were prepared in cold 50 mM tris-HCl buffer (pH 7.4) and protein concentration was adjusted to 3 mg/ml. Inhibition in lipid peroxidation by ethanolic solution of various phenolics at different concentrations was measured. Briefly, the reaction mixture contained tris-HCl buffer (50 mM, pH 7.4), t-butylhydroperoxide (t-BHP, 500 µM in ethanol) and FeSO$_4$ (1 mM in distilled water). Control samples contained equal amount of ethanol. After incubating the samples at 37°C for 90 min, the reaction was stopped by adding 200 µl of 8.1% sodium dodecyl sulphate (SDS), followed with 1.5 ml of 20% acetic acid (pH 3.5). The amount of malondialdehyde (MDA) formed during incubation was estimated by adding 1.5 ml of 0.8% thiobarbituric acid (TBA) and heating the samples at 95°C for 45 min. Samples were cooled, centrifuged and TBA-reactive substances (TBARS) were measured in supernatants at 532 nm. TBARS were quantified using the extinction coefficient 1.53×10$^5$ M$^{-1}$ cm$^{-1}$. IC$_{50}$ was calculated from the plot drawn for the percentage inhibition vs. concentration of phenolic used.

Protein in the homogenates was estimated by the method of Lowry et al.

**Results**

Scavenging of stable free radicals by phenolics

Radical scavenging activity of resveratrol and other test compounds (Fig. 1), calculated at time intervals of 10 min and 2 hr and at different concentrations, by measuring optical density at 517 nm is shown in Fig. 2. From the curves, concentration of each test compound was calculated.
oxidant causing 50% radical scavenging (IC_{50}) against 250 µM DPPH was calculated. The results showed myricetin and quercetin to be the strongest antiradicals in term of number of molecules of DPPH scavenged per molecule of the compound, followed by fisetin and catechin. Also, the IC_{50} at 10 min for slow reacting phenolics was less than at IC_{50} at 2 hr (Table 1).

**Inhibition in lipid peroxidation**

Antiperoxidative property of resveratrol and other phenolics in biological systems, like liver and lung homogenates was also assessed. Liver was employed for the study, as it is the main storage site of phenolics. Further, we studied the effects on inhibition in lipid peroxidation in pulmonary homogenates, to have a generalized idea on the antiperoxidative property of phenolics in the biological systems. The assay system employed for the present work utilized Fe^{2+} and BHP (t-butylhydroperoxide) for the induction in lipid peroxidation. It induces lipid peroxidation through production of iron-oxygen complexes, such as ferryl and perferryl ions, which extract a hydrogen atom from the polyunsaturated fatty acids of membranes. The lipid carbon-centered radicals take up oxygen to produce lipid peroxy radicals, which propagate the chain reactions. Effects of various concentrations of resveratrol and of other phenolics on inhibition in hepatic lipid peroxidation *in vitro*, measured in terms of nmoles TBARS formed/mg protein/90 min are shown in Fig. 3. All the phenolic compounds inhibited lipid peroxidation in a dose-dependent manner. From concentration-inhibition curves, IC_{50} of different phenolics was calculated. IC_{50} of fisetin, α-tocopherol, quercetin, resveratrol, catechin, myricetin, kaempferol and naringenin were 20 ± 1.91, 27.5 ± 2.43, 35 ± 2.81, 50 ± 4.73, 80 ± 7.72, 90 ± 8.10 and more than 1000 µM, respectively. Comparative *in vitro* efficacy of various phenolics at 30 µM concentration on pulmonary lipid peroxidation inhibition is shown in Fig. 4. The trend in inhibition

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**Table 1—DPPH-scavenging activity of various phenolics**

[For the assay, 200 µl of ethanolic solution of phenolic was mixed with 800 µl tris-HCl buffer, pH 7.4. This was further thoroughly mixed with 1 ml 500 µM DPPH in ethanol to record changes in optical density with time at 517 nm. Values of IC_{50} are mean ± SD of 4 assays]

<table>
<thead>
<tr>
<th>Test compound</th>
<th>IC_{50} (µM)</th>
<th>Number of molecules of DPPH scavenged/molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myricetin</td>
<td>16.2±0.08</td>
<td>10</td>
</tr>
<tr>
<td>Quercetin</td>
<td>16.2±0.10</td>
<td>10</td>
</tr>
<tr>
<td>Fisetin</td>
<td>22.5±1.19</td>
<td>7.6</td>
</tr>
<tr>
<td>Catechin</td>
<td>32.5±2.23</td>
<td>5.0</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>62.5±4.16</td>
<td>3.6</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>60.0±2.25</td>
<td>2.0</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>117±7.47</td>
<td>1.7</td>
</tr>
<tr>
<td>Naringenin</td>
<td>&gt; 250</td>
<td>0.5</td>
</tr>
</tbody>
</table>
of pulmonary lipid peroxidation was similar to that of hepatic lipid peroxidation.

Discussion

The structure-activity relationship of the test compounds (Fig. 1) show that O-dihydroxy group on ring B of flavonoid moiety plays a crucial role in radical scavenging activity in DPPH assay. A double bond at 2-3 position conjugated with 4-oxo function and OH groups in position 3 and 5 also contribute towards antiradical activity. Scavenging properties of compounds are associated with their ability to form stable radicals and it is well-known that aromatic compounds containing hydroxyl groups, especially having an O-dihydroxy group on ring B, appear to be important scavengers as reported for flavonoids. Moreover, reduction potential of hydroxy moieties present in polyphenols is influenced by their positions on the molecule and the degree by which the oxygen p-orbitals can overlap with extended π-orbital systems on the carbon skeleton. Further, of different classes of flavonoids, the flavonol derivatives were most efficient. In flavonols, the stronger antiradical activity may be attributed due to the presence of one or more structural elements, as already discussed, which are involved in antiradical activity, viz. the presence of O-dihydroxy group on ring B, a 2-3 double bond conjugated with 4-oxo function and hydroxyl groups on position 3 and 5. These may also explain the higher antiradical activity of quercetin and myricetin, compared to catechin. Fisetin, though a flavonol, is not found to be equally potent (antiradical) as quercetin and myricetin, which might be due to the absence of hydroxyl group on position 5. The weaker antiradical activity of kaempferol, another flavonol could be explained by the absence of O-dihydroxy group in ring B. Flavonones, e.g. naringenin, were found to be even weaker than catechins, which can be due to the absence of O-dihydroxy group on ring B. Naringenin is found to be a very weak scavenger of DPPH radical in another study.

Resveratrol, a non-flavonoid was found to exhibit antiradical activity stronger than kaempferol and naringenin. In terms of IC₅₀, resveratrol was equivalent to α-tocopherol, but was stronger than α-tocopherol, in terms of molecules of DPPH scavenged per molecule of the compound. The strong antiradical activity of resveratrol could be due to the presence of conjugated double bond, which makes the electrons more delocalized. Polyphenols are reported to have a higher antioxidant activity than monophenols, due to effective delocalization of an unpaired electron. As the number of OH groups increases, antioxidant activity increases due to increase in electron donating groups of the compound, which explains the poor efficiency of a phenol as an antioxidant. Three hydroxyl groups in resveratrol may be responsible for its reasonably good antiradical activity.

In increasing order the effective concentrations of the test compounds causing half maximal inhibition (IC₅₀) of DPPH (250 µM) were quercetin, myricetin, fisetin, catechin, α-tocopherol, resveratrol, kaempferol and naringenin. IC₅₀ of α-tocopherol in our study was in agreement with that reported by Yamaguchi et al. In terms of the number of molecules of DPPH scavenged/molecule of phenolics, the results showed that quercetin and myricetin were the strongest and gave protons to 10 molecules of DPPH per molecule, followed by fisetin, catechin and resveratrol, which trapped 7.6, 5.0 and 3.6 molecules of DPPH per molecule, respectively. Analysis of IC₅₀ at 10 min and 2 hr revealed that in case of slow reacting phenolics, IC₅₀ at 2 hr was less than at 10 min. α-Tocopherol consumed DPPH in the ratio of 2:1. The reaction mechanism of DPPH with α-tocopherol can be explained by a two-steps reaction: in the first step, one molecule of DPPH reacts with one molecule of α-tocopherol to produce α-tocopheroxyl radical and then, one α-tocopheroxyl radical reacts with another molecule of DPPH to form α-tocopherolquinone. Hence, two molecules of DPPH are reduced by one molecule of α-tocopherol. However, for the other test compounds, the mechanism of reacting with more than one molecule of DPPH is not known.

Trends of IC₅₀ of all the test compounds for lipid peroxidation in the two assay systems, i.e., hepatic and pulmonary homogenates, were similar. In both systems, antioxidant activity of resveratrol was nearly half of the α-tocopherol. Fisetin was found to be the strongest antioxidant and kaempferol and naringenin, the weakest in both systems. In contrast, another study reported that effective concentration of kaempferol causing half maximal inhibition in lipid peroxidation (IC₅₀) was almost equal to quercetin in vitamin E deficient microsomes of rat liver. However, in our study kaempferol was found to be a very weak antioxidant (IC₅₀ 130 ± 11.5 µM), compared to quercetin (IC₅₀ 35 ± 2.84 µM). IC₅₀ of kaempferol was
3.7 times higher than quercetin. The difference in results could be due to different assay conditions. Resveratrol was more potent antioxidant than catechin, myricetin, kaempferol, ellagic acid and naringenin, but was weaker than quercetin, fisetin and $\alpha$-tocopherol. In an earlier study, quercetin was found to be a stronger antioxidant than resveratrol in Cu$^{2+}$-catalysed oxidation of human LDL. In rat liver microsomes, resveratrol was shown to strongly inhibit non-enzymatic and NADPH-dependent lipid peroxidation. Resveratrol might be causing inhibition in lipid peroxidation by physically interacting with free radicals as it was found to be scavenger of a stable free radical, like DPPH, by donating an electron in a chemical system. The reaction product of this reaction i.e. resveratrol phenoxy radical contains unpaired electrons, which are delocalized extensively over its two aromatic rings, resulting in poor reactivity, and causes inhibition in free radical chain reaction in lipid peroxidation.

The present study also showed that phenolics caused inhibition in lipid peroxidation in a concentration-dependent manner, which was in accordance with a study on inhibition of NADPH and ADP-Fe$^{3+}$-dependent microsomal lipid peroxidation. Further, comparison of the results of antiradical and antiperoxidative assays revealed that the efficiency of a polyphenol to scavenge DPPH and to inhibit lipid peroxidation was not always the same, which could be because of different specificity of a polyphenol to scavenge DPPH particularly and other free radicals involved in the process of lipid peroxidation, as DPPH test provides information only of the reactivity of phenolic compounds with only one nitrogen-centered radicals, i.e. DPPH, in buffered solution. Whereas in the antiperoxidative assay, the reaction mixtures was a combination of many molecules in the homogenate.

In conclusion, antiradical activity of resveratrol in DPPH assay was found to be equivalent to $\alpha$-tocopherol (both in terms of IC$_{50}$ at 2 hr and the number of DPPH molecules scavenged/molecule), but it was weaker antioxidant than $\alpha$-tocopherol. It suggests that resveratrol scavenges lipid carbon-centered radicals more efficiently than $\alpha$-tocopherol. Thus, these antiradical and antioxidant properties of resveratrol may contribute towards its anticarcinogenic activity. However, it is yet to be established, if there is any correlation between the antiperoxidative/antiradical activities and the extent of chemoprevention of compounds evaluated in this study.

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References


