Antioxidant Potential of galls of Quercus infectoria
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Citation

Abstract

INTRODUCTION

Nowadays, the fact of harmful effect of reactive oxygen species on human health is well-known. The capability of natural defense systems of living organisms against excess production of these species decreases when influenced with negative environmental factors or aging. As a result, different cellular and extracellular components, and especially nucleic acids, are damaged, causing or enhancing a number of degenerative diseases. Therefore, antioxidants that scavenge free radicals are of great value in preventing such “oxidative” pathologies. That is why natural products with antioxidant properties become more and more popular all over the world. Natural phenolic phytochemicals in fruits and vegetables have been receiving increased interest from consumers and researchers for their beneficial health effects on coronary heart diseases and cancers mainly due to their antioxidant activity.

As plants produce a lot of antioxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with antioxidant activity. Among natural antioxidants, phenolic antioxidants are in the forefront since all the phenolic classes (simple phenolics, phenolic acids, anthocyanins, hydroxycinnamic acid derivatives, and flavonoids) have the structural requirements of free radical scavengers and antioxidants.

Quercus infectoria Olivier (Fagaceae) is a small tree or a shrub mainly present in Greece, Asia Minor, Syria and Iran. The tree capitulates galls that emerge on its shoots as a consequence of assault of gall wasp, Cypnis gallae tincotoriae. The galls of Q. infectoria have a great medicinal value and have pharmacologically been deciphered to be astringent, antidiabetic, antitremorine, local anaesthetic, antipyretic and antiparkinsonian. In Asian countries, the galls of Q. infectoria have been used for centuries in oriental traditional medicines for treating inflammatory diseases. Gargle of hot water extract of galls is very effective against inflamed tonsils, while direct application of boiled and bruised galls on skin effectively cures any swelling or inflammation. The application of powdered galls in the form of ointment also cures hemorrhoids caused by inflammation of the skin.

Report suggest the presence of tannoid principles, which are known antioxidants, we studied the antioxidant potential of the extract and also the percentage of gallic acid by high performance thin layer chromatography (HPTLC), which may be responsible for the antioxidant activity. HPLC and GC are efficient but time consuming methods; HPTLC on the other hand is relatively simple and a non expensive assay method, which does not require any experience, equipment or complex derivatization process.

Thus present study aims to assess the antioxidant potential of methanolic extract of Quercus infectoria. We also report the presence HPTLC Densitometric quantification method of gallic acid, which contributes to the antioxidant activity of the galls. Plant extracts were tested for different free radical scavenging activities including the 1,1-diphenyl 2-picryl hydrazyl (DPPH), nitric oxide, hydrogen peroxide, their capacity to reduce lipid peroxidation in rat liver homogenate, radical scavenging potential using chemiluminescence and their total antioxidant capacity. Also the percentage of gallic acid present in the extract was evaluated using HPTLC.

MATERIAL AND METHOD

CHEMICALS

Gallic acid, 1,1-diphenyl 2-picryl hydrazyl (DPPH), 1,1,3,3-tetraethoxypropane, 2-nitrobenzoic acid (DTNB) and potassium superoxide were obtained from Sigma Chemical Co. (St. Louis, U.S.A.), ferrous sulphate (FeSO4), trichloroacetic acid (TCA), thiobarbituric acid (TBA), ascorbic acid, ethylenediaminetetraacetic acid (EDTA), sodium nitroprusside, sulfanilamide, phosphoric acid,
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naphthyl ethylene diamine, ammonium molybdate, sodium phosphate, sodium hypochlorite, hydrogen peroxide and dimethyl sulfoxide (DMSO) were obtained from Sd. fine chemicals (Mumbai, India). All other reagents used were of analytical grade.

PLANT MATERIAL AND EXTRACTION
The air-dried galls of Quercus infectoria were purchased from Local market and identified based on its physical characteristics. One kilogram of the plant material was air dried at room temperature and powdered coarsely. The powdered material (250 g) was macerated with petroleum ether to remove the fatty substances; the marc was further extracted with methanol and filtered. The extract was concentrated under reduced pressure and lyophilised (Labconco, U.S.A.) to get dry residue (23.6 g).

HYDROGEN- DONATING ACTIVITY
Hydrogen donating activity was quantified in presence of stable DPPH radical on the basis of Blois method. Briefly, to a methanolic solution of DPPH (100?M, 2.95 ml), 0.05 ml of test compounds dissolved in methanol was added at different concentrations (2—10 mg/ml). Reaction mixture was shaken and absorbance was measured at 517 nm at regular intervals of 30 s for 5 min. Ascorbic acid was used as standard. The degree of discoloration indicates the scavenging efficacy of the extract.

TOTAL ANTIOXIDANT ACTIVITY
Total antioxidant capacity was measured according to spectrophotometric method. 0.1 ml of the extract (10 mg/ml) dissolved in water was combined in an eppendorf tube with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 min. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. Ascorbic acid was used as standard. The degree of discoloration indicates the scavenging efficacy of the extract.

NITRIC OXIDE SCAVENGING
Nitric oxide scavenging activity was measured spectrophotometrically. Sodium nitroprusside (5mM) in phosphate buffered saline was mixed with different concentrations of extract (2—10 mg/ml) dissolved in methanol and incubated at 25°C for 30 min, then 1.5 ml of

LIPID PEROXIDATION INHIBITION. LIVER HOMOGENATE
Male Sprague-Dawley rats (160—180 g) were purchased from the animal house of the Gold Mohur Lipton India Ltd, India. Ethical clearance for the animal study was obtained from the institutional animal ethics committee. These were kept in the departmental animal house at 26 ± 2°C and relative humidity 44—55% light and dark cycles of 10 and 14 h respectively for one week before the experiment.
Animals were provided with rodent diet (Amruth, India) and water ad libitum. Randomly selected male rats were fasted overnight and were sacrificed by cervical dislocation, dissected and abdominal cavity was perfused with 0.9% saline. Whole liver was taken out and visible clots were removed and weighed amount of liver was processed to get 10% homogenate in cold phosphate buffered saline, pH 7.4 using glass teflon homogeniser and filtered to get a clear homogenate.

ASSAY OF LIPID PEROXIDATION

The degree of lipid peroxidation was assayed by estimating the thiobarbituric acid-reactive substances (TBARS) by using the standard method. In brief, different concentration of extracts (200—1000 m g/ml) in water was added to the liver homogenate. Lipid peroxidation was initiated by adding 100m l of 15mM FeSO₄ solution to 3 ml of liver homogenate (final concentration was 0.5mM). After 30 min, 100m l of this reaction mixture was taken in a tube containing 1.5 ml of 10% TCA. After 10 min tubes were centrifuged and supernatant was separated and mixed with 1.5 ml of 0.67% TBA in 50% acetic acid. The mixture was heated in a hot water bath at 85 °C for 30 min to complete the reaction. The intensity of pink coloured complex formed was measured at 535 nm. The values of TBARS were calculated from a standard curve (absorption against concentration of Tetraethoxy propane) and expressed as nmol/mg of protein. The percentage inhibition of lipid peroxidation was calculated by comparing the results of the test with those of controls not treated with the extracts.

PHOTOCHROMILUMINESCENCE ASSAY

For the determination of the integral antioxidant capacity (AC) of the water soluble substances in Quercus infectoria extract the method of photochemiluminescence (PCL) was used. Apparatus used was Photochem® with Standard kit ACW (Analitik jena AG), where the luminescent plays a double role of photosensitizer as well as the radical detecting agent. Lysophilized extract was measured at 10 ?g g/ml concentration. A standard plot was plotted and the results were calculated in ascorbic acid equivalents (?mol/g).

QUANTITATIVE DENSITOMETRIC ASSAY FOR ESTIMATION OF GALLIC ACID

1 mg of pure gallic acid standard was dissolved in 10 ml of methanol. 1 g of powdered drug material was macerated with 10ml of 80% methanol. This mixture was refluxed at 60 °C for 3h and decanted through filter paper. Same process was repeated for 2 more times and all the filtrates were pooled and made up to 250 ml. From this 10?l was applied on TLC plates. 10?l of the standard and the sample respectively were then applied using the CAMAG Linomat V applicator onto the precoated silica gel F 254 plates (Merck) of 0.2 mm thickness plates. The plate was then eluted in solvent system. Toluene: Ethyl acetate: Formic acid (5:4:1). After elution, the plate was dried and scanned densitometrically using CAMAG TLC scanner 3 at 272 nm. The percentage of gallic acid in the extract was calculated by calibration using peak height ratio.

RESULT AND DISCUSSION

Free radical oxidative stress has been implicated in the pathogenesis of a wide variety of clinical disorders, resulting usually from deficient natural antioxidant defenses. In most diseases, increased oxidant formation is a consequence of the disease activity. Potential antioxidant therapy therefore should include either natural free radical scavenging antioxidant principles or agents, which are capable of augmenting the activity of the antioxidant enzymes. ROS are capable of damaging biological macromolecules such as DNA, carbohydrates or proteins. ROS is a collective term, which includes not only the oxygen radicals (O₂⁻, and OH) but also some non-radical derivatives of oxygen these include H₂O₂, HOCl and ozone (O₃). If human disease is believed to be due to the imbalance between oxidative stress and antioxidative defense, it is possible to limit oxidative tissue damage and hence prevent disease progression by antioxidant defense supplements.

DPPH is stable nitrogen centered free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radicals react with suitable reducing agents, then losing colour stoichiometrically with the number of electrons consumed, which is measured spectrophotometrically at 517 nm. As shown in Table 2, Quercus infectoria extract strongly scavenged DPPH radical with the IC₅₀ being 0.25 mg/ml. The scavenging was found to be dose dependent.

The total antioxidant capacity of the extract was calculated based on the formation of the phosphomolybdenum complex which was measured spectrophotometrically at 695 nm. The total antioxidant capacity of the extract was found to be 152.91 nmol/g ascorbic acid. Thus establishing the extract as an antioxidant. Quercus infectoria extract also moderately inhibited nitric oxide in dose dependent manner (Table 2) with the IC₅₀ being 0.258 mg/ml. Nitric oxide (NO) is a
potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities. Studies in animal models have suggested a role for NO in the pathogenesis of inflammation and pain and NOS inhibitors have been shown to have beneficial effects on some aspects of the inflammation and tissue changes seen in models of inflammatory bowel disease. Thus establishing the usage of the plant in the Indian indigenous system as an anti-inflammatory agent.

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H$_2$O$_2$ can probably react with Fe$^{2+}$ and possibly Cu$^{2+}$ ions to form hydroxyl radical and this may be the origin of many of its toxic effects. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. Two types of enzymes exist to remove hydrogen peroxide within cells. They are the catalases and the peroxidases, which leads to ground state oxygen without any singlet oxygen. The extract may have decomposition of H$_2$O$_2$ activity due to any of these enzymes.

Initiation of the lipid peroxidation by ferrous sulphate takes place either through ferryl–perferryl complex or through OH$^-$ radical by Fenton's reaction. Table 1 show that the 50% aqueous alcoholic extract of Quercus infectoria inhibited FeSO$_4$ induced lipid peroxidation in a dose dependent manner. IC$_{50}$ values were found to be 0.124 mg/ml. The inhibition could be caused by absence of ferryl–perferryl complex or by scavenging the OH radical or the superoxide radicals or by changing the Fe$^{3+}$/Fe$^{2+}$ or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself. Iron catalyses the generation of hydroxyl radicals from hydrogen peroxide and superoxide radicals. The hydroxyl radical is highly reactive and can damage biological molecules, when it reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids, lipid hydroperoxides is produced. Lipid hydro peroxide can be decomposed to produce alkoxy and peroxy radical they eventually yield numerous carbonyl products such as malondialdehyde (MDA). The carbonyl products are responsible for DNA damage, generation of cancer and aging related diseases. Thus the decrease in the MDA level with the increase in the concentration of the extracts indicates the role of the extract as an antioxidant. The extract also moderately scavenged superoxide radical with the IC$_{50}$ values of 1.024 mg/ml. Free radicals are generated in the instrument by means of photosensitizer. The free radicals thus generated were detected by their reaction with a chemiluminogenic substance. In the presence of radical scavengers in the extract the intensity of the PCL was attenuated as a function of concentration. In this way the antioxidative capacity of the extract could be quantified. The antioxidative capacity was found to be 1.014 nmol ascorbic acid/g equivalents. Since the plant has been reported to contain tannoid principles, we tried to investigate the presence the gallic acid in the extract and the gallic acid was found to be 0.68% w/w Table 3 by HPTLC (Fig. 1) in the Methanolic extract. The high percentage of the gallic acid in the extract justifies the potent antioxidant activity exhibited.

**CONCLUSION**

The present study aimed to evaluate the possible antioxidant activity of the Quercus infectoria extract but with no reported pharmacological data. The results obtained indicate that Quercus infectoria extract has potent antioxidant activity, achieved by scavenging abilities observed against DPPH, and lipid peroxidation. It showed high-H donating ability shown by the scavenging of DPPH radical. Thus the ethnobotanical claims of the plant being used in the skin disorders and as a tonic may be in part due to the antioxidant activity. Further the pharmacological activity of the extract is to be carried out to validate the ethnobotanical claims.

**Table 1.** Effect of Methanolic Extract of Quercus infectoria at Various Concentrations on Ferrous Sulphate Induced Lipid Peroxidation in Rat Liver Homogenates.

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>TBARS (nmol/mg protein)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.60 ± 0.63</td>
<td>1000</td>
</tr>
<tr>
<td>200</td>
<td>1.75 ± 0.68</td>
<td>52.36 ± 8.1</td>
</tr>
<tr>
<td>400</td>
<td>1.61 ± 0.90</td>
<td>53.97 ± 1.90</td>
</tr>
<tr>
<td>800</td>
<td>1.55 ± 0.37</td>
<td>57.38 ± 2.48</td>
</tr>
<tr>
<td>1600</td>
<td>1.38 ± 0.05</td>
<td>63.39 ± 3.12</td>
</tr>
<tr>
<td>3200</td>
<td>1.19 ± 0.47</td>
<td>67.51 ± 3.09</td>
</tr>
<tr>
<td>Tocopherol</td>
<td>0.06 ± 0.01</td>
<td>99.39 ± 2.16</td>
</tr>
</tbody>
</table>

*Values are mean ± S.E. of 6 replicates.*
Figure 2
Table 2: Radical Scavenging Capacity of Methanolic Extract of

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>DPPH Scavenging</th>
<th>Nitric Oxide Scavenging</th>
<th>Super Oxide Scavenging</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid (100 μM)</td>
<td>93.69 ± 1.07</td>
<td>88.77 ± 1.18</td>
<td>90.26 ± 2.86</td>
</tr>
<tr>
<td>200</td>
<td>65.74 ± 2.07</td>
<td>44.54 ± 2.37</td>
<td>54.44 ± 1.88</td>
</tr>
<tr>
<td>400</td>
<td>76.39 ± 1.88</td>
<td>68.36 ± 3.01</td>
<td>23.66 ± 2.56</td>
</tr>
<tr>
<td>800</td>
<td>88.32 ± 1.70</td>
<td>99.67 ± 3.18</td>
<td>29.26 ± 1.25</td>
</tr>
<tr>
<td>1000</td>
<td>91.52 ± 2.05</td>
<td>99.51 ± 2.40</td>
<td>98.78 ± 1.92</td>
</tr>
</tbody>
</table>

Values are mean ± S.E. of 6 replicates.

Figure 3
Table 3: Quantification of the active principle against the applied standard

Densitometric scan at 272 nm in 3D representation

References
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