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Antioxidant Constituents from Stem Galls of *Quercus Infectoria* Olivier†

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Gallic acid and methyl gallate have been identified as antioxidant constituents of stem galls of *Quercus infectoria* through bioactivity guided fractionation. All the antioxidant fractions were tested for the activity in various mechanisms, like superoxide, DPPH and hydroxyl radical assays. The activities are corroborative to gallic acid and methyl gallate content of corresponding fractions. *Quercus infectoria* extracts also showed moderate activity in brine shrimp lethality assay.

Key Words: Antioxidant activity, *Quercus infectoria*, Methyl gallate, Gallic acid, *Artemia salina*, Brine shrimp lethality.

INTRODUCTION

Quercus infectoria Olivier (Fagaceae) is commonly known as Manjuphal, Gall Oak or Dyer's Oak. Manjuphal is a small tree or shrub, native of Greece, Asia Minor, Syria and Iran. The galls are formed by excrescences of young twigs due to the eggs laid by a small insect *Adleria gallae-tinctoriae* Olivier. The galls are spherical or pear-shaped and measure 6–50 mm in diameter. The surface of mature dry gall is smooth and shining but more usually rough and of grayish brown colour[1]. Manjuphal is traditionally used as a gargle for the treatment of diarrhoea, dysentery and externally used for the treatment of piles and in plasters. It has analgesic, anesthetic, CNS depressant, antitremor, astringent, hypoglycemic, haemostatic, antiseptic, molluscicidal activities[2–4]. It is also used in dentifrice, tanning, dyeing industry and in production of ink[2]. Amentoflavone hexamethyl ether, hexagalloylglucose, isocryptomerin, β-sitosterol, tannic, gallic and ellagic acids, methyl gallate, starch, sugars, essential oils and anthocyanins have been isolated earlier[3–5].

The bioactivity guided fractionation of *Quercus infectoria* extracts based on antioxidant activity has been worked out and the results are presented in this paper.

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EXPERIMENTAL

Nitro blue tetrazolium (NBT), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), gallic acid and thiobarbituric acid were obtained from Sigma Chemicals Co., (USA). Brine shrimp (*Artemia salina* cysts) eggs were obtained from Argent Chemical Laboratories, Redmond, (USA). All other reagents are of AR grade and were procured from Qualigens Fine Chemicals, Mumbai, India. Silica gel (100–200 mesh) chromatography grade was obtained form Acme Laboratory Chemicals, Mumbai, India. Melting points were determined on a Mel-Temp apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer Spectrum-BX FTIR-spectrometer; ^{1}H NMR spectra on an AMX 400 MHz NMR spectrometer. HPLC was carried out using Shimadzu LC-10AT pumps, SIL-10A auto injector, SCL-10A system controller, SPD M-10AVP-PDA detector, class M10A software and mass spectra were obtained on an LC-MS Agilent 1,100 series VL model MS detector, 1,100 series binary pump 7725i Rheodyne injector, Chemstation software.

Extraction: Powdered raw material (600 g) of *Q. infectoria*, supplied by Laila Impex, was extracted with hexane (2.5 L), ethyl acetate (2 L), methanol (2 L) using a Soxhlet apparatus. Aqueous methanol (80%, 2 L) and water (2 L) extracts were obtained by refluxing at 90°C for 2 h. All the extractives were concentrated independently, under reduced pressure to obtain the crude residues 1, 28, 230, 78 and 45 g, respectively.

The residues from ethyl acetate and methanol extracts were subjected, separately, to bioassay guided fractionation (antioxidant activity) using silica gel column with chloroform and mixtures of chloroform and methanol as eluants with increasing polarity. Combined fractions were tested for their antioxidant activity and selected fractions which showed potent antioxidant activity were purified further to obtain gallic acid (yield 1.5%) and methyl gallate (yield 0.06%).

Identitification of known compounds: Gallic acid (3,4,5-trihydroxy benzoic acid), m.p.: 258–260°C, HPLC conditions: Column: Altima C_{18} 5μ (250 × 4.6 mm), mobile phase 0.1% (v/v) H_3PO_4 in water, Acetonitrile, system gradient (0.01–7.00 min 10%, 7.01–15.00 min linear gradient from 10–50% and 15.01–17.00 min linear gradient from 50–100% of acetonitrile in mobile phase), RRT: 5.23 and LC-MS (negative ion mode) m/z (%): 170 (7.0) M^+, 169 (100). Identity was ascertained further by direct comparision with gallic acid obtained from Sigma Chemical Company (mmp, co-HPLC and spectral data). Methyl gallate (methyl-3,4,5-trihydroxybenzoate), m.p.: 200–201°C, HPLC conditions: Same as described above for gallic acid, RRT: 15.02 min. Identity was ascertained further by direct comparision with synthetic sample (mmp, co-HPLC and spectral data).

Synthesis of methyl gallate: Gallic acid (4.5 g, 26.4 mM) was dissolved in dry methanol (40 mL) and treated with concentrated sulphuric acid (2.5 mL). The mixture was stirred at 60°C for 8 h. The reaction mixture was poured on to crushed ice (150 g) and stirred for 15 min. The mixture was extracted with ethyl acetate (100 mL × 2). The organic layer was washed successively with water, brine, dried over Na_2SO_4 and concentrated under reduced pressure to yield crude product (4.1 g). The residue was crystallized from methanol to give methyl gallate (3.5 g).

Purity by HPLC 99%; IR, (KBr): 3463, 3326, 1694, 1618, 1540, 1469, 1314, 1196, 1037, 1003, 745, 756 cm^{-1}. LC-MS (negative ion mode) m/z (%): 184 (8.4) M^+, 183 (100), 124 (6.2), 113 (5.1). ^1H NMR (400 MHz, DMSO-d_6) δ: 8.98 (2H, s), 8.65 (1H, s), 6.70 (2H, s) and 3.50 (3H, s).

Determination of superoxide radical scavenging activity: Superoxide radical scavenging activity of various extracts of *Q. infectoria* was determined by the method of McCord and Fridovich[6]. The assay mixture contained EDTA (6.6 μM) containing 3 μg NaCN, riboflavin (2 μM), NBT (50 μM), various concentrations of test substances and phosphate buffer (58 mM, pH 7.8) in a final volume of 3 mL. The tubes were mixed well and optical densities were measured at 560 nm. The tubes were uniformly illuminated with an incandescent lamp for 15 min and the optical densities were measured again at 560 nm. The percentage inhibition of superoxide radical generation was measured by comparing the absorbance values of control and those of the test substances. IC_{50} values were obtained from the best-fit line drawn concentration (μg) *vs.* percentage inhibition.

Determination of DPPH free radical scavenging activity: DPPH radical scavenging activity was determined by the method of Lamaison *et al.*[7], based on the reduction of coloured methanolic solution of DPPH. Free radical scavenging ability of the test substances added to the methanolic solution of DPPH is inversely proportional to the difference in initial and final absorption of DPPH solution at 517 nm. Drug activity is expressed as the 50% inhibitory concentration (IC_{50}). The reaction mixture contained 1×10^{-4} mM methanolic solution of DPPH and various concentrations of the test substances. Percentage inhibition was determined by comparing the absorbance values of test and control tubes. IC_{50} values were obtained from the best-fit line drawn concentration (μg) *vs.* percentage inhibition.

Determination of hydroxyl free radical scavenging activity: Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the test compounds for hydroxyl radicals generated by Fenton reaction. The hydroxyl radicals attack deoxyribose that eventually result in (thiobarbituric acid reactive substances) TBARS formation. The reaction mixture contained deoxyribose (2.8 mM), $FeCl_3$ (0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), ascorbic acid (0.1 mM), sodium phosphate buffer (20 mM, pH 7.4) and various concentrations of test substance in a final volume of 1 mL. The reaction mixture was incubated for 1 h at 37°C. Deoxyribose degradation was measured as TBARS by the method of Ohkawa *et al.*[8] The percentage inhibition was determined by comparing the absorbance values of test and control tubes. IC_{50} values were obtained from the plot drawn concentration (μg) *vs.* percentage inhibition.

Determination of brine shrimp lethality: Brine shrimp (*Artemia salina*) shrimps were hatched using brine shrimp eggs in a conical shaped vessel (1 L), filled with sterile artificial seawater (prepared using sea salt 38 g/L and adjusted to pH 8.5 using 1 N NaOH) under constant aeration for 48 h. After hatching, 10 nauplii were drawn through a glass capillary and placed in each vial containing 4.5 mol brine solution and added various concentrations of drug solutions and

volume was made up to 5 mol using brine solution and maintained at 37°C for 24 h under the light of incandescent lamps and surviving larvae were counted[9, 10]. Each experiment was conducted along with control (vehicle treated), different concentrations of the test substance in a set of three tubes per dose. The percentage lethality was determined by comparing the mean surviving larvae of test and control tubes. LC_{50} values were obtained from the plot drawn concentration (μg) *vs.* percentage inhibition. Podophyllotoxin was used as a positive control.

RESULTS AND DISCUSSION

Antioxidant activity data of *Q. infectoria* extracts in various methods like superoxide, DPPH and hydroxyl radical scavenging assay have been presented in Table-1. These extracts have shown potent antioxidant activity in all three antioxidant mechanisms, tested in comparision with known antioxidants, namely, vitamin C and vitamin E. Bioactivity guided fractionation of the active fractions yielded gallic acid and methyl gallate, as active constituents. The results of antioxidant activity were found to corroborate with the concentrations of gallic acid and methyl gallate in the fractions (Table-1). Fraction-II of the ethyl acetate extract has shown highest antioxidant activity and its activity is comparable to that of gallic acid, whereas Fraction-I has shown lower antioxidant activity.

TABLE-1
ANTIOXIDANT ACTIVITY DATA*

Test substances	Content of		Superoxide radical scavenging activity (IC_{50} μg/mL)	DPPH radical scavenging activity (IC_{50} μg/mL)	Hydroxyl radical scavenging activity (IC_{50} μg/mL)
	GA	MG			
Hexane ext.	0.00	0.00	>100	>100	>100
Ethyl acetate ext.	30.74	0.03	1.9	1.8	3.7
MeOH ext.	10.32	3.20	2.0	1.92	4.85
Aq. MeOH ext.	8.15	2.98	2.05	1.95	3.4
Aqueous ext.	8.99	6.98	1.9	1.85	3.05
Fraction-I	23.59	1.28	4.5	2.35	>10
Fraction-II	71.36	1.78	1.36	1.29	5.5
Commercial aqueous extract	16.48	0.058	3.05	2.6	8.7
Gallic acid	—	—	1.3	1.25	5.2
Methyl gallate	—	—	1.2	1.27	2.8
Vitamin C	—	—	1.50	2.5	—
Vitamin E	—	—	3,125	5,500	—

*Values are mean of 3 tubes for each dose, GA = gallic acid, MG = methyl gallate.

TABLE-2
BRINE SHRIMP LETHALITY DATA*

Test substance	Concentration in µg/mL	0	25	50	75	100	150	200	250	300	400	500	LC_{50}
Hexane ext.	Mean viable larvae	8.66	7.67	4.33	1.67	—	—	—	—	—	—	—	50
	% lethality	C[a]	11.5	50	80.8	—	—	—	—	—	—	—	
EtOAc ext.	Mean viable larvae	8.87	—	7.67	—	6	5	3.33	0.33	—	—	—	170
	% lethality	C[a]	—	11.53	—	30.8	42.3	61.53	96.15	—	—	—	
MeOH ext.	Mean viable larvae	9	—	—	—	8	—	5.67	—	4	0	0	250
	% lethality	C[a]	—	—	—	11	—	37.04	—	55.56	100	100	
AqMeOH ext.	Mean viable larvae	9	—	—	—	6.67	—	4.67	—	4	0	0	220
	% lethality	C[a]	—	0	—	25.93	—	48.15	—	55.56	100	100	
Aqueous ext.	Mean viable larvae	8.67	—	—	—	7.33	—	5.33	—	5	3	0.67	295
	% lethality	C[a]	—	0	—	15.38	—	38.46	—	42.31	65	92	
Commerical ext.	Mean viable larvae	9.67	8.67	6.17	—	4.67	2.0	0	—	—	—	—	92
	% lethality	C[a]	10.34	36.21	—	51.72	79.31	100	—	—	—	—	
Test substance	Concentration in µg/mL	0	1	2.5	5	10	25	50	100	—	—	—	LC_{50}
Podophyllotoxin	Mean viable larvae	8.33	—	5.6	4.8	1	0	0	0	—	—	—	3.1
	% lethality	C[a]	—	32.8	42	94	100	100	100	—	—	—	

*Values are mean of three tubes, [a]Considered as zero percent lethality

Q. infectoria extracts were tested for brine shrimp lethality and results are summarized in Table-2. All extracts have shown moderate brine shrimp lethality, but lower activity in comparison to that of the podophyllotoxin standard. Commercial *Q. infectoria* extracts have shown better activity than individual *Q. infectoria* extracts and the activity is, perhaps, distributed in different extracts.

Conclusion

Q. infectoria extracts exhibited weak activity in brine shrimp lethality assay but showed potent antioxidant activity in comparison to the known standards. The antioxidant activity of the extracts is in corroboration to gallic acid and methyl gallate content. Methyl gallate and gallic acid appear to be responsible for the observed antioxidant activity of *Q. infectoria.*

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