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Antioxidant activity of stem bark of *Thespesia populnea* Soland ex Corr.

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Abstract

Objective: To evaluate the antioxidant activity of stem bark of *Thespesia populnea*. Materials and Method: Antioxidant activity of *T. populnea* stem bark was studied in four *in vitro / ex vivo* models, viz., DPPH radical scavenging activity, superoxide scavenging activity, reducing power assay and lipid peroxidation. Results: Preliminary phytochemical screening showed the presence of high amount of phenolics, tannins and flavonoids. Subsequent quantification by Folin Ciocalteau method showed the presence of high amount of total phenolics (10.11 % w/w, calculated as gallic acid). Methanolic extract of stem bark of *T. populnea* showed a very good DPPH radical scavenging activity (EC_{50} of 12.08 µg) and superoxide scavenging activity (EC_{50} of 73.53 µg) in a dose dependent manner. The reduction ability of the extract, (Fe^{3+} to Fe^{2+} transformation) was found to increase with increasing concentrations where a maximum absorbance of 1.012 was obtained at a concentration of 300 µg. It also inhibited lipid peroxidation in a dose dependent manner with an EC_{50} of 20.76 µg, which was comparable to α-tocopherol (EC_{50} of 27.35 µg). Conclusion: The methanolic extract of the stem bark of *T. populnea* showed very good free radical scavenging activity, including inhibition of lipid peroxidation and reducing power.

1. Introduction

Stem bark of *Thespesia populnea*, commonly known as the Tulip tree or the Portia tree (Family; Malvaceae) is used in many ayurvedic formulations like *Panchvalkaladi kawath, Yastimadhukadi tailum* [1]. Decoction of the stem bark is used for skin diseases like psoriasis and scabies. Oil from the bark is used in cases of urethritis and gonorrhoea [2]. Infusion of the stem bark is used to treat dysmenorrhoea, infertility, and secondary amenorrhoea [3]. It is also used in the treatment of cough, asthma, fever, seizures, fungal and viral infections [4]. The stem bark showed antioxidant activity against CCl_4-induced liver injury [5] and antihepatotoxic activity [6] in rats.

The stem bark is reported to contain different iso forms of gossypol, *viz*., gossypol [l7-10] (+)-gossypo [l7-10] and (DL) gossypol [l8]. Gossypol is reported to show antioxidant [9-12] and pro-oxidant activity [10,11]. Some biologically active phytochemicals like phenols, flavonoids, coumarins, benzyl-isothiocynate are known to scavenge and/or stabilize free radicals

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[15,16]. Many plant extracts and phytochemicals have been shown to have antioxidant/free radical scavenging properties [16,19-21]. In the present paper, we report our work on the evaluation of free radical scavenging properties of the stem bark of *T. populnea*.

2. Materials and Methods

2.1 Plant material

Stem bark of *T. populnea* was collected from Ahmedabad in January 2004. The sample was authenticated by our taxonomist and a voucher specimen was preserved in the Pharmacognosy and Phytochemistry department.

2.2 Chemicals

Ethylene diamine tetra acetate (EDTA), Folin Ciocalteu's reagent, glacial acetic acid and sodium dodecyl sulphate (SDS) were purchased from SD Fine Chemicals, India. 1,1-Diphenyl-2-picryl hydrazyl (DPPH), riboflavin, adenyl diphosphate (ADP), nitro blue tetrazolium chloride (NBT), thiobarbituric acid (TBA) and pyrogallol were purchased from HIMEDIA Ltd, India. Ascorbic acid was a gift sample from Cadila Pharmaceuticals Ltd., Ahmedabad, India. Potassium ferricyanide was purchased from Qualigens Fine Chemicals, India; Trichloroacetic acid (TCA) and Iron (III) chloride ($FeCl_3$) from E. Merck India Ltd., India. Gallic acid and tannic acid were gift samples from Tetrahedron Ltd., India. (+)-Gossypol (Fig. 1) was isolated in the laboratory by the reported method [17] and characterized by recording MS, IR, NMR spectra and identified by comparing with the reported data [17].

2.3 Equipment

UV/VIS Spectrophotometer (Elico-India; SL-164).

2.4 Preparation of methanolic extract

10 g of the powdered stem bark of *T. populnea* was extracted with methanol (4 x 50 ml) under reflux. The extract was filtered, pooled and the solvent was removed under reduced pressure.

2.5 Phytochemical evaluation of the methanolic extract

500 mg of the dried methanolic extract was reconstituted in 10 ml of methanol and it was used for preliminary phytochemical testing for the presence of different chemical groups of compounds.

2.5.1 Estimation of total phenolics

The total phenolic content of the extract was estimated according to the method described by Singleton & Rossi [18]. Briefly the method is as follows: A stock solution (1 mg/ml) of the methanolic extract was prepared in methanol. From the stock solution, suitable quantity of the extract was taken into a 25 ml volumetric flask and 10 ml of water and 1.5 ml of Folin Ciocalteu's reagent were added to it. The mixture was kept for 5 min, and then 4 ml of 20 % sodium carbonate solution was added and made up to 25 ml with double distilled water. The mixture was kept for 30 min and the absorbance was recorded at 765 nm in a spectrophotometer. Percentage of total phenolics was calculated from calibration curve of gallic acid (50 µg – 250 µg) plotted by using the above procedure and total phenolics were expressed as % gallic acid.

2.6 Free radical scavenging activity

100 mg of dried methanolic extract was dissolved in 100 ml of methanol to make a stock solution of 1 mg/ml. Aliquots from this stock solution was further diluted with methanol as per the concentrations required.

Free radical scavenging activity of the methanolic extract was tested in three *in vitro* models, *viz*., antiradical activity using DPPH [19-21], superoxide radical scavenging activity in riboflavin-light-NBT system [21,22] and reducing power assay and one *ex vivo* model,

lipid peroxidation. The reaction mixtures for the assays are given below:

2.6.1. Assay for antiradical activity with DPPH

Antiradical activity was measured by a decrease in absorbance at 516 nm of a methanolic solution of coloured DPPH brought about by the sample [19-21]. A stock solution of DPPH (1.3 mg/ml in methanol) was prepared such that 75 µl of it in 3 ml methanol gave an initial absorbance of 0.9. Decrease in the absorbance in the presence of sample extract at different concentrations was noted after 15 min. EC_{50} was calculated from % inhibition. A blank reading was obtained using methanol instead of the extract. Pyrogallol was used as positive control [20,21].

Suitably diluted stock solution of methanolic extracts of the stem bark and isolated (+)-gossypol (prepared by dissolving 2 mg of (+)-gossypol in 25 ml of methanol) were spotted on two TLC plates and were developed in two different solvent systems:

Solvent system – 1: Toluene : Ethyl acetate : Formic acid : Methanol (7 : 3 : 1 : 1).

Solvent system – 2: *n*-Butanol : Acetic acid : Water : Methanol : Ethyl acetate (5 : 1 : 2 : 2 : 3).

Then the plates were sprayed with 0.2 % DPPH in methanol. Bleaching of DPPH by the resolved bands was observed for 10 minutes and the details were recorded.

2.6.2. Assay for superoxide radical scavenging activity

The assay was based on the capacity of the sample to inhibit blue formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system [22]. The reaction mixture contained 50 mM phosphate buffer (pH 7.6), 20 µg riboflavin, 12 mM EDTA, NBT 0.1 mg/3ml, added in that sequence. The reaction was started by illuminating the reaction mixture with different concentrations of sample extract for 150 seconds. Immediately after illumination, the absorbance was measured at 590 nm and EC_{50} was calculated. Methanol was used for blank reading. Ascorbic acid was used as positive control [20,21].

2.6.3. Measurement of reducing power

The reducing capability of the sample extracts was measured by the transformation of Fe^{3+} to Fe^{2+} in the presence of the extracts at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power [23]. Different concentrations of extracts in 1ml of water were mixed with 2.5 ml of phosphate buffer and 2.5 ml of potassium ferricyanide. The mixture was incubated at 50ºC for 20 min. 2.5 ml of trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 RPM for 10 min. 2.5 ml of upper layer solution was mixed with 2.5 ml distilled water and 0.5 ml of $FeCl_3$ solution and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Gallic acid and tannic acid were used as positive controls.

2.6.4. Effect on lipid peroxidation in rat liver homogenate

Rat liver homogenate (10 % w/v) was prepared according to the procedure described by Tripathi *et al* [24]. Peroxidation was induced in liver tissue by Iron-ADP complex in the presence of ascorbic acid. The incubation medium constituted 0.5 ml of liver homogenate (10 % w/v), 100 M $FeCl_3$, 1.7 M ADP, 500 M of ascorbate and different concentrations of samples in 2 ml of total incubation medium. The medium was incubated for 20 min at 37°C and the extent of lipid peroxidation was measured by estimation of malondialdehyde (MDA) formed [19,20]. Results were expressed in terms of decrease in MDA formation by the sample extract. α-Tocopherol acetate was used as positive control [19].

Figure 1
Structure of (+)-Gossypol

Table 1. Preliminary phytochemical screening of *Thespesia populnea* stem bark.

Chemical group	Observation
Phenols	+++
Tannins	+++
Steroids/terpenoids	++
Alkaloids	++
Anthraquinones	-
Flavonoids	+++

+++, Abundant; ++, Average; - , Absent

Table 2. Antiradical activity of methanolic extract of *Thespesia populnea* stem bark observed with DPPH.

Sample	Concentration (µg)	% Inhibition*	EC_{50} (µg)
Methanolic	4	16.02 ± 0.64	
extract	8	22.65 ± 0.16	
	10	40.29 ± 0.80	12.08
	14	60.89 ± 0.24	
	18	65.80 ± 0.48	
	22	77.76 ± 0.16	
	26	79.23 ± 0.63	
Pyrogallol			4.85

* Mean ± SD (n=3)

Table 3. Superoxide anion scavenging activity of methanolic extract of *Thespesia populnea* stem bark observed with a riboflavin-light-NBT system.

Sample	Concentration (µg)	% Inhibition*	EC_{50} (µg)
Methanolic	20	07.94 ± 1.15	73.53
extract	40	39.95 ± 0.66	
	80	48.53 ± 1.73	
	100	60.92 ± 0.91	
	150	72.31 ± 0.49	
	200	78.79 ± 0.25	
Ascorbic acid			45.39

* Mean ± SD (n=3)

Figure 2.
TLC profile of methanolic extract of stem bark of *Thespesia populnea*, sprayed with 0.2 % methanolic DPPH. A. Solvent system-1; B. Solvent system-2; Track 1. Methanolic extract; Track 2. Gossypol standard

Table 4. Reducing power assay of methanolic extract of *Thespesia populnea* stem bark measured by the transformation of Fe^{3+} to Fe^{2+}.

Sample	Concentration (µg)	Absorbance*
Methanolic extract	20	0.076 ± 0.009
	50	0.149 ± 0.005
	100	0.400 ± 0.002
	150	0.566 ± 0.013
	200	0.848 ± 0.004
	300	1.012 ± 0.003
Gallic acid standard	5	0.088 ± 0.008
	10	0.183 ± 0.001
	20	0.523 ± 0.031
	50	1.218 ± 0.015
Tannic acid standard	5	0.146 ± 0.019
	10	0.306 ± 0.008
	20	0.710 ± 0.010
	50	1.482 ± 0.034

* Mean ± SD (n=3)

Table 5. Inhibition of lipid peroxidation induced by iron/ADP/ascorbate system in rat liver homogenate by *Thespesia populnea* stem bark.

Sample	Concentration (µg)	% Inhibition*	EC_{50} (µg)
Methanolic extract	5	11.25 ± 2.13	20.76
	10	21.52 ± 3.25	
	20	54.56 ± 1.79	
	30	60.01 ± 1.57	
	40	68.30 ± 2.33	
	50	82.22 ± 3.54	
α-Tochopherol			27.35

* Mean ± SD (n=3)

3. Results and discussion

Normally free radicals of different forms are generated at a low level in cells to help in the modulation of several physiological functions and are quenched by an integrated antioxidant system in the body. However if produced in excessive amounts they can be destructive leading to inflammation, ischemia, lung damage and other degenerative diseases [23]. Herbal drugs containing free radical scavengers like phenolics, tannins and flavonoids are known for their therapeutic activity [25].

Preliminary phytochemical screening of the stem bark of *T. populnea* showed the presence of alkaloids, terpenoids, high amount of phenolics, tannins and flavonoids (Table 1). Subsequent quantification of phenolics by Folin Ciocalteau method showed the presence of a good amount of total phenolics (10.11 % w/w calculated as gallic acid). High amount of phenolics prompted us to study the free radical scavenging activity of stem bark of *T. populnea*. Methanolic extract of the stem bark of *T. populnea* exhibited different levels of free radical scavenging activity in quenching DPPH and superoxide radical.

DPPH is one of the stable free radicals, generally used for testing radical scavenging activity of chemical compounds or plant extracts. The method is based on the reduction of alcoholic DPPH radical solution in the presence of hydrogen donating antioxidant due to the formation of the non-radical from DPPH; the remaining DPPH radical measured after a certain time corresponds inversely to the radical scavenging activity of the antioxidant [26]. The methanolic extract showed a concentration dependant DPPH radical scavenging activity by bleaching it, with an EC_{50} of 12.08 µg. Beyond 26 µg, there was no increase in quenching with increase in concentration of the extract upto 40 µg tested. The activity was comparable to that of pyrogallol (EC_{50} of 4.85 µg) (Table 2). TLC of the methanolic extract when developed in a relatively less polar solvent system resolved six compounds at R_f 0.11, 0.24, 0.33, 0.66 ((+)-gossypol), 0.70 and 0.76 that showed antiradical activity by bleaching DPPH (possibly phenolic

compounds, as observed from their bluish-green colouration upon derivatization with 5 % methanolic ferric chloride solution). Furthermore, when a more polar solvent system (solvent system 2) was used, from the point of sample application to the last band (R_f = 0.04 to R_f = 0.97) it showed a streak of discolouration of DPPH, covering the entire area, which shows that all the polar components of the extract have very good antiradical activity (Fig.2).

Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxygen species [25]. The assay for studying superoxide scavenging activity is based on the capacity of the sample extract or test compounds to inhibit the formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system [22]. Illumination of either UV or visible light on riboflavin in the presence of EDTA will generate superoxide radical. The generated superoxide radicals react with nitro blue tetrazolium to form blue coloured complex, which can be measured at 590 nm. When the superoxide radicals are scavanged by the sample, there is decrease in the intensity of test solution [19]. The methanolic extract of the stem bark of *T. populnea* showed very good superoxide scavenging activity with an EC_{50} of 73.53 µg (Table 3).

Reducing power assay is a convenient and rapid screening method for measuring the antioxidant potential [23]. The reduction ability, i.e., "Fe^{3+} to Fe^{2+} transformation" (in terms of increase in absorbance at 700 nm), was found to increase with increasing concentration of the extract. A maximum absorbance of 1.012 was obtained at a concentration of 300 µg of extract. Gallic acid and tannic acid were used as positive control which gave maximum absorbance of 1.218 and 1.482 respectively, at a concentration of 50 µg (Table 4).

In lipid peroxidation, hydroxyl radicals are generated which are considered to be the most reactive species of all, initiating the peroxidation of the cell membranes [27]. The lipid radicals thus generated would initiate chain reaction in the presence of oxygen, giving rise to lipid peroxide, which breaks down to aldehydes such as malondialdehyde, which are known to be mutagenic and carcinogenic [28]. The methanolic extract of *T. populnea* stem bark protected hepatocytes from damage due to lipid peroxidation, induced by ferric-ADP-ascorbate in rat liver homogenate in a dose dependent manner as indicated by the reduction in malondialdehyde formation (EC_{50} of 20.76 g), and its activity was found to be comparable to that of α-tochopherol (Table 5).

T. populnea stem bark has been reported to have antioxidant activity, where it was shown to increase the levels of glutathione peroxidase, glutathione S-transferase, glutathione reductase, superoxide dismutase and catalase and decreased the level of lipid peroxidation CCl_4 induced liver toxicity in rat [5]. From this it is clear that the stem bark of *T. populnea* not only enhances the inherent antioxidant system of the body, but also scavenges the free radicals. This dual pronged system works more efficiently in combating the damage due to free radical generation during disease process.

4. Conclusion

The above *in vitro* studies reveal that the stem bark of *T. populnea* had a very good free radical scavenging activity which can be attributed to the presence of various chemical components including phenolics [20,21].

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