Phytochemical screening and *in vitro* antioxidant study of chloroform soluble fraction of *Thespesia populnea* bark extract

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Abstract

In the present study chloroform soluble fraction (CSF) separated from methanolic extract of *T. populnea* bark has been investigated for the presence of various phytochemicals. The *in vitro* antioxidant potential of the fraction was also evaluated using superoxide and nitric oxide radical scavenging assays. Phytochemical screening revealed the presence of steroids, alkaloids, tannins, flavonoids and triterpenes whereas glycosides, diterpenes and saponins were absent. CSF of *T. populnea* exhibited marked scavenging activity against superoxide anion radical (O_2.-) and nitric oxide radical (NO.) in a dose dependent manner comparable to the effect produced by vitamin C standard. The EC_{50} value for superoxide radical inhibition by CSF of *T. populnea* was 41.01 ± 4.82 µg /ml and for nitric oxide radical inhibition EC_{50} obtained was 40.87 +/5.95. So the present study identified that chloroform soluble fraction of methanolic extract of *T. populnea* bark is rich in potent phytochemicals and it possesses marked antioxidant activity in a dose dependent manner.

Key words: *Thespesia populnea*; Chloroform soluble fraction; Phytochemicals; Antioxidant

Introduction

Since historical era plants have been used as therapeutic agents for various ailments across the world. Several plant extracts and plant derived compounds are proven to possess antioxidant, anti-inflammatory, anthelminthic, antibacterial and anticancer activities in various experimental studies. Antioxidant activity or free radical scavenging activity of plant extracts are gaining special attention in the present scenario since free radicals are being identified as a major contributing factor for several diseases including cancer (Hoye *et.al*., 2008).

Thespesia populnea belonging to the family Malvaceae is a medium sized evergreen tree found in tropics and is distributed throughout the coastal regions of India. *T. populnea* bark was used traditionally for ailments like dysentery, haemorrhoids, skin and liver diseases and also in wound healing (Ilavarasan *et.al*., 2003). *T. populnea* has been scientifically proved for its astringent effect, antibacterial effect, antioxidant and hepatoprotective activity, anti-inflammatory activities etc (Gollapalle *et.al.,* 2011). The present study aims at qualitative estimation of the phytochemicals present in the chloroform soluble fraction of *T. populnea* bark extract and evaluation of its antioxidant potential.

Materials and Methods

Collection of plant material and authentication

Bark of *Thespesia populnea* was collected from Thiruvanathapuram district of Kerala by the month of August 2016. The plant material was taxonomically identified and authenticated by The Head, Raw Materials, Herbarium and Museum Division, National Institute of Science Communication and Information Resources (NISCAIR), New Delhi (India). Voucher specimen (NISCAIR/RHMD/2014/2794/173-2) of the plant has been deposited at the herbarium of NISCAIR.

Methanolic extraction and separation of chloroform soluble fraction

Fresh bark of *T. populnea* was chopped into small pieces, shade dried and powdered coarsely in a temperature controlled plant sample pulveriser. Powdered bark (500g) was extracted with methanol in soxhlet extractor at room temperature. The crude methanolic extract of *T. populnea* bark was further fractionated using chloroform and water in a separating funnel, to obtain chloroform soluble fraction (CSF), aqueous fraction (AF) and insoluble residual fraction (RF) of the extract. Chloroform was removed from CSF using rotary vaccum evaporator and then dried completely by keeping at room temperature to use for further experiments.

Phytochemical screening

The CSF of methanolic extract of *T. populnea* bark was tested qualitatively for the presence of various phytochemical constituents namely steroids, alkaloids, tannins, flavonoids, glycosides, diterpenes, triterpenes, saponins and cardiac glycosides as per the standard protocols (Harbone, 1991).

Superoxide scavenging assay

Superoxide anion scavenging activity of the fraction was measured according to the method of Robak and Gryglewski, (1988) with minor modifications. All the solutions were prepared in 0.1 M phosphate buffer (pH 7.4). 1 mL of nitrobluetetrazolium (NBT, 156 µM), 1 mL of reduced nicotinamide adenine dinucleotide (NADH, 468 µM) and 3 mL of the extracts at 0.95, 1.95, 3.9, 7.81, 15.625, 31.25, 62.5, 125, 250 and 500 µg/mL concentrations were mixed. The reaction was initiated by adding 100 µL of phenazine methosulphate (PMS, 60 µM). The reaction mixture was incubated at 25^0C for 5 min and absorbance was measured thereafter at 560 nm using UV/VIS/NIR Spectrophotometer, Lambda 750, Perkin Elmer, Singapore. The reference standard taken for comparison was ascorbic acid. The percentage of inhibition was calculated by using the formula; Percentage inhibition of superoxide = [(AC – AT) / AC] x 100. Where, AC: Absorbance of the control and AT: Absorbance of the extracts/standard.

Nitric oxide scavenging assay

The nitric oxide scavenging activity of the fraction was measured according to the modified method of Sreejayan and Rao, (1997). To 2 ml of different concentrations of extract (0.95, 1.95, 3.9, 7.81, 15.625, 31.25, 62.5, 125, 250 and 500 µg/mL), 0.5 ml of 5mM sodium nitroprusside (SNP) solution in PBS (pH-7.4) was added and incubated for 2hr at room temperature. After incubation added 1.2 ml of Griess reagent (equal volume of 1 percent sulfanilamide in 5 percent H_3PO_4 and 0.1 percent naphthylethylene diamine dihydrochloride in distilled water) to the reaction mixture. The absorbance was read immediately at 546 nm against PBS blank and compared with vitamin C standard. Nitric oxide scavenging activity (percent) = [(AC – AT) / AC] x 100

Where, AC: Absorbance of the control and AT: Absorbance of the extracts/standard.

Statistical analysis

The data of antioxidant assays are expressed as mean± SEM. The Effective Concentration 50 (EC_{50}) values of CSF of methanolic extract of *T. populnea* bark were calculated using the online software "Very Simple IC_{50} Tool Kit".

Results and Discussion

The results of phytochemical screening of CSF of *T. populnea* bark extract are presented in table 1. Phytochemical screening revealed the presence of steroids, alkaloids, tannins, flavonoids and triterpenes in CSF of *T. populnea* bark extract whereas glycosides, diterpenes and saponins were absent. Previous study done by Parthasarathy *et.al.*, (2010) also indicated the presence of carbohydrate, protein, tannins, phenol, flavonoids, terpenes, saponins and gums in the ethanolic extract of *T. populnea* bark.

Table. 1 Phytochemical contstituents in Chloroform Soluble Fraction of *T. populnea bark* extract

Phytochemicals	**Tests**	**CSF of methanolic extract of *T. populnea* bark**
Steroids	Salkowski test	+
	Lieberman Burchardt test	+
Alkaloids	Mayer's test	-
	Wagner's test	+
	Hager's test	+
	Dragendroff's test	+
Tannins	Ferric chloride test	+
	Gelatin test	-
Flavonoids	Ferric chloride test	+
	Lead acetate test	+
Glycosides	Sodium hydroxide test	-
	Benedict's test	-
Diterpenes	Test for diterpenes	-
Triterpenes	Salkowski test	-
	Lieberman Burchardt test	+
Saponins	Foam test	-
Cardiac glycosides	Keller-Kiliani test	-

Table 2. The percentage inhibition of superoxide and nitric oxide radical generation by Chloroform Soluble Fraction of *T. populnea bark* extract

Concentrations µg/ml	**Percent inhibition of superoxide radical**		**Percent inhibition of nitric oxide radical**	
	CSF of *T. populnea*	**Vitamin C standard**	**CSF of *T. populnea***	**Vitamin C standard**
500	94.50±0.96	97.03±0.58	87.31±1.69	79.08±0.98
250	94.43±2.71	96.81±1.16	79.36±1.35	71.98±1.35
125	91.14±1.01	88.08±1.08	73.28±2.03	66.93±2.01
62.5	63.37±3.32	81.94±2.09	65.13±1.44	59.78±3.11
31.25	42.85±2.47	54.63±2.20	52.04±3.52	43.04±1.92
15.62	29.31±4.18	28.89±2.74	46.09±1.70	42.38±2.06
7.81	19.42±2.98	27.01±2.09	34.11±1.50	40.61±1.55
3.91	12.87±4.92	16.82±1.65	29.35±1.35	39.07±0.69
1.95	9.95±2.92	15.32±2.03	31.83±2.16	37.52±2.73
0.98	8.33±2.14	9.36±0.87	26.37±1.17	35.08±1.46
EC_{50}	**41.01 ± 4.82**	**30.97 ± 3.45**	**40.87 +/5.95**	**69.91 ± 6.83**

Values expressed as Mean ± SEM

The percent inhibition of superoxide and nitric oxide radical generation by CSF of *T. populnea* bark extract at different concentrations ranging from 0.98 to 500 µg/ml are presented in table 2. CSF of *T. populnea* exhibited marked superoxide and nitric oxide radical scavenging activity *in vitro* in a dose dependent manner. The EC 50 (Effective Concentration 50; the concentration of extract required to scavenge 50 percent of initial concentration of the free radical generated) value of superoxide radical inhibition obtained for *T. populnea* was 41.01 ± 4.82 whereas for nitric oxide radical inhibition the EC50 value was 40.87 ± 5.95 which was significantly lower than the EC50 value 69.91 ± 6.83 noted for vitamin C standard. Hence CSF of *T. populnea* bark extract was found to be more potent in scavenging *in vitro* nitric oxide radical compared to vitamin C

standard. In earlier studies Silva and Soysa, (2011) showed that decotion prepared of *T. populnea* bark markedly inhibited DPPH, nitric oxide and hydroxyl radicals *in vitro*. Anandjiwala *et.al*., (2008) also demonstrated a significant *in vitro* antioxidant effect of methanolic extract of *T. populnea* with an EC 50 value of 12.08 µg/ml in DPPH radical scavenging assay. The results of the present study confirm the antioxidant potential of *T. populnea* bark and also showed that the CSF obtained from methanolic extract of *T. populnea* bark has potent *in vitro* antioxidant effect in a dose dependent manner. On phytochemical estimation CSF was found to be rich in phytoconstituents like flavonoids and this may be the reason for its marked antioxidant activity as phenolic compounds and flavonoids can exert free radical scavenging action because of the redox properties of their hydroxyl groups and chemical structure (Burda and Oleszek, 2001).

Hence the present study was able to identify that chloroform soluble fraction separated from methanolic extract of *T. populnea* bark have excellent antioxidant action and it can be considered as a source for isolating naturally occuring free radical scavenging compounds.

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