FREE RADICAL SCAVENGING POTENTIAL OF ETHANOLIC EXTRACT OF *URARIA PICTA* LINN.

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Summary

The main objective of the present study is to access the free radical scavenging potential of ethanolic extract of *Uraria picta*. The different *in-vitro* models were studied for its free radical scavenging property viz. DPPH radical scavenging assay, ABTS radical scavenging assay, O-phenanthroline assay, lipid peroxidation assay, superoxide scavenging assay, total antioxidant and non-enzymatic haemoglobin glycosylation assay. The results were analyzed statistically by regression method. Its antioxidant activity was estimated by IC_{50} value and the values were 198.1 µg/ml (DPPH radical scavenging), 96.86 µg/ml (ABTS radical scavenging), 40 µg/ml (O-phenanthroline assay), 27.18 µg/ml (lipid peroxidation), and 40.52 µg/ml (superoxide scavenging). Total antioxidant capacity of ethanolic extract of *Uraria picta* (10mg/ml) is equivalent to 63.31 mg/ml of ascorbic acid. It showed 84.89% inhibition in non-enzymatic haemoglobin glycosylation assay. In conclusion, significant antioxidant activity was associated with presence of phenolic, flavonoid, sterol and terpene derivatives.

Keywords: DPPH, ABTS, lipid peroxidation, Papilionaceae

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Introduction

Oxygen is essential for the survival for all living things and during the process of its utilization in normal physiological and metabolic processes, approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals like superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals [1, 2]. The necessity of compounds with antioxidant activity is increasing as there is a realization that the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been linked in the pathogenesis of several human diseases such as atherosclerosis, diabetes mellitus, chronic inflammation, neurodegenerative disorders and certain types of cancer [3, 4].When generation of ROS overtakes the antioxidant defense of the cells, the free radicals start attacking the cell proteins, lipids and carbohydrates and this leads to a number of physiological disorders [5-7].Oxygen free radicals disintegrate DNA, destroy cell membranes, and create havoc among cell's basic enzymatic metabolic processes [8,9].Many plants often contain substantial amounts of antioxidants including vitamin C and E, carotenoids, flavonoids and tannins etc., and thus can be utilized to scavenge the excess free radicals from human body [10].

Uraria picta Desv. (Fam. Papilionaceae), a suffruticose sparingly branched perennial herb having a height of 0.9–1.8 m, is distributed throughout Bangladesh, India, Sri Lanka, Tropical Africa, Malay Islands and the Philippines [11-13]. Traditionally, the plant is used as an antidote to the venom of a dangerous Indian snake, Echis carinata [12]. Its leaves are a good antiseptic and are used against gonorrhoea. The fruits and pods are effective against oral sores in children and the roots have use against cough, chills and fever [12-13]. Two isoflavanones, 5,7-dihydroxy-20-methoxy-30,40-methylenedioxyisoflavanone and 40,5-dihydroxy-20,30-dimethoxy-7-(5-hydroxyoxychromen-7yl)-isoflavanone along with six known compounds including isoflavanones, triterpenes and steroids were isolated from the roots of Uraria picta. The compounds showing the antimicrobial activities against bacteria (both Gram positive and Gram negative) and fungi. [14]

Materials and methods

Chemicals

1, 1-diphenyl, 2-picryl hydrazyl (DPPH) was obtained from Sigma Chemicals, USA. TBA (Thiobarbituric acid), TCA (Trichloro acetic acid) and BHT (Butylated hydroxytoluene) obtained from Himedia, Mumbai. The other chemicals used were 2,2- azinobis- (3-ethylbenzothiazoline- 6- sulphonate) (ABTS), O-Phenanthroline, ferric

chloride, ascorbic acid, dimethyl sulphoxide, NBT(Nitro blue tetrazolium chloride), Folin Ciocalteu's reagent, Gallic acid, sodium carbonate, sodium hydroxide and potassium chloride. All other chemicals and solvents used in the experiment were of analytical grade.
The instruments used were UV spectrophotometer (Shimadzu 1650), homogenizer (Remi, India), centrifuge (Remi, India) and pH meter (Elico Ltd., India).

Plant Material

The whole plant of *Uraria picta* was collected from the Valsad district, Gujarat, India. It is authenticated by Dr. Gopalakrishna Bhatt, Botanist, Poorna Prajna College, Udupi. A voucher specimen has been deposited for future reference in the Department of Pharmacognosy, K.B.Raval College of Pharmacy, Gandhinagar.

Preparation of Ethanolic extract

About 200 g of the entire plant powder of *Uraria picta* were extracted thoroughly with ethanol for 5 to 6 hrs at 60-70'C. The ethanol from the extract was removed under reduced pressure and finally dried in a desiccator. Its ethanolic extract was used for in- vitro antioxidant activity.

Preparation of U. Picta stock solution

Uraria picta stock solution was prepared in concentration of 1000µg/ml in methanol. From the stock solution different concentration viz. 2, 4, 8, 16, 32, 64, 128, 256, 512, 1000 µg/ml were prepared in distilled water and used for antioxidant studies.

Preparation of stock solution (Standard drugs)

Ascorbic acid was used as standard. Ascorbic acid stock solution was prepared in concentration of 1000µg/ml in methanol. From the stock solution different concentration viz. 2, 4, 8, 16, 32, 64, 128, 256, 512, 1000 µg/ml were prepared in methanol and used for antioxidant studies.
Vitamin E (Tocopherol) was used as standard for antioxidant haemoglobin glycosylation method.

Invitro Antioxidant methods

DPPH radical scavenging assay [15, 16]

To the methanolic solution of DPPH (1 mM) an equal volume of the test compound dissolved in methanol was added at various concentrations from 2 to 1000 µg/ml in a final volume of 1 ml. An equal amount of methanol was added to the control. After 20 min, absorbance was recorded at 517 nm. Experiment was performed in triplicate.

ABTS radical scavenging assay [15, 16]

To the reaction mixture containing 0.3 ml of ABTS radical, 1.7 ml phosphate buffer and 0.5 ml drug was added at various concentrations from 2 to 500 µg/ml. Blank was carried out without drug. Absorbance was recorded at 734 nm. Experiment was performed in triplicate.

O-phenanthroline assay [15-17]

The reaction mixture containing 1ml O-Phenanthroline, 2ml Ferric chloride, and 2ml drug at various concentrations ranging from 2 to 1000 µg/ml in a final volume of 5 ml was incubated for 10 minutes at ambient temperature. The absorbance at 510 nm was recorded. Ascorbic acid was added instead of test compound and Absorbance obtained taken as equivalent to 100% reduction of all ferric ions. Blank was carried out without drug. Experiment was performed in triplicate.

Lipid peroxidation assay [18]

Egg phosphotidylcholine (20mg) in chloroform (2ml) was dried under vacuum in a rotary evaporator to give a thin homogenous film and further dispersed in normal saline (5ml) with a vortex mixture. The mixture was sonicated to get a homogeneous suspension of liposome. Lipid peroxidation was initiated by adding 0.05 mM ascorbic acid to a mixture containing liposome (0.1ml). 150 mM potassium chloride, 0.2 mM ferric chloride, drug solution (2-100 µg/ml) were added separately in a total volume of 1ml. The reaction mixture was incubated for 40 min at 37ºC. After incubation, the reaction was terminated by adding 1ml of ice cold 0.25M sodium hydroxide containing 20% w/v TCA, 0.4% w/v TBA and 0.05% w/v BHT. After keeping in boiling water bath for 20 min, the samples were cooled. The pink chromogen was extracted with constant volume of n-butanol and absorbance of the upper organic layer was measured at 532nm. The experiment was performed in triplicate.

Superoxide scavenging assay [19]

Alkaline DMSO was used as a super oxide generating system. To 0.5 ml of different concentrations of the test compound, 1 ml of alkaline DMSO and 0.2 ml of NBT 20 mM in phosphate buffer pH 7.4 was added. The experiment was performed in triplicate.

Total Antioxidant Capacity [15]

Total antioxidant capacity was measured by spectrophometric method. 0.1 ml of the extract (10 mg/ml) dissolved in water was combined in eppendorf tube with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95ºC for 90 min. After cooling to room temperature, the absorbance of the methanolic solution of each was measured at 695 nm against blank. Ascorbic acid was used as the standard and the total antioxidant capacity is expressed as equivalents of ascorbic acid. The experiment was performed in triplicate.

Non-enzymatic haemoglobin glycosylation assay [20, 21]

The antioxidant activities of different extracts were investigated by estimating degree of nonenzymatic haemoglobin glycosylation, measured colorimetrically. Haemoglobin, 60 mg/100 ml in 0.01 M phosphate buffer (pH 7.4) was incubated in presence of 2 g/100 ml

concentration of glucose for 72 h, in order to find out the best condition for haemoglobin glycosylation. The assay was performed by adding 1 ml of glucose solution, 1 ml of haemoglobin solution, and 1 ml of gentamycin (20 mg/ 100 ml), in 0.01 M phosphate buffer (pH 7.4). The mixture was incubated in dark at room temperature for 72 h. The degree of glycosylation of hemoglobin in the presence of different concentration of extracts and their absence were measured colorimetrically at 520 nm. The experiment was performed in triplicate.

Statistical analysis

All results are expressed as mean ± S.E.M. Linear regression analysis (Origin 6.0 version) was used to calculate the IC_{50} values.

Results

Concentrations ranging from 2-1000µg/ml of the ethanolic extract of *Uraria picta* were tested for their antioxidant activity in different *invitro* models. It was observed that free radicals were scavenged by the extract in concentration dependent manner up to the given concentration in all the models. The percentage scavenging and IC_{50} values were calculated for all the models. In DPPH method, the maximum scavenging activity was found at a concentration 512µg/ml and the minimum scavenging activity at a concentration of 2µg/ml (fig.1). IC_{50} value of DPPH was found to be 198.1 µg/ml. In ABTS method, maximum activity was exhibited at 256 µg/ml with 90.44% scavenging (fig 2). IC_{50} value of ABTS was found to be 96.86 µg/ml. Iron chelating activity, maximum scavenging was found at 1000 µg/ml with IC_{50} of 40.0 µg/ml (fig.3). In superoxide scavenging assay, the maximum activity was observed at 160.0 µg/ml with scavenging of 76.76% (fig. 4). Maximum scavenging activity was observed at 100 µg/ml with an IC_{50} value of 27.18 µg/ml in lipid peroxidation (fig.5). Antioxidant haemoglobin glycosylation, the % scavenging was found to be 76.74% and 84.89% at concentration 0.5 µg/ml and 1.0 µg/ml of extract respectively. Total antioxidant capacity of ethanolic extract of *Uraria picta* (10mg/ml) is equivalent to 63.31 mg/ml of ascorbic acid. The IC_{50} values of different assay were reported (Table-1).

Table-1: Results of different in vitro antioxidant assay

Sr. no.	Model	IC_{50} (µg/ml)	
		Ethanolic extract	**Ascorbic acid/ α-tocopherol**
1	DPPH scavenging assay	198.1	4.55
2	ABTS scavenging assay	96.86	12.46
3	O-Phenanthroline assay	40.00	2.02
4	Lipid peroxidation assay	27.18	30.78*
5	Super oxide scavenging assay	40.52	15.79
6	Antioxidant haemoglobin glycosylation		
	Extract/Standard	*Conc. (µg/ml)*	*% Scavenging*
	Ethanolic extract	0.5	76.74
		1	84.89
	Vitamin-E	0.5	73.68
		1	90.89
7	Total Antioxidant: 10 mg/ml ethanolic extract of *U. picta* is equivalent to 63.31 µg/ml of ascorbic acid.		

*In lipid peroxidation α-tocopherol was taken as standard drug.

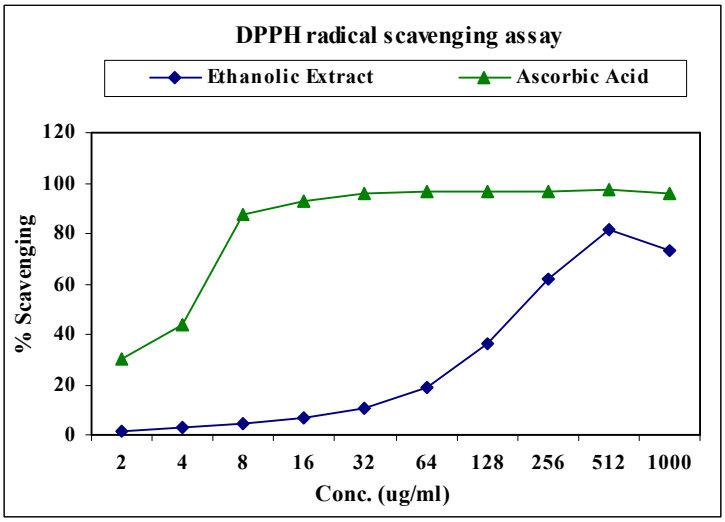

Figure. 1. DPPH radical scavenging activity of different concentrations of *Uraria picta* and ascorbic acid. The results are expressed in terms of concentration vs % scavenging. Each value represents mean ±S.E.M.

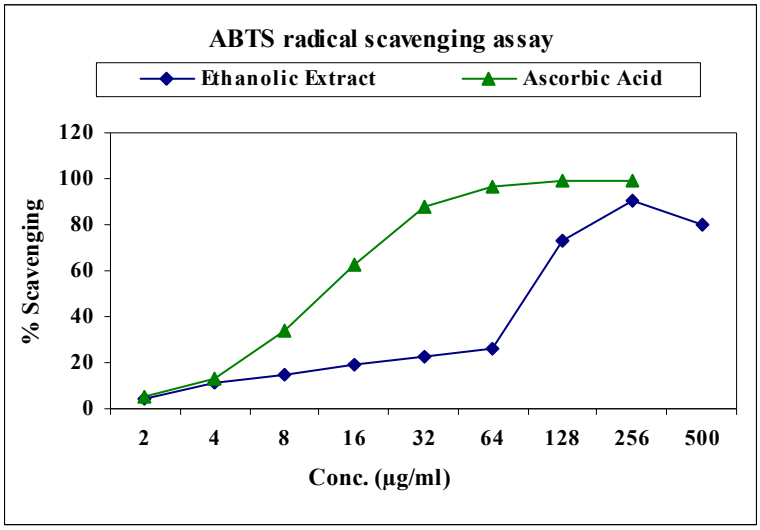

Figure. 2. ABTS radical scavenging activity of different concentrations of *Uraria picta* and ascorbic acid. The results are expressed in terms of concentration vs % scavenging. Each value represents mean ±S.E.M.

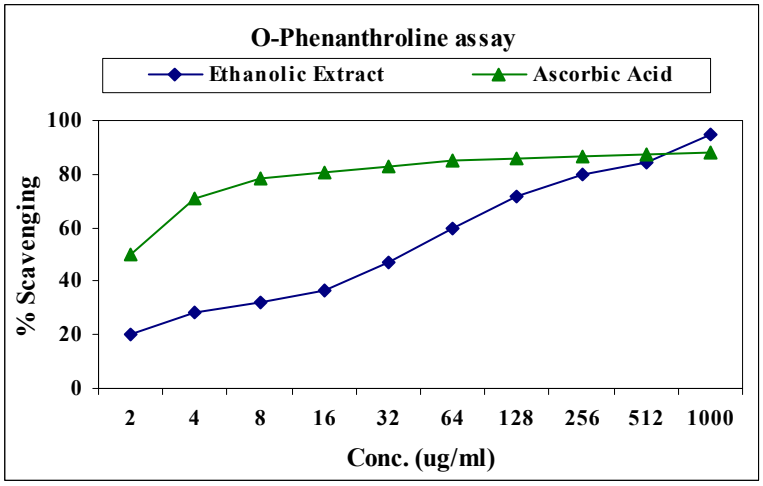

Figure. 3. O-Phenanthroline assay of different concentrations of *Uraria picta* and ascorbic acid. The results are expressed in terms of concentration vs % scavenging. Each value represents mean ±S.E.M.

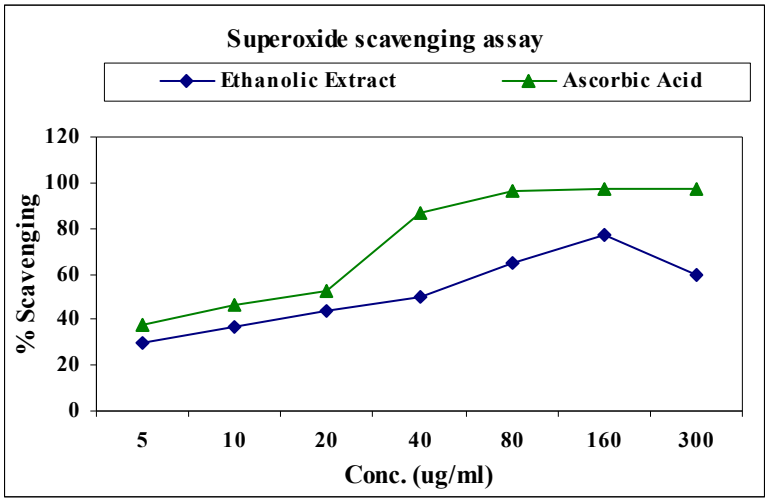

Figure. 4. Super oxide anion radical scavenging activity of different concentrations of *Uraria picta* and ascorbic acid. The results are expressed in terms of concentration vs % scavenging. Each value represents mean ±S.E.M.

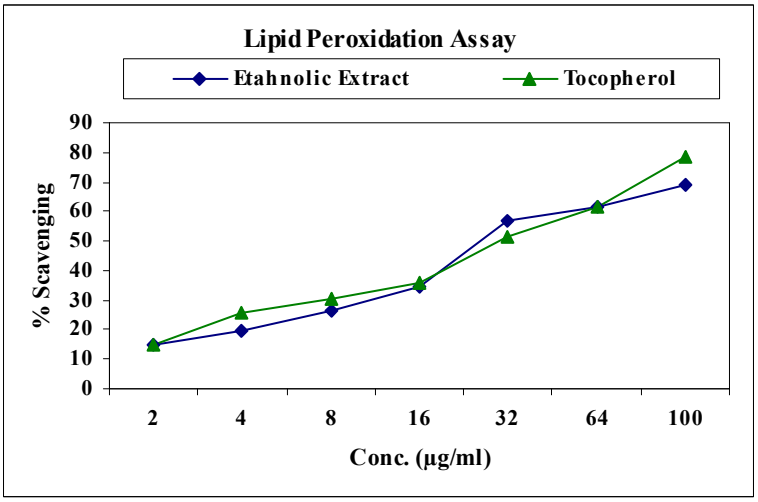

Figure. 5. Antioxidant activity of different concentrations of Uraria picta and α-tocoferol in lipid peroxidation assay. The results are expressed in terms of concentration vs % scavenging. Each value represents mean ±S.E.M.

Discussion

There is an extensive evidence to implicate free radicals in the development of degenerative diseases. Free radicals have been implicated in the causation of ailments such as diabetes, liver cirrhosis, nephrotoxicity, cancer etc. Together with other derivatives of oxygen, they are inevitable by products of biological redox reactions. Reactive oxygen species such as superoxide anions, hydroxyl radical, and nitric oxide inactivate enzymes and damage important cellular components causing tissue injury through covalent binding and lipid peroxidation. [22-25]

DPPH is a stable free radical. When antioxidant reacts with this stable radical, the electron becomes paired off and bleaching of the colour stochiometrically depends on the number of electrons taken up. From our finding, it may be postulated that *Uraria picta* reduces the radical moderately to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principles. [26, 27]

ABTS assay is a decolorizing assay, which involves the direct generation of ABTS radical into monocation, which has a long wavelength absorption spectrum without involvement of any intermediary radical. The antioxidant activity of the extract by this assay implies that the action may be either inhibiting or scavenging radicals since both inhibition and scavenging properties of antioxidant towards this radical have been reported in earlier studies.[28]

Ortho substituted phenolic compounds may exert prooxidant effects by interacting with iron. O-phenanthroline quantitatively forms complexes with $Fe^{2+,}$ which get disrupted in the presence of chelating agents. The ethanolic extract interfered with the formation of ferrous-o-phenanthroline complex, thereby suggesting that the extract has metal chelating activity. Iron stimulates lipid peroxidation by Fenton reaction and also accelerate peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals which themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation. The metal chelating capacity is important, since it reduces concentration of the catalyzing transition metal in lipid peroxidation. It has been reported that the chelating agents, form bonds with a metal are effective as secondary antioxidant because they reduce the redox potential, thereby stimulating the oxidized form of the metal ion. The observed results demonstrate a marked capacity of the extract for iron binding, suggesting that their action as a peroxidation protector may be related to its iron binding capacity. [29-33]

Superoxide is a highly reactive molecule that can react with many substrates produce in various metabolic processes including

phagocytosis. It can cause oxidation or reduction of solutes depending on their reduction potential. Both aerobic and anaerobic organisms possess superoxide dismutase enzyme, which catalyze the breakdown of superoxide radical. In our study, alkaline DMSO used for superoxide generation indicates that *Uraria picta* is a potent superoxide scavenger. [34]

Lipid peroxidation has been implicated in the pathogenesis of number of diseases and clinical conditions. Malondialdehyde and other aldehydes have been identified as products of lipid peroxidation that reacts with the thiobarbituric acid to give pink coloured species. The aldehyde products are responsible for DNA damage, generation of cancer and aging related diseases. The decrease in the concentration of the malondialdehyde leveled with the increase in the concentration of *Lepidium sativum* extract indicates the antioxidant role of the extract. [35]

Haemoglobin glycosylation is an *invitro* non-enzymatic method. Being an oxidation reaction, an antioxidant is expected to inhibit the reaction. The degree of haemoglycosylation *invitro* in the presence of different concentration of extract can be measured spectrophotometrically. [36]

Total antioxidant capacity of the extract was calculated based on the formation of phosphomolybdenum complex which was measured spectrophotometrically. [37]

Uraria picta ethanolic extract exhibits its antioxidant action in several ways: removal of oxygen, scavenging of ROS, binding of metal ions needed for catalysis of ROS and upregulation of endogenous antioxidant defences. Our finding shows that the ethanolic extract of *Uraria picta* has significant antioxidant potential in all the models and to a lesser extent in DPPH assay. The significant antioxidant activity was associated with presence of phenolic, flavonoid, sterol and terpene derivatives.

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