

Antioxidant activity of methanolic and ethanolic extracts of *Pueraria tuberosa* plant

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Abstract

Objective: As plants produce significant amount of antioxidants to prevent the oxidative stress caused by photons and oxygen, they represent a potential source of new compounds with antioxidant activity. *In vitro* antioxidant activity of tuber *Pueraria tuberosa* was investigated.

Methods: Total phenolic content was estimated using the folin-ciocalteu method. The flavonoid content was determined by aluminium chloride method. *In vitro* antioxidant activity viz., DPPH, hydroxyl, superoxide, ABTs radical cation scavenging activity and reducing power of methanol and ethanol extract of tuber of *Pueraria tuberosa* were determined using standard methods.

Results: The total phenolics and flavonoids in methanol extract were found to be 0.68 g 100 g⁻¹ and 1.21 g 100 g⁻¹ respectively. The methanolic extract of *Pueraria tuberosa* showed potent hydroxyl, superoxide, ABTs radical cation scavenging activities. Ethanolic extract of tuber showed strong DPPH radical scavenging activity. The maximum inhibitory concentration (IC₅₀) in all models viz., superoxide, hydroxyl, DPPH and ABTs radical cation scavenging activity of tuber of *Pueraria tuberosa* were found to be 27.16, 26.12, 30.65 and 25.53 µg/mL respectively at 1 µg/mL concentration.

Conclusion: The findings of the present study confirmed the presence of total phenolics and flavonoids and possess *in vitro* antioxidant activity.

Keywords: Antioxidant activity, flavonoid, DPPH, ABTs, *Pueraria tuberosa*

Introduction

Antioxidants are vital substances which possess the ability to protect the body from damages caused by free radical-induced oxidative stress [1]. Reactive oxygen species (ROS) formed *in vivo*, such as superoxide anion, hydroxyl radical and hydrogen peroxide are highly reactive and potentially damaging transient chemical species. Free radicals are produced continuously in various metabolic processes and exist in biological systems. They are important for maintaining normal physiological function. Reactive oxygen species (ROS) (e.g. superoxide anion, hydroxyl radical and hydrogen peroxide) increases the possibility of a wide range of common degenerative diseases. Oxygen is a highly reactive atom that is capable of becoming a part of potentially damaging molecules commonly called “free radicals.” Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function. Many medicinal plants contain large amounts of antioxidants other than vitamin C, vitamin E and carotenoids. The free radical may cause lipid peroxidation, aggregation of protein and degradation of DNA, protein and polysaccharide [1], which eventually destroys cell membranes and kills cells [2].

Recently, more investigations are about the antioxidant activity of medicinal plant extracts, the potentially cancer-inducing oxidative damage might be prevented or limited by

dietary antioxidants found in fruits and vegetables. Antioxidants derived from fruits, vegetables, spices and cereals are very effective and have reduced interference with the body's ability to use free radicals constructively. Natural antioxidants mainly come from plants in the form of phenolic compounds (flavonoids, phenolic acids and alcohols) ascorbic acid and carotenoids. The quest for natural antioxidants for dietary, cosmetic and pharmaceutical uses has become a major industrial and scientific research challenge over the last two decades [2].

In this context, we have attempted to find the bioactive components that might be responsible for the medicinal properties medicinal plants namely, *Pueraria tuberosa*. This plant has been employed in traditional medicine since time immemorial. The tuberous roots of this plant are used in Indian traditional medicine (Ayurveda) in general debility, nervous breakdown, spermatorrhoea, burning sensation, heart diseases, intrinsic hemorrhage, tuberculosis etc. Therefore, we study the antioxidant potential of *Pueraria tuberosa* by carrying out different *in vitro* free radical-scavenging assays namely DPPH and reducing power. The extracts are also screened for total flavonoid and polyphenol content [3]. In view of the above medicinal properties of genus *Pueraria*, the present study was designed to evaluate

the *in vitro* antioxidant activity of methanol and ethanol extracts of tuber of *Pueraria tuberosa*.

Materials and methods

Collection, authenticity and processing of plant material

The tubers of *Pueraria tuberosa* (family: Fabaceae) were collected from the Satpura plateau region of central India (Betul) in the month of September-October 2015. The agro-climatic conditions prevailing in the region provide an ideal habitat for the natural growth of a variety of plants and herbs, which provide raw materials for phytochemical, pharmaceutical and cosmetic industries. The plant material was authenticated by Department of Botany, J.H. Govt College Betul (MP); voucher specimens were identified and preserved in the ethnopharmacology unit.

Extraction of plant material

Tubers were cleaned with purified water and dried under shade then the material was added in mechanical grinder for size reduction and stored in air tight container. The powdered crude drug was extracted with methanol and ethanol by soxhlation process. Suitable amount of drug was filled in soxhlation apparatus and the drug was extracted for 12-15 cycles or until the drug was completely extracted. The extract was collected in a tarred conical flask. The solvent was removed by distillation. The last traces of solvent being removed under vacuum and finally dried extracts were obtained. The final extract thus obtained was used for *in vitro* antioxidants activity.

In-vitro antioxidant activity

Literature concludes that a compound with the presence of tannin and flavonoids gives antioxidant activity. As free radicals causes inflammatory changes in dermal papilla resulting in hair loss, these compounds stabilizes the free radicals and prevents hair loss. So a plant with good

antioxidant activity can also possess good hair growth activity. The antioxidant activity of plants was determined by using gallic acid equivalent, DPPH free radical scavenging method and reducing power method [3].

Determination of total phenolic content

The Folin–Ciocalteu reagent assay was used to determine the total phenolic content. Samples (100 μ L) extract was mixed with 0.5 ml folin–Ciocalteu reagent previously diluted with 7ml of deionized water. The solution was allowed to stand for 3 min at 25 $^{\circ}$ C, after 3 min add 0.2 ml of saturated sodium carbonate solution. The mixture was allowed to stand for another 120 min. The absorbance was measured at 725 nm. Gallic acid was used as standard for the calibration curve. The total phenolic content of the extract was calculated in terms of gallic acid equivalent (GAE).

$$C = c \times \frac{V}{m}$$

Where, C= total content of phenolic compound in mg/g of the extract; c= concentration of gallic acid established via calibration curve; V = volume of extract in ml. Total phenolic content was expressed as gram of gallic equivalents per 100 gram of dry weight ($g\ 100\ g^{-1}\ DW$) of the plant samples.

Determination of total flavonoid content

The content of total flavonoid was determined by aluminium chloride colorimetric method. The content of flavonoid was determined as quercetin equivalent. 1 ml of plant extract in respective solvent (stock solution SS) was mixed with 2 ml $AlCl_3$ (2% w/v) in methanol and the solution was made up to 25 ml with methanolic solution of acetic acid (0.5% v/v) (Probe solution PS). 1 ml of SS was made up to 25 ml with methanolic solution of acetic acid (contrast solution CS). The absorbance of PS and CS was measured at 420 nm after 30 min. The result expressed as percentage of total flavonoid content [3-5].

Table 1: Total phenolic and flavonoid content

Sr. No.	Extract	Total phenolic content ($g\ 100\ g^{-1}$)	Total flavonoid content ($g\ 100\ g^{-1}$)
1.	<i>Pueraria tuberosa</i> (Methanol)	1.23	5.9
2.	<i>Pueraria tuberosa</i> (Ethanol)	0.72	0.93

Evaluation of anti-oxidant activity

DPPH free radical scavenging assay

Free radical scavenging effect was determined by using the free radical generator DPPH (2, 2-diphenyl-1- picrylhydrazyl) by a similar method to Brand-Williams, Cuvelier, & Berset, 1956 [6]. 0.1 mm solution of DPPH in methanol was prepared and 1mL of this solution was added to different concentrations of plant extract to 3 ml of the solution of all extracts in methanol at different concentration (125, 250, 500 & 1000 μ g/mL). Standard DPPH solution was then mixed with test extract with the dilution in a ratio 1:3. The mixtures were kept in the dark at a room temperature for 90 min. The

absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10S UV: Thermo electron corporation). Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenging the DPPH radical was calculated by using the following formula:

$$DPPH\ scavenging\ activity = \{(A_0 - A_1)/A_0\} \times 100\}$$

Where, A_0 is the absorbance of the control reaction and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Hydroxyl radical scavenging activity

Stock solutions of ascorbic acid (1mM), EDTA (1mM), H₂O₂ (10 mM) FeCl₃ (10 mM) and deoxyribose (10 mM), were prepared in distilled deionized water. The assay was performed by adding 0.1mL EDTA, 0.01mL of FeCl₃, 0.1mL H₂O₂, 0.36 mL of deoxyribose, 1.0 mL of the *Pureria tuberosa* extract of different concentration (125, 250, 500 & 1000 µg/mL) dissolved in distilled water, 0.33 mL of phosphate buffer (50 mM, pH 7.9), 0.1mL of ascorbic acid in sequence then mixture was incubated at 37 °C for 60 min. 1.0 mL portion of the incubated mixture was mixed with 1.0 mL of 10% TCA and 1.0 mL of 0.5% TBA (in 0.025M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532 nm [7].

$$\text{Hydroxyl radical scavenging activity} = \{(A_0 - A_1)/A_0\} \times 100\}$$

Where, A₀ is the absorbance of the control reaction and A₁ is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Superoxide anion scavenging activity

The superoxide anion scavenging activity was measured as described by Srinivasan [8]. The superoxide anion radicals were generated in 3.0 ml of Tris-HCL buffer (16 mM, pH 8.0), containing 0.5 mL of NBT (0.3 mM), 0.5 ml NADH (0.936 mM) solution, 1.0 mL extract of different concentration (125, 250, 500 & 1000 µg/ml) and 0.5 mL Tris-HCl buffer (16 mM, PH 8.0). The reaction was started by adding 0.5 mL PMS solution (0.12 mM) to the mixture, incubated at 25 °C for 5 min and the absorbance was measured at 560 nm against a blank sample, ascorbic acid. The percentage inhibition was calculated by using the following equation

$$\text{Superoxide radical scavenging activity} = \{(A_0 - A_1)/A_0\} \times 100\}$$

Where, A₀ is the absorbance of the control reaction and A₁ is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Antioxidant activity by radical cation

ABTS assay was based on the slightly modified method of Huang [9]. ABTS radical cation (ABTS⁺) was produced by reacting 7mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS⁺ solution were diluted with ethanol to an absorbance of 0.70±0.02 at 734 nm. After addition of 100µL of sample or trolox standard to 3.9 mL of diluted ABTS⁺ solution, absorbance was measured at 734 nm by Genesys 10S UV-VIS (Thermo scientific) exactly after 6 min. Results were expressed as trolox equivalent antioxidant capacity (TEAC).

$$\text{ABTS radical cation activity} = \{(A_0 - A_1)/A_0\} \times 100\}$$

Where, A₀ is the absorbance of the control reaction and A₁ is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Reducing power of herbal plant extracts

The reducing power of extract was determined according to the method [10]. 1.0 mL of solution containing 125, 250, 500 & 1000µg/mL of extract was mixed with phosphate buffer (2.5 ml, 2M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The resulting mixture was incubated at 50 °C for 20 min. 2.5 ml of trichloroacetic acid (TCA, 10%) was added to the mixture and centrifuged at 1500 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Results and discussions

The total phenolic contents of ethanolic and methanolic extract of *Pureria tuberosa* tuber were found to be 0.72 g 100 g⁻¹ and 1.23 g 100g⁻¹ respectively. The flavonoid content of ethanol and methanol extract of *Pureria tuberosa* tuber was found to be 1.45 g 100 g⁻¹ and 0.93 g 100 g⁻¹ respectively. The result of DPPH radical scavenging activity of the ethanolic and methanolic extract and the standard ascorbic acid were presented in figure 1. At a concentration of 1000 µg/ml, the hydroxyl scavenging activity of the ethanol and methanol extract reached 49.09% and 46.12% respectively while at the same concentration that of the ascorbic acid was found to be 52.73%. Superoxide radical scavenging activity of the ethanolic and methanolic extract of *Pureria tuberosa* tuber (1000 µg/ml) exhibited the maximum scavenging activity of 71.32% and 73.32% which is lower than the standard i.e. is 75.56%. ABTs radical cation scavenging potential of the extracts of *Pureria tuberosa* tuber was presented in figure 4. The percentage of IC₅₀ and scavenging activity value of the investigated extract at 1 min of the reaction time was calculated. The highest percentage of activity of 78.23% (1000 µg/ml) was found for methanolic extract of tuber. The reducing powers of the plant extract compared to ascorbic acid were presented in figure 5. The IC₅₀ values of DPPH, hydroxyl, superoxide and ABTs radical scavenging activity were presented in figure 6.

In the present study, *Pureria tuberosa* tuber extract showed potent hydroxyl radical scavenging activity which is comparable to the standard. The hydroxyl activity may be due to the presence of phenolics and flavonoids in ethanol extract of *Pureria tuberosa* tuber. Although superoxide anion is a weak oxidant, it gives rise to powerful and dangerous hydroxyl radicals and singlet oxygen, both of which contribute to oxidative stress. Numerous biological reactions generate superoxide anions. The IC₅₀ values were found to be 56.12 µg/mL, 66.12 µg/mL and 72.45 µg/mL respectively for ethanolic and methanolic extract of *Pureria tuberosa* tuber and ascorbic acid. The results indicate that the *Pureria tuberosa* tuber extracts have a noticeable effect as scavenging superoxide radical. The decolorization of ABTs

radical reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to inactivate this radical species. Hydroxyl radical is the most reactive free radical and formed from superoxide anion and hydrogen peroxide in the presence of copper or iron. Hydroxyl radicals react with lipid, polypeptides, proteins, thiamine, guanosine and reacts with polyunsaturated fatty acid moieties and causes damage to cell [11]. Many studies focused on the biological activities of phenolic compounds, which have potential antioxidants and free radical scavengers [12]. Phenolic compounds are well known as scavenging agents and antioxidant free radicals associated with oxidative damage. Phenolic compounds have attracted much interest recently because *in vitro* studies suggest that they have a variety of beneficial biological properties. Studies have attributed that antioxidant properties are due to the presence of phenols and flavonoids. Antioxidant activity of phenolic compounds is based on their ability to donate hydrogen atoms. In addition, they possess ideal structural properties for free radical scavenging properties. Flavonoids are important secondary metabolites of plant modulating lipid peroxidation involved in atherogenesis,

thrombosis and carcinogenesis. The reducing power of a compound depends on the presence of reductases which have been exhibited antioxidant potential by breaking the free radical chain, donating. The reducing capacity of *Pueraria tuberosa* tuber is a significant indicator of this potential antioxidant activity. The methanol and ethanol extracts of *Pueraria tuberosa* tuber showed good antioxidant activity in various *in vitro* systems tested.

In this study, it is evident that the extracts of *Pueraria tuberosa* tuber possess effective antioxidant activity. *Pueraria tuberosa* tuber extract exhibited potent *in vitro* antioxidant activity in DPPH radical scavenging assay, hydroxyl radical scavenging assay, superoxide free radical scavenging assay, ABTS radical cation scavenging activity and reducing power in comparison to the known antioxidants such as ascorbic acid. In conclusion, the high antioxidant activity exhibited by the extracts of *Pueraria tuberosa* provided justification for the therapeutic use of this plant due to the phytochemical constituents. The present study suggests that this extract could be of great importance for the treatment of radical related diseases and oxidative damage.

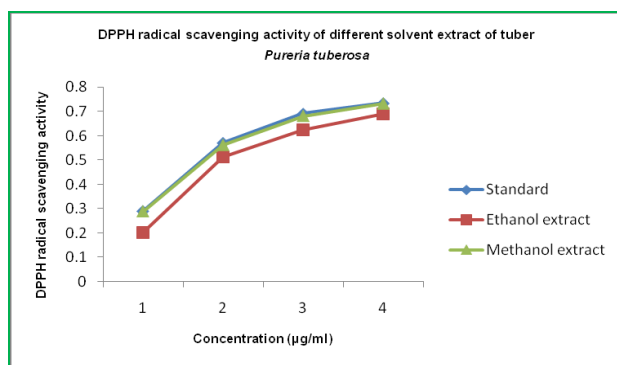


Figure 1: DPPH radical scavenging activity of different solvent extract of tuber *Pueraria tuberosa*

Results are given in mean of triplicate. Error bars was omitted for the simple presentation of graph.

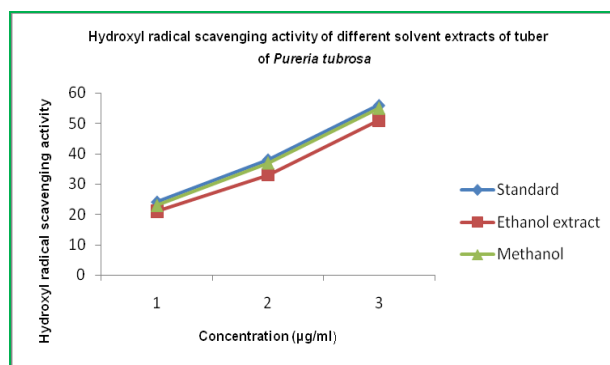


Figure 2: Hydroxyl radical scavenging activity of different solvent extracts of tuber of *Pueraria tuberosa*

Results are given in mean of triplicate. Error bars was omitted for the simple presentation of graph.

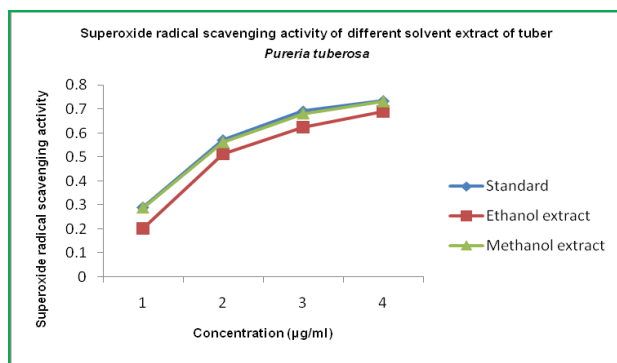


Figure 3: Superoxide radical scavenging activity of different solvent extract of tuber *Pueraria tuberosa*

Results are given in mean of triplicate. Error bars was omitted for the simple presentation of graph.

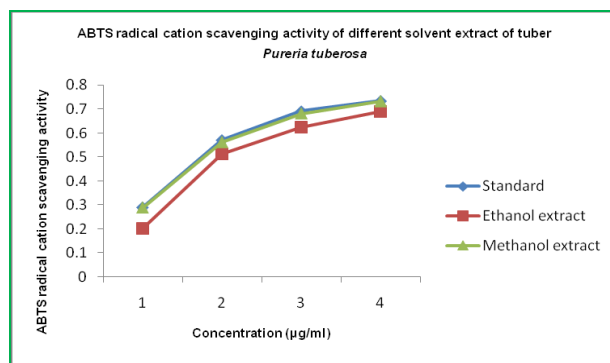


Figure 4: ABTS radical cation scavenging activity of different solvent extracts of tuber of *Pueraria tuberosa*

Results are given in mean of triplicate. Error bars was omitted for the simple presentation of graph.

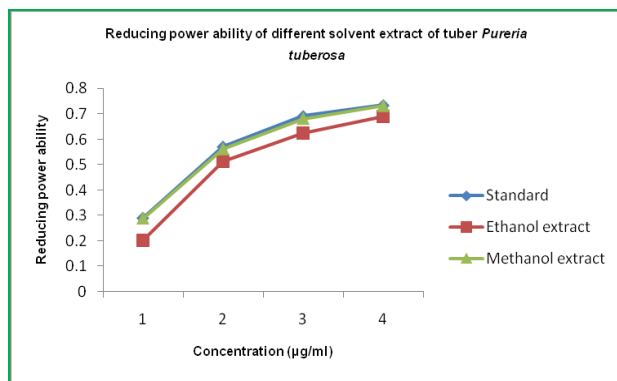


Figure 5: Reducing power ability of different solvent extract of tuber *Pueraria tuberosa*

Results are given in mean of triplicate. Error bars was omitted for the simple presentation of graph.

Conclusion

The comparative study revealed that methanolic extract showed more significant free radical scavenging and reducing activities compared to ethanolic extract of *Pueraria tuberosa*. According to this study, a significant and linear relationship was found between the antioxidant activity and phenolic contents, indicating that phenolic compounds could be major contributors to antioxidant activity. The conclusions if established by in vivo studies on biological systems can open up new avenues in the search for natural antioxidants that can be employed successfully in further clinical trials. These results may have implications in the use of the extract as a therapeutic agent in the prevention of oxidative stress related diseases.

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Conflict of Interest: None declared,

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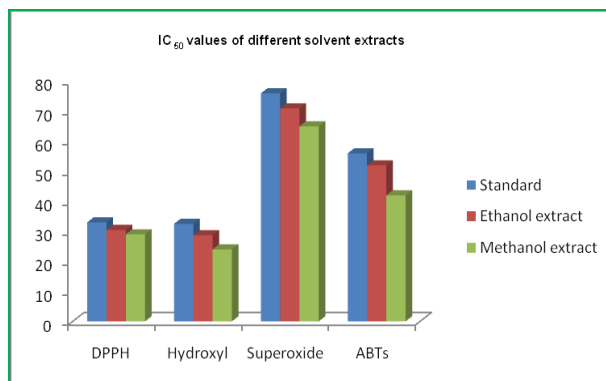


Figure 6: IC₅₀ values of different solvent extracts

Results are given in mean of triplicate. Error bars was omitted for the simple presentation of graph.

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