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In-Vitro Antioxidant Properties and Total Phenolic, Flavonoid and Tannin Contents of *Pueraria Tuberosa* (Roxb. Ex Willd.) DC.

Zereena Viji[1], and Paulsamy[2]*.

[1]Department of Botany, NSS College, Nemmara, Palakkad - 678508, India.
[2]Department of Botany, Kongunadu Arts and Science College, Coimbatore-641029, Tamil Nadu, India.

ABSTRACT

The aim of this study is to evaluate the total phenolics,flavonoids,and tannin content and determine antioxidant activity of different fractions of *P. tuberosa* extracts. Antioxidant potential of petroleumether, chloroform, acetone, methanol and hot water extracts by using FRAP, metal chelating, phosphomolybdenum and free radical scavenging by using $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ were determined by using standard protocols with slight modifications. Quantification assays were carried out for the determination of phenols by Folin-ciocalteu method, tannins by using polyvinylpyrrolidine (PVPP) and flavonoids by aluminum chloride colorimetric method. Higher amount of phenols were found in acetone extract that is why itshows higher antioxidant activity. In $ABTS^{+}$ assay higher scavenging activity of the tuber was observed in methanolic extract(9328.44±1923.77µmoles TE/g extract). In the present study the chloroform fraction was found to be more potent hydroxyl radical scavenger ,with an IC_{50} value of 119.59µg/ml compared to other extracts.Acetone extract showed good antioxidant potential using FRAP(1796.67±146.21mmolesFeII/mg, metal chelating (112.78 ± 3.7512.78mg EDTA/gmextract, phosphomolybdenum (15.52 g AA/100g extract).). The total phenolic and tannin content was found to be higher in acetone extracts of tuber (443.87mg GAE/g and290.41mgGAE/g. The present results suggests that acetone extracts of *P. tuberosa* has significant antioxidant activity that it is effective against free radical mediated diseases and may be considered as natural source of antioxidant .

Keywords: *Pueraria tuberosa*, Fabaceae, phytoconstituents, antioxidant activity.

Corresponding author

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INTRODUCTION

Antioxidants are compounds that protect organisms against oxidative damage which is involved in various lifestyle diseases like diabetes mellitus, cancer, arteriosclerosis, Alzheimer's disease etc. [1]. Free radical production occursinall cells continuously as a result of normal cellular functions. Antioxidants prevent free radical induced tissue damage by preventing theformation of free radicals, scavenging them or promoting their decomposition. Reactive oxygen species like hydroxylradical, superoxide anion, hydrogen peroxide can cause injuries which may cause DNA and protein damage and also oxidation of enzymes in human body. The efficacy of plant extracts as antioxidants were well studied and based on the results obtained by commonly accepted assays [2, 3].Even though large numberof synthetic antioxidants like BHT and BHA are available, they are unsafe and are toxic. Therefore developing plant based antioxidants without any side effects must be developed which can be used as preventive medicine and as nutraceuticals for human consumption.

Pueraria tuberosa (Roxb.ex willd.) DC belongs to Fabaceae family. It is an important plant used in Indian medicine, commonly called as Vidarikand or Indian Kudzu. The plant is described as rasayana and tonic in Ayurvedic Pharmacopoeia of India. The tubers are sweet to taste and it is used in indigenous system of Indian medicine as tonic, aphrodisiac, anti rheumatic, diuretic and galactogue [4]. Tubers possess hypolipidaemic, and cardio protective effects [5, 6].

Due to the changing lifestyle and eating habits, the incidence of lifestyle diseases among the population is increasing. The intake of antioxidant rich foods may prevent the damage caused by free radicals. *P. tuberosa* is used by the tribal people as a general tonic and a rejuvenant. The present investigation is to analyse the biological contents of the root tuber and to evaluate the antioxidant potential of different extracts of *P. tuberosa* using five different methods like ABTS, DPPH, FRAP, metal chelating and phosphomolybdenum.

MATERIALS AND METHODS

Description of the study plant

Pueraria tuberosa, commonly known as kudzu, Indian kudzu,or Nepalese kudzu, is a climber with woody tuberculated stem.The tubers are globose or pot-like, about 25 centimeters (9.8 in) across and the insides are white, starchy and mildly sweet. Flowers are bisexual, around 1.5 cm (0.59 in) across and blue or purplish-blue in color. The fruit pods are linear, about 2–5 cm (0.79–1.97 in) long and constricted densely between the seeds. Seeds vary from 3 to 6 in number.

Preparation of extracts

P. tuberosa root tubers were collected from Nelliampathy and identified. The collected tubers were cut into small pieces; the shade dried tubers of the plant were crushed into powder with a blender. The powdered material was extracted with with organic solvents like petroleum ether, chloroform, acetone, methanol and hot water in the increasing order of polarity using a soxhlet apparatus. The filtrates of extracts were dried under vacuum in a rotary evaporator at 40°C to pursue further analysis.

Chemicals

Gallic acid, BHT, Folin–Ciocalteu, DPPH and Quercetin were procured from Himedia, The rest of the chemicals and solvents used were of analytical grade and were procured from Sigma or Merck.

Quantification assay

Measurement of total phenolics

Total phenolics concentration was measured by Folin-ciocalteu assay by siddhuraju and Becker [7]. Fifty microlitre aliquots of the extracts were taken in test tubes and made up to 1 ml with distilled water. To this 0.5 ml of folin-ciocalteu phenol regent (1:1 with water) was added followed by the addition of 2.5M of sodium carbonate solution (20%).the reaction mixture is vortexed and the test tubes are incubated in dark for

40 minutes and the absorbance was measured at 725 nm against the reagent blank analysis was performed in triplicates and the results were expressed as Tannic acid equivalents.

Determination of Tannins

The same extracts were used in tannin estimation using PVPP by siddhuraju and Manian [8].100 mg of PVPP was weighed into eppendorf tubes and to this 1mLof distilled water and then 1mL of sample extracts were added the content was vortexed and kept in the freezer at 4^{o} C for 15 minutes. Then the sample was centrifuged at4000rpm for 10 minutes and the supernatant was collected. The supernatants have simple phenolics, while tannins would have been precipitated along with PVPP. The phenolic content of the supernatant was measured and expressed as the content of non tannin phenolics on a dry matter basis.
The tannin content of the sample was calculated as:

Tannins %= Total phenolics% - Non tannin phenolics %

Determination of flavonoids

Flavonoids content of the extracts were quantified by the method of Zhishen [9]. About500 µL of the plant extract was taken in different test tubes, to this 2mL of distilled water was added. Blank was prepared by taking 2.5 ml of distilled water .Then ,150µL of $NaNO_2$ as added to all the test tubes and incubated at room temperature for 6 minutes. After incubation 150µLof$AlCl_3$was added to the entire test tubes including blank. All the test tubes were incubated again for 6 minutes at room temperature. Then 2ml of 4% NaOH was added to all the test tubes which were then made up to 5 mL using distilled water. The contents in all the test tubes were vortexed well and allowed to stand for 15 minutes. Pink color was developed due to the presence of flavonoids. The absorbancy was measured at 510 nm. Rutin was used as standard the experiments were done in triplicates and the results were expressed as Rutin equivalents (RE).

***In-vitro* antioxidant assays**

Ferric reducing oxidant assay

The antioxidant capacities of different extracts of sample were estimated according to the procedure of Pulido et.al. [10]. Frap reagent is freshly prepared and incubated at 37o C and was mixed with 90 mL of distilled water and 30mL of test sample or methanol(for blank)The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5 ml of 20 mM (2, 4, 6 tripyridyl S triazine)TPTZ solution in 40mM HCl plus 2.5 ml of 20 mM $FeCl_3.6H_2O$ and 25 ml of 0.3 M acetate buffer, pH 3.6 .After incubation the absorbance was measured at 593nm against reagent blank. Methanolic solutions of known Fe (II) concentrations in the range of 100-2000 mM ($FeSO_4.7H_2O$) were used for calibration curve preparation. The parameter Equivalent Concentration or EC1 was defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mM $FeSO_4.7H_2O$. EC1 was calculated as the concentration of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 mM concentration of Fe (II) solution.

Phosphomolybdenum assay

The antioxidant capacities of different extracts of sample were estimated according to the procedure described by Prieto *et al.* [11]. An aliquot of 10-40 µL of sample or ascorbic acid in 1mM dimethyl sulphoxide (standard) and distilled water (blank) was added with 1Ml of reagent solution (prepared by 0.6M H_2SO_4, 28 mM sodium phosphate and 4 mM ammonium molybdate) in a test tube. The test tubes are covered with foil and incubated in water bath at95^{o} C for 90 minutes. The samples are then cooled at room temperature and the absorbance was measured at 695nm against reagent blank .The results are reported as mean values expressed as milligrams of ascorbic acid equivalents per gram extract.

Metal Chelating activity

The chelation of ferrous ions by various extracts of P.tuberosa was estimated by the method described by Dinis *et al.* [12] 400µL of sample and BHT (standard) were added to 50µL solution of 2 mM $FeCl_2$

followed by the addition of 200µL of 5 mM ferrozine and the mixture was shaken vigorously and left to stand at room temperature for 10 minutes. absorbance of the solution was the measured at 562 nm against deionised water as blank. The metal chelating capacityof the extracts were evaluated using the equation:

$$\text{Metal chelating capacity \%} = [(A_0 - A_1) / A_0] \times 100$$

Where A_0 =Absorbance of control and A_1 is the absorbance of the sample extract/standard

DPPH radical scavenging activity

The antioxidant activity of different extracts of *P. tuberosa* was determined in terms of hydrogen donating and radical scavenging ability using the stable radical DPPH according to the method described by Blois [13]. Sample extracts of various concentrations was taken and the volume was adjusted to100µL with methanol. Aliquots of samples were taken, to this 5mL of 0.1 mM methanolic $DPPH^{\bullet}$ was added. Same quantity of $DPPH^{\bullet}$ was added to standard Rutin and shaken vigorously. Negative control was prepared by adding 100µL of methanol in 5mL of 0.1 mM methanolic solution $DPPH^{\bullet}$. The tubes were allowed to stand for 20 minutes at 27^{o} C. The absorbance of the samples were measure at 517 nm against the blank (methanol).Radical scavenging activity of the samples was expressed as IC_{50}, which is the concentration of the sample required to inhibit 50% $DPPH^{\bullet}$ concentration.

$ABTS^{\bullet+}$ assay

$ABTS^{\bullet+}$ radical- scavenging activity of *P. tuberosa* extracts was determined according to Re *et.al.* [14]. $ABTS^{\bullet+}$ was produced by reacting 7Mm ABTS aqueous solution with2.4mM potassium persulphate in the dark for12-16hours at room temperature. Prior to assay, this solution was diluted with ethanol(about1:89v/v)and equilibrated at 30^{o}Ctogive an absorbance of 0.700±0.02 at 734nm. The stock solution of the sample extracts were diluted so that after introduction of 10µL aliquots in to the assay, they produced between20%and 80% inhibition of the blank absorbance. After the additionof1µL of diluted ABTSsolutionto10µLofsample or Trolox (finalconcentration0-15µM) in ethanol, absorbance was measured at30^{o}C exactly 30 minutes after the initial mixing .Triplicate determinations were made at each dilution of the standard percentage inhibition was calculated against the blank (ethanol) absorbance at 734nm and then was plotted as a function of Trolox concentration. The unit of antioxidant activity (TAA) is defined as the concentration of Trolox having equivalent antioxidant activity expressed as µmoles/g sample extract

RESULTS AND DISCUSSION

Determination of total phenolics

The amount of total phenolics was analyzed and shown (Fig.1).The total phenolic content was found to be higher in acetone extracts of tuber(443.87mg GAE/g extract),followed by methanolic extract(149.07mg GAE/g extract).from the results we can conclude that the antioxidant activity of *P.tuberosa* tubers is due to these phenolic compounds. Phenols are a class of antioxidants which can scavenge free radicals [15].

Determination of tannins

The root extracts of *P.tuberosa* was analysed for its tannin content. The total tannins were found to be higher in acetone extract290.41mgGAE/g extract (Fig. 2). Higher the content of tannins greater will be the capacity to quench free radical. Tannins, poly phenols including flavonoids have been reported to exhibit a wide range of activity and prevent the attack of free radicals in human body [16].Tannins inhibit the absorption of Iron which may lead to anaemia [17].Tannins are metal chelators and the chelated metal ions are not bio available [18].Therefore higher amount of tannins of *P. tuberosa* have greater capacity to chelate metal ions.

Determination of flavonoids

Flavonoid contents were analyzed in the root tubers of *P tuberose* and were shown in (Fig .3). The methanolic extract of tubers posses high (673.76mgRE/g extract) followed by chloroform (355.19mgRE/g extract). Flavonoids are group of natural phenolics which generate H_2O_2, that can scavenge free radicals.

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Flavonoids not only scavenge free radicals but also posses the capability of chelating metal ions and inhibition of enzymes like NADPH [19]. The higher contents of isoflavonoids in *Pueraria lobata* were inferred to be responsible for its more potent antioxidant activity as compared with that of *Pueraria thomsonii* [20].

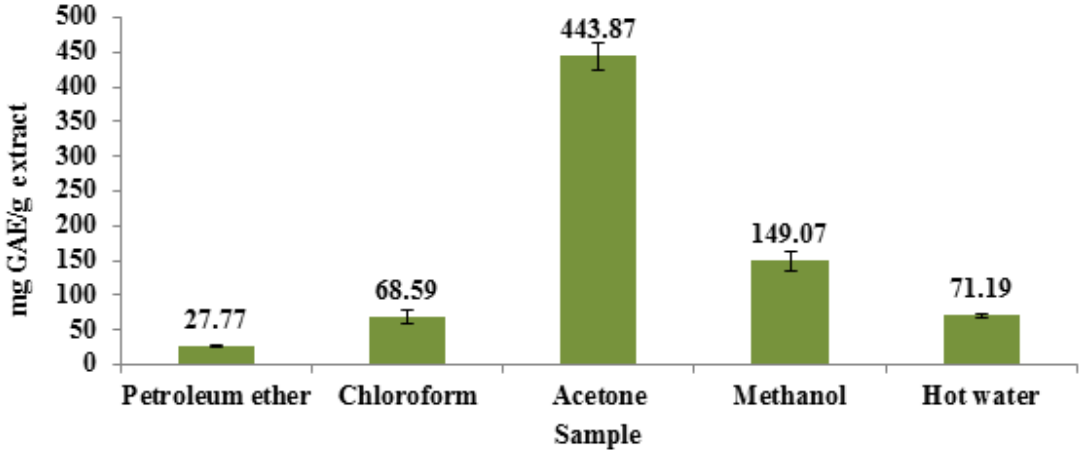

Figure 1: Total phenolic content of *P. tuberosa* root extract.
Values are expressed as mean (n=3) ± Standard Deviation (SD). GAE – Gallic Acid Equivalents

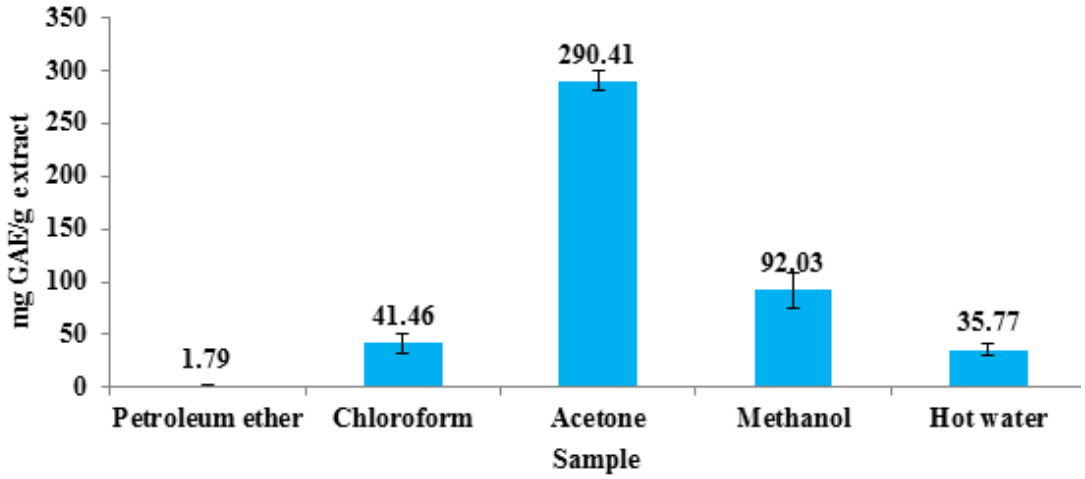

Figure 2: Tannin content of *P. tuberosa* root extracts.
Values are expressed as mean (n=3) ± Standard Deviation (SD).GAE – Gallic Acid Equivalents

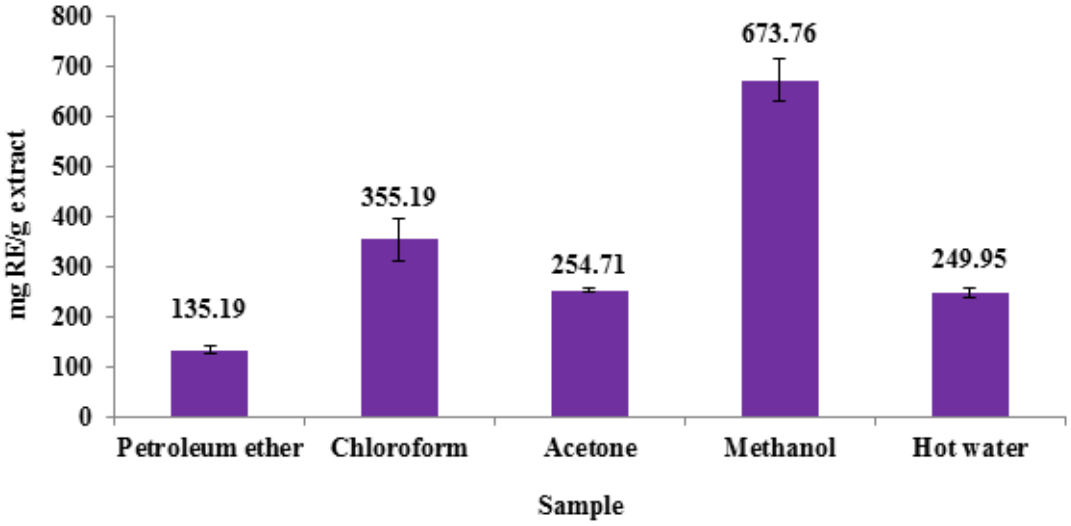

Figure 3: Flavonoid content of *P. tuberosa* root extracts
Values are expressed as mean (n=3) ± Standard Deviation (SD). RE – Rutin Equivalents

***In-vitro* antioxidant assays**

Ferric reducing antioxidant assay

FRAP is a simple and reliable test to measure the reducing potential of an antioxidant reacting with ferric2, 4, 6 tripyridyl S triazine (Fe (III) TPTZ complex and producing a coloured ferrous 2, 4, 6 tripyridyl S triazine (Fe (II) TPTZ complex at low pH

Higher the absorbance higher will be the reducing power.It was found that FRAP values were consistently higher in acetone fraction (Table1)when referred to the corresponding Fe (II) methanolic fraction.

Phosphomolybdenum assay

The phosphomolybdenum assay is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound which turns green Mo (V) complex with absorption maximum at 695nm.

Total antioxidant capacity of different extracts were analyzed and shown in Table 1.The better antioxidant capacity was shown by acetone fraction (15.52 gAA/100g extract).This can be correlated with the free radical scavenging activity of natural antioxidant Ascorbic acid.

Metal chelating activity

Metal chelating action is based on chelation of Fe^{2+} ions by the reagent Ferrozine , which results in the formation of a complex with Fe^{2+} ions [21].When other chelating agents are present it would decrease the formation of red coloured complex. Measurement of the rate of reduction of colour, therefore allows estimation of chelating activity.

In the present study *P. tuberosa* root extracts interact with ferrous and ferrozine complex suggesting that they have chelating properties which may capture Fe^{2+} ions before ferrozine. Metal chelating capacity of acetone extract of tuber was found to be 112.78mgEDTA/g (Table 1).from the result we can say that the extracts have protective role against oxidative damage by sequestering iron ions that may catalyse hydroperoxide decomposition reaction.

Table1: Phosphomolybdenum, FRAP, $ABTS^+$ metalion radical scavenging activity of *P.tuberosa* root extract

Sample extract	PhosphomolybdenumgAA /100g	FRAP (mmolesFeII/mg)	$ABTS^+$ (µmolesTE/g)	Metal ion chelating (mgEDTA/g extract)
Petroleum Ether	5.23±0.32	99.22±7.27	4056±785.66	92.71±5.11
Chloroform	3.99±0.47	526.56±52.94	4589.97±166.47	23.59 ± 7.16
Acetone	15.52±1.23	1796.67±146.21	7735.45±4989.04	112.78 ± 3.75
Methanol	9.37±0.54	1161.48±86.18	9328.44±1923.77	102.53 ± 5.65
Hot water	3.85±0.29	533.33±31.04	4610.22±1154.48	109.92 ± 4.29

Values are mean of triplicate determination (n=3) ± standard deviation

DPPH radical scavenging activity

The principle of DPPH method is based on the reduction of DPPH in the presence of a hydrogen donating antioxidant .Extracts reduce the colour of DPPH due to the power of hydrogen donating ability[9] DPPH is one of the compounds that possess a proton free radical with a characteristic absorption which decreases significantly on exposure to proton radical scavengers[22]Anti oxidants may guard against reactive oxygen species(ROS) toxicities by scavenging reactive metabolites and converting them to less reactive molecules.

Importantly IC_{50} value of the extracts was also calculated to determine the amount of extract needed to quench 50% of radicals. The results of DPPH were expressed inIC_{50} value. Lower theIC_{50} value, higher will be the antioxidant activity. The free radical scavenging activity of the extracts were estimated by comparing with

standards such as BHT, BHA, quercetin and rutin and the result were shown in (Table2) In the present study the chloroform fraction was found to be more potent hydroxyl radical scavenger,with an IC_{50} value of 119.59µg/mL compared to other extracts.

Table 2: DPPH radical scavenging activity of *P. tuberosa* root extracts

Extract	IC_{50} (µg/mL)
Petroleum ether	676.88 ± 12.36
Chloroform	119.59 ± 9.18
Acetone	227.06 ± 15.34
Methanol	498.17 ± 21.84
Hot water	882.22 ± 26.71
BHA	5.10 ± 1.21
BHT	9.10 ± 2.14
Quercetin	3.81 ± 1.81
Rutin	4.94 ± 1.39

Values are expressed as mean (n=3) ± Standard Deviation

ABTS radical scavenging activity

The Trolox Equivalent Antioxidant Capacity (TEAC) was measured using improved $ABTS^+$ radical decolouration assay. This measures the ability of a compound to scavenge $ABTS^+$ radical [23].The results were expressed as µmoles Trolox/gm dry weight of plant material. The results of$ABTS^+$ cation radical scavenging activity of different solvent extracts of *P.tuberosa* was shown in Table.1. Higher scavenging activity of the tuber was observed in methanolic extract (9328.44µmolesTE/gextract)

High molecular weight phenolics have more ability to quench free radicals ($ABTS^{+)}$ and their effectiveness depends on molecular weight, number of aromatic rings etc [19].

CONCLUSION

In the present study it was found that the root tubers of *Pueraria tuberosa* showed higher amount of phenolics and flavonoids which may be responsible for higher antioxidant activity. The toxicity of synthetic antioxidants like BHA,BHT are a problem of concern and are found to be unsafe ,investigations must be directed towards the identification of natural or plant based antioxidants that may be better used for human consumption.

Abbreviations

$DPPH^{\bullet}$: Diphenylpicrylhydrazy
$ABTS^{\bullet+}$: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
FRAP: Ferric reducing oxidant assay
BHA: Butylated hydroxyanisole
BHT: Butylated hydroxy toluene
PVPP: Polyvinylpyrrolidine

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