

Determination of polyphenols and free radical scavenging activity of *Tephrosia purpurea* linn leaves (Leguminosae)

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ABSTRACT

Background: Leaves of *Tephrosia purpurea* Linn. (sarpankh), belonging to the family Leguminaceae, are used for the treatment of jaundice and are also claimed to be effective in many other diseases. This research work was undertaken to investigate the *in vitro* antioxidant activity of aqueous and ethanolic extracts of the leaves. **Method:** The therapeutic effects of tannins and flavonoids can be largely attributed to their antioxidant properties. So, the quantitative determinations were undertaken. All the methods are based on UV-spectrophotometric determination. **Result:** The total phenolic content of aqueous and ethanolic extracts showed the content values of $9.44 \pm 0.22\%$ w/w and $18.44 \pm 0.13\%$ w/w, respectively, and total flavonoid estimation of aqueous and ethanolic extracts showed the content values of $0.91 \pm 0.08\%$ w/w and $1.56 \pm 0.12\%$ w/w, respectively, for quercetin and $1.85 \pm 0.08\%$ w/w and $2.54 \pm 0.12\%$ w/w, respectively, for rutin. Further investigations were carried out for *in vitro* antioxidant activity and radical scavenging activity by calculating its percentage inhibition by means of IC_{50} values, all the extracts' concentrations were adjusted to fall under the linearity range and here many reference standards like tannic acid, gallic acid, quercetin, ascorbic acid were taken for the method suitability. **Conclusion:** The results revealed that leaves of this plant have antioxidant potential. The results also show the ethanolic extract to be more potent than the aqueous decoction which is claimed traditionally. In conclusion, *T. purpurea* Linn. (Leguminosae) leaves possess the antioxidant substance which may be responsible for the treatment of jaundice and other oxidative stress-related diseases.

Key words: Ferric reducing antioxidant power, 1, 1-diphenyl-2-picrylhydrazyl–radical scavenging assay, *Tephrosia purpurea* Linn. (Leguminosae), total phenolic content

INTRODUCTION

Tephrosia purpurea Linn (Leguminosae) is commonly known as Sarpankh. Synonyms of *Tephrosia purpurea* Linn are Thila (in Gujarati), Sarponkh (in Hindi), Vempali (in Telugu). This drug is also not official in Ayurvedic Pharmacopoeia.^[1] It is one of the excellent plants gifted by the nature for human beings, and is composed of all the essential constituents that are required for normal and good human health. Leaves of *T. purpurea* are taken as

emetic in the form of leaf juice or decoction. This along with sugar is also used in jaundice. *T. purpurea*, commonly known in Sanskrit as Sharapunkha, is a highly branched, suberect, herbaceous perennial herb.^[2] According to Ayurveda literature, this plant has also been given the name of “wranvishapaka” which means that it has the property of healing all types of wounds.^[3] It is an important component of some preparations such as Tephroli and Yakrifit used for liver disorders.^[4] In Ayurvedic system of medicine, various parts of this plant are used as remedy for impotency, asthma, diarrhea, gonorrhoea, rheumatism, ulcer and urinary disorders. The plant has been claimed to cure diseases of kidney, liver spleen, heart and blood.^[5] The dried herb is effective as a tonic laxative, diuretic and deobstruent. It is also used in the treatment of bronchitis, bilious febrile attack, boils, pimples and bleeding piles. The roots and seeds are reported to have insecticidal and piscicidal properties and are also used as vermifuge. The roots are also reported to be effective in leprosy wound and their juice for the eruption on skin. An extract of pods

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is effective for pain and inflammation, and their decoction is used in vomiting.^[6] The ethanolic extract of seeds has shown significant *in vivo* hypoglycemic activity in diabetic rabbits.^[7] The ethanolic extracts of *T. purpurea* possess potent antibacterial activity. The flavanoids were found to have antimicrobial activity.^[8] Phytochemical investigations on *T. purpurea* have revealed the presence of glycosides, rotenoids, isoflavones, flavanones, chalcones, flavanols and sterols.^[9] It is also a good source of minerals and amino acids. High performance liquid chromatography (HPLC) analysis of the extract of flowering plant of *T. purpurea* shows the presence of rutin and quercetin. Although the *T. purpurea* plant leaves carry high potential uses, especially for the treatment of jaundice, not many proper scientific studies have been carried out on the leaves of this plant, especially in an antioxidant perspective.

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols.^[10] As oxidative stress might be an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied, particularly in the treatment for stroke and neurodegenerative diseases. However, it is unknown whether oxidative stress is the cause or the consequence of disease. Antioxidants are also widely used as ingredients in dietary supplements in the hope of maintaining health and preventing diseases such as cancer and coronary heart disease. Although initial studies suggested that antioxidant supplements might promote health, large clinical trials conducted later did not detect any benefit and suggested instead that excess supplementation may be harmful. In addition to these uses of natural antioxidants in medicine, these compounds have many industrial uses, such as preservatives in food and cosmetics.^[11] So, we made an attempt to fulfill the paucity of studies by carrying out preliminary *in vitro* antioxidant work in the leaves of *T. purpurea*. Here is our initiation for the future drug.

MATERIAL AND METHODS

Chemicals and reagents

Chemicals used in this study were 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA), phosphomolybdic acid, sodium tungstate, potassium ferricyanide and sodium nitroprusside,

naphthylethylenediamine dihydrochloride, sodium nitrite, trichloroacetic acid, ascorbic acid, ethylenediamine tetraacetic acid, phosphoric acid, nitroblue tetrazolium, phenazine methosulfate and ferrous ammonium sulfate (Sd Fine Chemicals Ltd, Bombay, India). All other reagents and solvents used in the study were of analytical grade.

Plant material

Leaves of *T. purpurea* were collected with flowering top during the month of August, from a local cultivating field area of Mehsana district, Gujarat, India. The plant material was authenticated at the Department of Botany, Govt. Arts and Science College, Modasa, Gujarat. A voucher specimen as a herbarium (NMP/08/28082009) has been kept in our museum for future reference. The leaf parts were chopped and dried at room temperature for 10 days and used as raw material. The dried leaves of the drug were powdered using mechanical method and the resulting powder was passed through a 40 # sieve and stored in an airtight container.

Preparation of raw material

Preparation of crude aqueous extract

Accurately 100 g of powder was weighed accurately and taken in a stainless steel vessel and mixed with 2000 ml (1:20) of distilled water. Then, the mixture was boiled for about 2 hours using a gas burner. After that, the mixture was filtered through cotton bag and then using vacuum filter assembly, the filtrate was poured in a borosilicate 500 ml beaker. Then, the filtrate was evaporated on a hot plate until it reaches the concentrated quantity (not in viscous state).

Preparation of dry powder form of extract

The dry powder of this extract was prepared using the simple saloon water sprayer by spraying the extract on stainless steel evaporating plate, after this the thick solution is poured on sprayer, after that it was heated on hot plate at constant temperature of about 60°C. The clumpy dry powder obtained was scraped by the knife and made into fine powder form by using pretreated mortar and pestle (glass type) and packed in airtight plastic container. Every step must be carried out at above the room temperature and the powder was stored in vacuum desiccators as such or in the form of stock solution prepared by the same solvent until further use. The preconditioned set method can be optimized quantitatively by evaluating the presence of any constituent like tannins, flavonoids or any marker, by suitable validation methods. This study was undertaken using the spectrophotometric method.

Preparation of ethanolic extracts

Then, 55 g of the drug powder was weighed and packed in a thimble flask, and 550 ml of ethanol (70%) was added in 11 round bottom flask. Then the Soxhlet assembly was set up to complete 10–15 cycles. After this, the extract was

filtered and filtrate was concentrated up to 50 ml using a water bath. From the concentrate, 10 ml of the extract was taken in an evaporating dish (borosilicate glass) that was previously weighed. The total weight of evaporating dish containing 10 ml extract was recorded and the extract was evaporated till a thick liquid was obtained. The difference in weight was calculated at every 10 minutes until a constant weight was obtained. The residue at the constant weight (it can be obtained from the graph % Loss on Drying) was used as a dry extractive^[12] to prepare the stock solutions (w/v) and the percentage yield was 11.2% w/w and this residue was stored in the freezer until further use.

Estimation of total phenolic content by spectrophotometer

By Folin–Denis method

The method is based on the oxidation of molecule containing –OH groups. Tannin and tannin like compounds reduce phosphotungstomolybdic acid in an alkaline solution to produce a highly blue colored solution.^[13,14] One milliliter of the aqueous and ethanolic extracts, adjusted to fall under the linearity range, i.e., 50 µg/ml of both the drugs, was withdrawn in 10 ml volumetric flasks separately. To each flask, 0.5 ml of Folin-Denis reagent and 1 ml of sodium carbonate were added and the volume was made up to 10 ml with distilled water. The absorbance was measured at absorption maxima 700 nm within 30 minutes of reaction against the blank. The total phenolic content (TPC) was determined by using calibration curve (5–30 µg/ml). Three readings were taken for each and every solution to get reproducible and accurate results. The results are shown in Table 1 and Figure 1. The intensity of the solution is directly proportional to the amount of polyphenols present in solution. This test is done by using Tannic acid

Concentration of extracts	% w/w of total tannin
L.S aqueous 50 µg/ml	9.44 ± 0.22
L.S ethanolic 50 µg/ml	18.44 ± 1.23

Values are mean ± SEM, n = 3

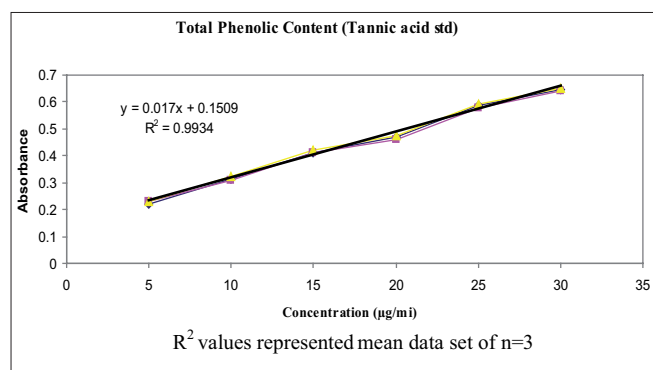


Figure 1: Results of total phenolic content

as standard. The Total Phenolic Content was expressed as mg tannic acid equivalents per 100 g dry weight of sample.

Total flavonoid content by spectrophotometer Aluminum chloride colorimetric assay method

Total flavonoid contents were measured using aluminum chloride colorimetric assay.^[15] Aqueous and ethanolic extracts that had been adjusted to fall under the linearity range, i.e., 400 µg/ml, and different dilutions of standard solution of quercetin (10–100 µg/ml) were added to 10 ml volumetric flask containing 4 ml of water. To the above mixture, 0.3 ml of 5% NaNO₂ was added. After 5 minutes, 0.3 ml of 10% AlCl₃ was added. After 6 minutes, 2 ml of 1 M NaOH was added and the total volume was made up to 10 ml with distilled water. Then, the solution was mixed well and the absorbance was measured against a freshly prepared reagent blank at 510 nm. Results are shown in Table 2 and Figure 2. Total flavonoid content of the extracts was expressed as percentage of quercetin equivalent per 100 g dry weight of sample.

In vitro antioxidant study

Ferric reducing antioxidant power method

The ferric reducing property of the extract was determined by taking 1 ml of different dilutions of standard solutions

Concentration of extracts	% w/w of total flavonoid	
	Quercetin	Rutin
L.S aqueous 500 µg/ml	0.91 ± 0.08	1.85 ± 0.23
L.S ethanolic 500 µg/ml	1.56 ± 0.32	2.54 ± 0.68

Values are mean ± SEM, n = 3; r₂ values represented mean data set of n = 3

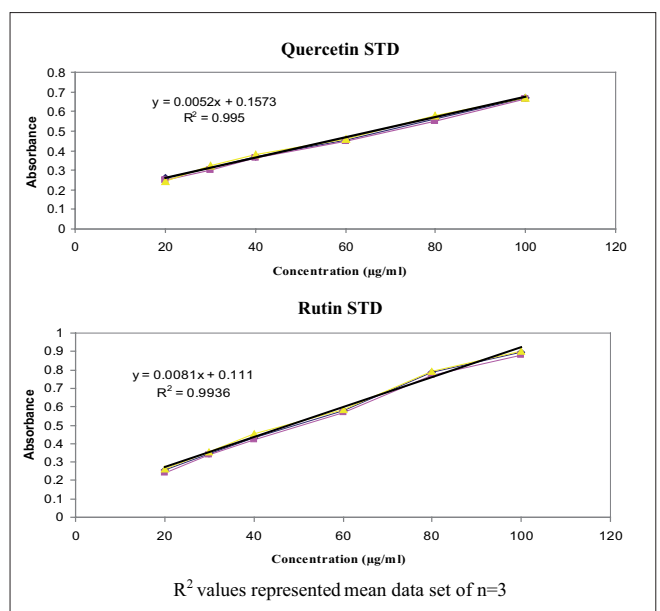


Figure 2: Results of flavonoid content

of gallic acid (10–100 µg/ml). Aqueous and ethanolic extracts that had been adjusted to fall under the linearity range, 500 µg/ml, were taken in 10 ml volumetric flasks and mixed with 2.5 ml of potassium buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide.^[16] The mixture was incubated at 50°C for 20 minutes. Then, 2.5 ml of 10% trichloroacetic acid was added to the mixture to stop the reaction. To 2.5 ml of above solution, 2.5 ml of distilled water was added and then 0.5 ml of 0.1% FeCl₃ was added and allowed to stand for 30 minutes before measuring the absorbance at 593 nm. Results are shown in Table 3 and Figure 3. The absorbance obtained was converted to gallic acid equivalent as milligrams per gram of dry material (GAE/g), using gallic acid standard curve.

Scavenging activity assays

Nitric oxide scavenging assay

Nitric oxide radical inhibition was estimated using Griess Illosvory reaction.^[10,17] In this investigation, Griess Illosvory reagent was generally modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-naphthylamine (5%). The reaction mixture (3 ml) containing 2 ml of 10 mM sodium nitroprusside, 0.5 ml saline phosphate buffer and 0.5 ml of standard solution or aqueous and ethanolic extracts (500–1000 µg/ml) were incubated at 25°C for 150 minutes. After incubation, 0.5 ml of the reaction mixture was mixed with 1 ml sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 minutes for the completion of the reaction of diazotization. After this, a further 1 ml of the naphthyl ethylene diamine dihydrochloride was added, mixed and was allowed to stand for 30 minutes at 25°C. The concentration of nitrite was assayed at 546 nm and was calculated with the control absorbance of the standard

nitrite solution (without extracts or standards, but the same condition should be followed). Here buffer was used as blank solution and Ascorbic acid and quercetin were taken as standard solution. Results are given in Figures 4–7. The percentage inhibition was calculated using the formula:

$$\% \text{ scavenging activity} = [(A_{\text{control}} - A_{\text{test}} \text{ or } A_{\text{std}}) / A_{\text{control}}] * 100$$
 where A_{control} is the absorbance of control and A_{test} or A_{std} is the absorbance of test or standard, respectively.

Hydrogen peroxide scavenging assay

The ability of extracts to scavenge hydrogen peroxide was determined with a little modification. Here, the solution of hydrogen peroxide (100 mM) was prepared by the addition of various concentrations of aqueous

Table 3: Results of FRAP

Concentration of extracts	mg GAE/g of extracts
T.P aqueous 500 µg/ml	64.94 ± 0.28
T.P ethanolic 500 µg/ml	76.56 ± 0.54

Values are mean ± SEM, n = 3

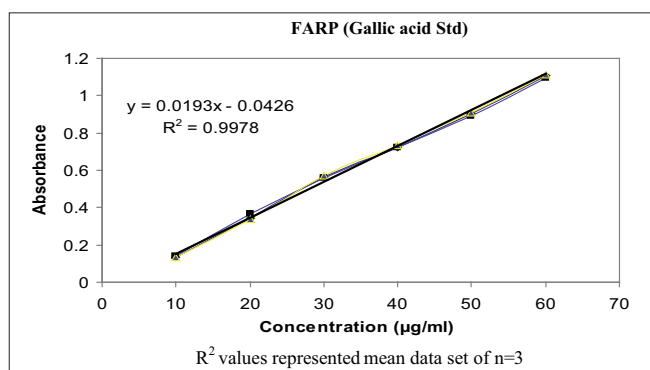


Figure 3: Results of FRAP

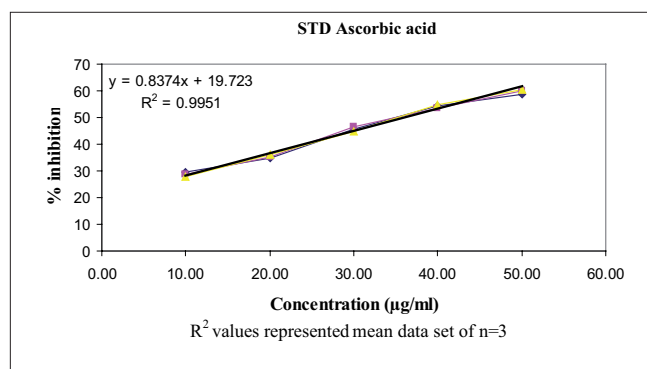


Figure 4: Results of nitric oxide radical scavenging assay

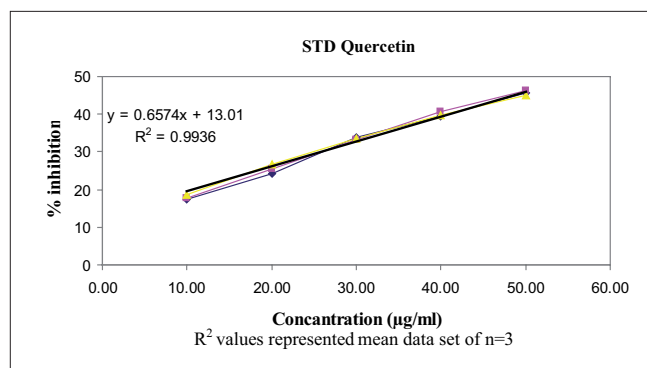


Figure 5: Results of Nitric oxide radical scavenging assay

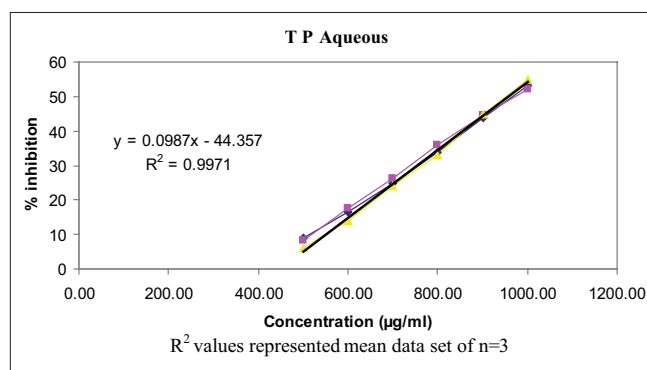


Figure 6: Results of Nitric oxide radical scavenging assay

and ethanolic extracts (100–1000 µg/ml) to hydrogen peroxide solution (2 ml), instead of preparing it in 40 mM phosphate buffer saline of pH 7.4.^[18] Absorbance of hydrogen peroxide at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. For each concentration, a separate blank sample was used for background subtraction. For control sample take Hydrogen Peroxide solution and take absorbance at 230 nm. Results are shown in Figures 8–10. The percentage inhibition activity was calculated from the formula $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of extract/standard taken as gallic acid (10–100 µg/ml).

1, 1-diphenyl-2-picrylhydrazyl–radical scavenging assay method

The free radical scavenging activities of aqueous and ethanolic extracts and the standard L-ascorbic acid (vitamin C) were measured in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH.^[19,20] Here, 0.1 mM solution of DPPH in alcohol was prepared and it must be protected from light influence by maintaining the dark condition and was folded with a aluminum foil and 3 ml of this solution was added to 1 ml various concentrations of (100–2000 µg/ml) extracts or standard solution of 10–100 µg/ml. Absorbance was taken after 30

minutes at 517 nm. Results are shown in Figures 11–13. The percentage inhibition activity was calculated using the formula $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of extract/standard taken as ascorbic acid.

Statistical analysis

Values were represented as mean \pm SEM of three parallel data.

RESULTS AND DISCUSSION

Effect of total phenolic content and flavonoid content

The quantitative determination of the TPC, expressed as milligrams of tannic acid equivalents and per 100 g dry weight of the sample TPC of T.P. aqueous and ethanolic extracts, showed the values of $9.44 \pm 0.22\%$ w/w and $18.44 \pm 1.23\%$ w/w, respectively, and total flavonoid content of the extracts was expressed as percentage of quercetin and rutin equivalent per 100 g dry weight of sample. The total flavonoid estimation of aqueous and ethanolic extracts showed the content values of $0.91 \pm 0.08\%$ w/w and $1.56 \pm 0.32\%$ w/w, respectively, for quercetin and $1.85 \pm 0.28\%$ w/w and $2.54 \pm 0.43\%$ w/w, respectively, for rutin. The above results show that the aqueous extract contains less tannins and flavonoid than the alcoholic extract. This is

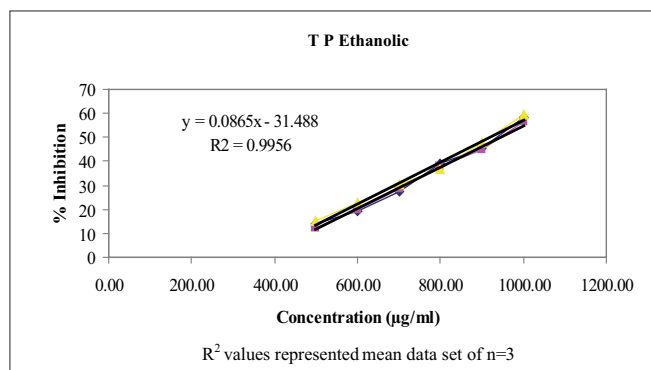


Figure 7: Results of Nitric oxide radical scavenging assay

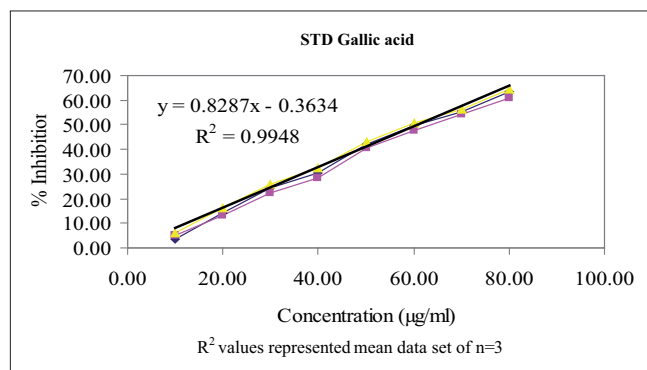


Figure 8: Results of Hydrogen Peroxide scavenging Assay

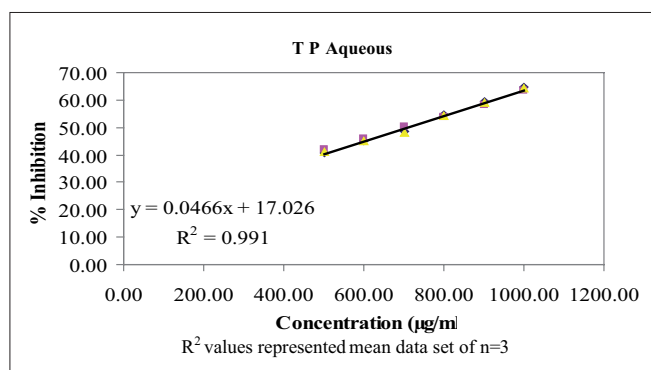


Figure 9: Results of Hydrogen Peroxide scavenging Assay

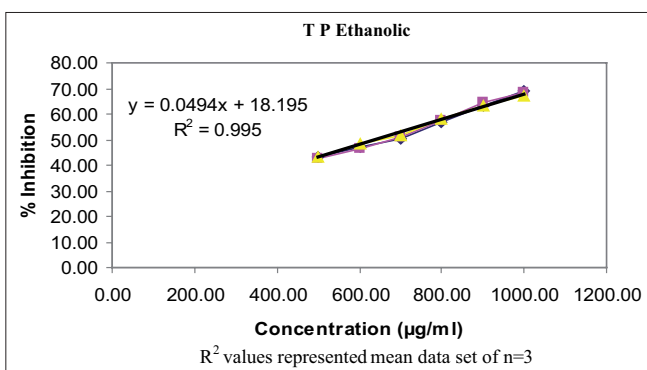


Figure 10: Results of Hydrogen Peroxide scavenging Assay

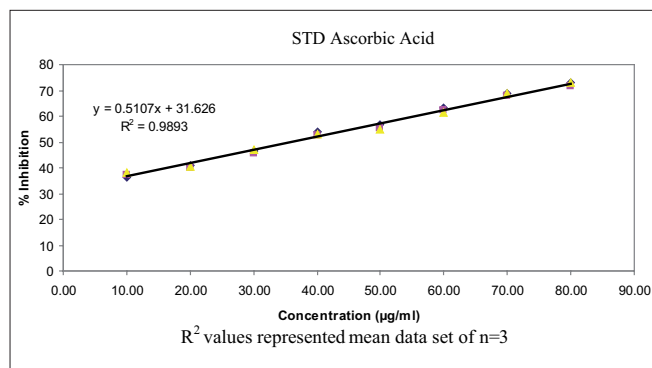


Figure 11: Results of DPPH radical scavenging assay

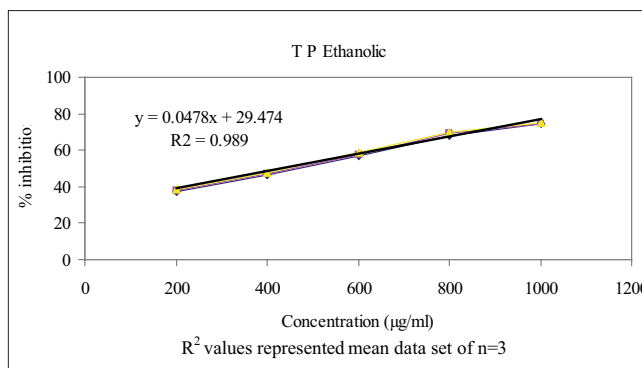


Figure 13: Results of DPPH radical scavenging assay

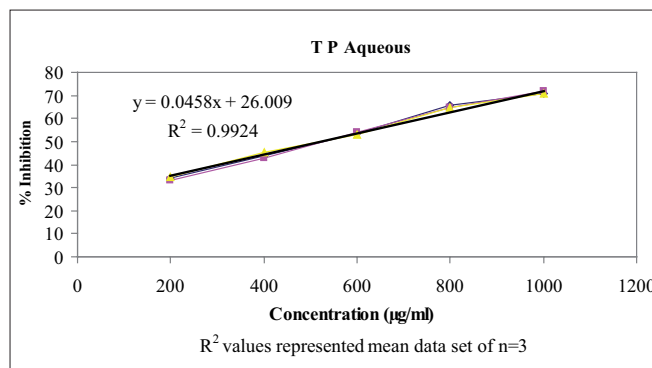


Figure 12: Results of DPPH radical scavenging assay

because of principle active constituents present in higher amount in alcoholic solvent. Thus, it has been accepted as a universal solvent for the extraction of plant constituents.

Results of ferric reducing antioxidant power method

At low pH, measuring the change in absorption at 593 nm can monitor reduction of a ferric complex to the ferrous form which has an intense bluish green color. The change in absorbance is directly related to the combined or "total" reducing power of the electron-donating antioxidants present in the reaction mixture. Here, the ferric reducing antioxidant power (FRAP) showed the results of aqueous and ethanolic extracts that of 64.94 ± 0.28 mg equivalent to gallic acid (GAE)/g of aqueous sample and

76.56 ± 2.54 mg GAE/g of ethanolic sample, respectively.

Results of nitric oxide scavenging assay

Nitric oxide is a very unstable species under the aerobic condition. It reacts with O_2 to produce the stable product nitrates and nitrite through the intermediates NO_2 , N_2O_4 and N_3O_4 . It is estimated by using the Griess reagent. In the presence of test compound, which is a scavenger, the amount of nitrous acid decreases. The extent of decrease reflects the extent of scavenging. The percentage inhibition of aqueous and ethanolic extracts of three parallel readings ($r^2 = 0.9971$) showed IC_{50} values of 956.00 and 942.06 $\mu\text{g/ml}$ ($r^2 = 0.9956$), respectively, as compared to the standards ascorbic acid and quercetin, whose values were 36.16 $\mu\text{g/ml}$ ($r^2 = 0.9951$) and 56.27 $\mu\text{g/ml}$ ($r^2 = 0.9936$), respectively.

Results of hydrogen peroxide scavenging

H_2O_2 itself is not very reactive, but it can sometimes be toxic to the cell because it may give rise to hydroxyl radical in the cells. Thus, removal of H_2O_2 is very important for protection of food systems. Scavenging of hydrogen peroxide and its percentage inhibition of aqueous and ethanolic extracts showed IC_{50} values of 707.60 $\mu\text{g/ml}$ ($r^2 = 0.991$) and 834.21 $\mu\text{g/ml}$ ($r^2 = 0.995$), respectively. Gallic acid taken as reference showed 62.33 $\mu\text{g/ml}$ ($r^2 = 0.9948$).

Results of 1, 1-diphenyl-2-picrylhydrazyl–radical scavenging assay

Table 4: Results and discussion of all the % inhibition studies

Results	Samples	Equation	*r ² values	IC ₅₀ values (µg/ml)
% Inhibition by nitric oxide assay	Standard ascorbic acid	y = 0.8374x + 19.723	0.9951	43.71
	Standard quercetin	y = 0.6574x + 13.01	0.9936	28.73
	L.S aqueous	y = 0.0987x - 44.357	0.9971	938.92
	L.S ethanolic	y = 0.0865x - 31.488	0.9956	805.85
% Inhibition by hydrogen peroxide assay	Standard gallic acid	y = 0.8287x - 0.3634	0.9948	62.28
	L.S aqueous	y = 0.0466x + 17.026	0.9932	653.28
	L.S ethanolic	y = 0.5107x + 31.626	0.991	341.55
% Inhibition by DPPH-RSA	Standard ascorbic acid	y = 0.0494x + 18.195	0.995	58.96
	L.S aqueous	y = 0.0458x + 26.009	0.9924	831.36
	L.S ethanolic	y = 0.0478x + 29.474	0.989	561.18

*Data set of n = 3 and mean r² values obtained from the graphs

The antioxidant reacts with stable free radical, DPPH and converts it to 1, 1-diphenyl-2-picryl hydrazine. The ability to scavenge the free radical, DPPH, was measured at an absorbance of 517 nm. So the 1, 1-diphenyl-2-picrylhydrazyl–radical scavenging assay (DPPH–RSA) and its percentage inhibition of aqueous and ethanolic extracts showed IC₅₀ values of 523.82 µg/ml ($r^2 = 0.9924$) and 429.41 µg/ml ($r^2 = 0.989$), respectively. Ascorbic acid was taken as reference which showed 35.98 µg/ml. ($r^2 = 0.9893$). These results show the ethanolic extract to be more potent than traditionally claiming decoction.

The overall results of percentage inhibition as shown in Table 4 with respect to IC₅₀ values and regression r^2 value. Repeatability is done for 3 times.

CONCLUSION

T. purpurea leaves possess the antioxidant substance which may be potentially responsible for the treatment of jaundice. This leaves a lot of scope to conduct numerous studies on antioxidative stress, hepatoprotective, anticancer activities, etc., of the leaves of the plant. In future we look forward to check the potency of the leaves by means of *in vivo* antioxidant studies.

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