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RESEARCH ARTICLE

In vitro Free Radical Scavenging Activity, Phytochemical Screening and Quantitative Analysis of *Passiflora ligularis* Seed

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Abstract: The medicinal properties of plants are due to the presence of chemical substances that produce certain definite physiological action on the human body. The qualitative analysis as well as quantification of phytochemicals of a medicinal plant is regarded as fundamental step in any kind of medicinal plant research. Ethanolic extract of *Passiflora ligularis* seed was screened for secondary metabolites such as flavonoids, tannins and carbohydrates. Total alkaloid, flavonoid, phenol, saponin and tannin contents were also analysed quantitatively. The preliminary phytochemical screening revealed presence of flavonoids, saponins, tannins, phenols and glycosides. Analysis of the free radical scavenging activity of the extract exposed a concentration dependent antiradical activity resulting from the reduction of radicals to non-radical forms. Findings of the study provided support that crude extract of the plant species contains medicinally important bioactive compounds and justify the use of the plant in the traditional medicine for the treatment of different diseases.

Key words: Antioxidants, Free radicals, alkaloids.

INTRODUCTION:

Nature has provided many things for humankind over the years, including the tools for the first attempts at therapeutic intervention and ancient civilization depended on plant extracts for the treatment of various ailments [1]. Free radicals are incessantly produced in the human body, as they are essential for energy supply, detoxification, chemical signaling and immune function [2]. In humans antioxidant enzymes are produced to neutralize the free radicals [3]. When the generation of reactive oxygen species (ROS) overtakes the antioxidant defense of the cells the free radicals start attacking cellular proteins, lipids and carbohydrates leading to the pathogenesis of many disorders including arthritis and connective tissue disorders, liver disorders, neurodegenerative disorders, cardiovascular disorders, diabetes, chronic inflammation, mutagenesis, carcinogenesis and in the process of ageing [4]. Antioxidants provide protection for living organisms from damage caused by uncontrolled production of ROS and the concomitant lipid peroxidation, protein damage and DNA strand breaking [5].

Antioxidants reduce the oxidative stress in cells and are therefore useful in the treatment of many human diseases, including cancer, cardiovascular diseases and inflammatory diseases. This activity is due to the ability of antioxidants to reduce oxidative stress by neutralizing or scavenging of reactive species by hydrogen donation [6].Recent studies have confirmed that free radicals would damage nearby structures including DNA, proteins or lipids. Radical scavenging antioxidants are mainly significant in protecting cells from the injury of free radical [7]. Thus, antioxidants with free radical scavenging activities may have enormous significance in the prevention and therapeutics of diseases [8]. Phytochemicals are divided into two groups, which are primary and secondary constituents; according to their functions in plant metabolism. Primary constituents comprise common sugars, amino acids, proteins and chlorophyll while secondary constituents consists of alkaloids, terpenoids and phenolic compounds and many more such as flavonoids and tannins etc.[9].

Plants synthesize compounds with biological activity, namely antioxidant, as secondary products, which are mainly phenolic compounds serving in plant defense mechanisms to counteract reactive oxygen species (ROS) in order to avoid oxidative damage. Phenolics are secondary plant metabolites ranging from simple to highly polymerized compounds [10]. Many epidemiological studies have shown that the consumption of phenolic rich foods is associated with the prevention of chronic diseases [11]. In

addition to their antioxidant properties, these compounds have been reported to be potential candidates in lowering cardiovascular diseases [12] and anticarcinogenic activities [13, 14] antiallergenic, anti-arthrogenic, anti-inflammatory, antimicrobial and antithrombotic effects [15]. Plant phenolics, in particular phenolic acids, tannins and flavonoids are known to be potent antioxidants and occur in vegetables, fruits, nuts, seeds, roots and barks [16]. In the case of phenolic compounds, the ability of the phenolics to act as antioxidants depends on the redox potential of their phenolic hydroxyl groups that allow them to act as reducing agents, hydrogen-donating antioxidants and oxygen quenchers [17].

Flavonoids, glycosides, alkaloids, phenolic compounds and volatile constituents have been reported as the major phyto-constituents of the Passiflora species [18]. Health promoting properties are attributed to the phytochemicals (e.g., phenolic antioxidants, vitamins, minerals, fiber among others) present in these natural sources [19, 20].

MATERIALS AND METHODS

Plant material

The fresh fruits of plant specimens were collected from Cunoor in Nilgiri Hills and it was authenticated (No.BSI/SC/5/23/09-10/Tech.323) as *Passiflora ligularis* and Family: Passifloraceae in Botanical Survey of India, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

Preparation of seed extract

The dried seed powder of *Passiflora ligularis* was extracted with ethanol by using Soxhlet apparatus for 5 days and it was concentrated by evaporation. The brown oily substance obtained was weighed and stored in an air tight container for future use.

Phytochemical Screening

The qualitative analysis of secondary metabolites in ethanolic seed extract of *P. ligularis* was carried out by the method of Trease and Evans (1996) and Harborne (1987), which is described below [21, 22].

Test for alkaloids

a) 2 ml aliquot of the extract was treated with the Dragenderoff's reagent. An orange red precipitate is produced immediately indicating the presence of alkaloids.
b) 1 ml aliquot of the extract was treated with few drops of Mayer's reagent. Formation of white or pale yellow precipitate showed the presence of alkaloids

Test for flavonoids

a) 1 ml of the extract was treated with magnesium turnings and 1-2 drops of concentrated HCl. Formation of pink or red color shows the presence of flavonoids.
b) 1 ml of the extract was treated with one ml of ferric chloride. The formation of brown color confirms the presence of flavonoids.

Test for tannins and phenolic compounds

a) 1 ml of the extract was treated with few ml of 5% neutral ferric chloride. A dark blue or bluish black color product shows the presence of tannins.
b) 1 ml of the extract was treated with few ml of gelatin solution; a white precipitate is formed revealing the presence of tannins and phenolic compounds
c) 1 ml of the extract was treated with few ml of lead tetra acetate solution. A precipitate production shows the presence of tannins and phenolic compounds.

Test for amino acids and proteins

a) To 1 ml of extract, 2 drops of freshly prepared 0.2% ninhydrin reagent was added and heated. Development of purple color indicates the presence of proteins.
b) The extract was treated with one ml of 40% sodium hydroxide solution and two drops of 1% copper sulphate reagent. Appearance of violet color indicates the presence of proteins.

Test for carbohydrates

a) Fehling's test
The extract was treated with 5 ml of fehling's solution (A and B) and kept in boiling water bath for 5 min. Formation of yellow or red color precipitate indicates the presence of reducing sugar.
b) Benedict's test
To 1 ml of the extract, added 5 ml of Benedict's solution and kept at boiling water bath for 5 min. Red, yellow or green precipitate indicates the presence of reducing sugars.

Test for glycosides

To the solution of the extract in glacial acetic acid, few drops of ferric chloride and concentrated sulphuric acid are added, and observed for a reddish brown coloration at the junction of two layers and the bluish green color in the upper layer which indicates the presence of glycosides.

Test for saponins

a) About 1 ml of alcoholic extract was diluted separately with 20 ml of distilled water and shaken in a graduated cylinder for 15 minutes. A one cm layer of foam indicates the presence of saponins.
b) To 1 ml of the extract, 1 ml of alcoholic vanillin solution was added which was followed by the addition of few drops of concentrated sulphuric acid. A deep violet color confirms the presence of saponins.

Test for fixed oils and fats

a) Spot test:

A small quantity of extract is pressed between two-filter papers. Oil stains on the filter paper indicates the presence of fixed oil.

Test for terpenoids

a) Horizon test

To 1 ml of extract, 2 ml of trichloroacetic acid was added. The formation of yellow to red precipitate shows the presence of terpenoids.

b) Libermann test

To 1ml of extract 3 ml of acetic acid and few drops of concentrated sulphuric acid were added. Color changed from red to blue indicating the presence of terpenoids.

Test for steroids

a) Libermann-Burchards test

To 1.0 ml plant extract, 1.0 ml of concentrated sulphuric acid was added followed by the addition of 2.0 ml of acetic anhydride solution. A greenish colour developed and it turned blue to indicate the presence of steroids.

b) Salkowski reaction

To 2.0 ml sample extract, 1.0 ml of concentrated sulphuric acid was added carefully along the sides of the tube. A red colour was produced in the chloroform layers.

***In vitro* free radical scavenging activity**

DPPH radical scavenging assay was estimated by Blois method [23], 2,2'-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical activity was estimated by the method of Re, et al., [24] the hydroxyl radical scavenging activity was measured according to the method of Klein, et al., [25] Hydrogen peroxide radical scavenging assay was determined by Ruch method [26], Nitric oxide (NO) scavenging activity of the extract was determined by the method of Green, et al., [27] Measurement of superoxide radical scavenging activity was done using the standard method of Liu, et al., [28] and metal chelating activity was performed by the method Dinis [29]. The reducing power of the whole plant extract was quantified according to the method of Oyaizu [30] and the total antioxidant potential of sample was determined using ferric reducing antioxidant power (FRAP) by the method of Benzie and Strain [31].

Quantitative analysis

Determination of total phenolics

The total phenolic contents of leaves, stem and flower extracts of *P. ligularis* were determined according to the method described by Malik and Singh [32]. Aliquots of the extracts were taken in a 10 ml glass tube and made up to a volume of 3 ml with distilled water. Then 0.5 ml folin ciocalteau reagent (1:1 with water) and 2 ml Na_2CO_3 (20%) were added sequentially in each tube. A blue color was developed in each tube because the phenols undergo a complex redox reaction with phosphomolibdic acid in folin ciocalteau reagent in alkaline medium which resulted in a blue colored complex, molybdenum blue. The test solutions were warmed for 1 minute, cooled and absorbance was measured at 650 nm against the reagent used as a blank. A standard calibration plot was generated (Figure-1) at 650 nm using known concentrations of catechol. The concentrations of phenols in the test samples were calculated from the calibration plot and expressed as mg catechol equivalent of phenol/g of sample.

Determination of alkaloids

Alkaloid determination was done using Harborne (1973) method [33]. 5 g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

Determination of flavonoid

Flavonoid determination was done using the method of Bohm and Kocipai- Abyazan (1994). 10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through whatman filter paper No 42 (125 mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight [34].

Determination of saponin

The method used was that of Obadoni and Ochuko (2001) [35]. 20 g of samples powder was put into a conical flask and 100 ml of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 4 h with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200 ml 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a waterbath. After evaporation the samples were dried in

the oven to a constant weight and the saponin content was calculated as percentage.

Determination of tannins content [36]

Dried plant material (0.5 g) was extracted with 300 ml of diethyl ether for 20 hours at room temperature. The residue was boiled for 2 h with 100 ml of distilled water, and then allowed to cool, and was filtered. The extract was adjusted to a volume of 100 ml in a volumetric flask. The content of tannins in the extract was determined colorimetrically using Folin–Denis reagent, and by measuring absorbance of the blue complex at 760 nm, using tannic acid solution as a standard solution.

RESULT AND DISCUSSION

Phytochemical screening

Natural antioxidants such as phenols, flavonoids, alkaloids and tannins are increasingly attracting attention because they are having qualities of disease-preventing, health-promoting and anti-ageing substances [37].The beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity [38]. Phenolics content are very important plant constituents because they can act as reducing agents, hydrogen donors and metal chelator [39]. They also act as radical scavenger due to their hydroxyl groups. Flavonoids show their antioxidant action through scavenging or chelating process [40]. Medicinal plants are rich sources of bioactive compounds gaining adequacy worldwide and thus provide as a significant raw material for drug invention and have become a target for the search of novel drugs[41-44].

Table 1: Phytochemical screening of ethanolic seed extract of *P. ligularis*

Phytochemicals	Ethanolic seed extract
Alkaloids	+
Flavonoids	+
Steroids	-
Tannins and Phenols	+
Saponins	+
Cardioglycosides	-
Fixed oil and fats	+
Amino acids and Proteins	-
Carbohydrates	+
Terponoids	-

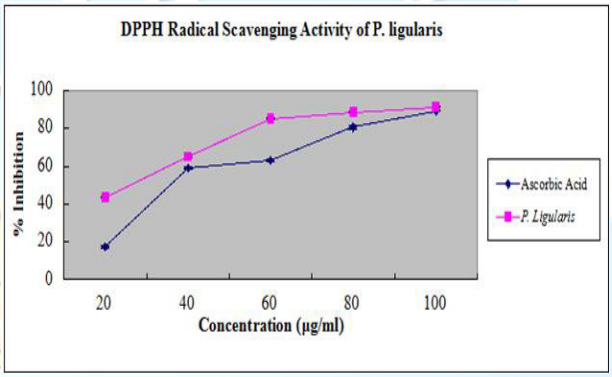
"+" : present; "–" : absent

In vitro radical scavenging activity

DPPH radical scavenging activity

DPPH antioxidant assay is based on the ability of 1,1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. DPPH is usually used as a substrate to evaluate the antioxidant activity of antioxidants [45]. It has been reported that oxidative stress, which occurs when free radical formation exceeds the body's ability to protect or scavenge them, forms the pathological basis of several chronic disease conditions [46, 47]. The IC_{50} value of seed ethanolic extract of *Passiflora ligularis* and ascorbic acid were found to be 27 µg/mL and 36 µg/mL respectively which are shown in Figure 1.

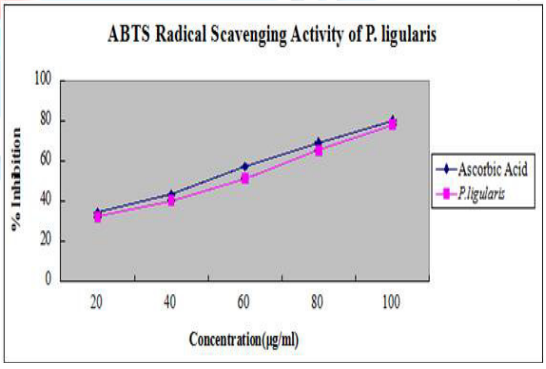
Figure 1: DPPH radical scavenging activity of P. ligularis



ABTS radical scavenging activity

ABTS radical, a protonated radical has characteristic absorbance maximum at 734 nm which decreases at the scavenging of proton radical which is known as excellent substrate for peroxidases frequently used to study antioxidant properties of natural compounds [48]. Figure 2 indicates that the ethanolic seed extract of *P. ligularis* on ABTS+ radical assay shows a significant antioxidant activity at a concentration of 100 µg/ml with an inhibitory activity of 78% as compared to that of standard ascorbic acid (80%). The percentage inhibition of the extract was found to be 58 µg/mL (seed) and 50 µg/mL (ascorbic acid).

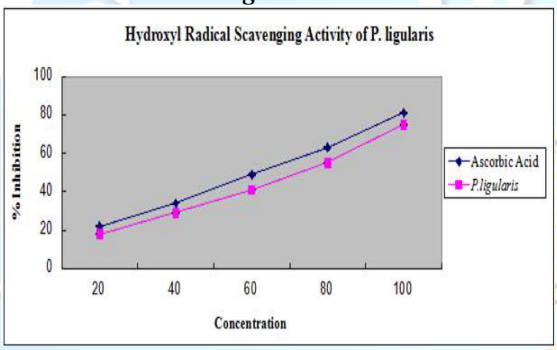
Figure 2: ABTS radical scavenging activity of *P. ligularis*



Hydroxyl radical scavenging activity

The presence of transition metal ions in a biological system could catalyse the Haber-Weiss and Fenton type reactions, resulting in generation of hydroxyl radicals (OH). However, these transition metal ions could form chelates with the antioxidants, which results in the suppression of OH generation and inhibition of peroxidation processes of biological molecules [49].The hydroxyl radical scavenging activities of ethanolic extract of *Passiflora ligularis* was showed in figure 3. At the concentration of 100 µg/ml, the ethanolic seed extract exhibited 75% inhibition whereas with standard antioxidant showed 81% inhibition respectively. The IC_{50} value of stem ethanolic extract of *Passiflora ligularis* and ascorbic acid were found to be 73 µg/mL and 61 µg/mL respectively.

Figure 3: Hydroxyl radical scavenging activity of *P. ligularis*



Hydrogen peroxide radical scavenging activity

Hydrogen peroxide is a weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups [50]. It can probably react with Fe2+ and possibly Cu2+ ions to form hydroxyl radicals and this may be the origin of many of its toxic effects [51]. Hydrogen peroxide scavenging depends upon the phenolic content of the extract which can donate electrons to H_2O_2 thus neutralizing it into water [52]. The scavenging of hydrogen peroxide by the extract increased in a dose dependent manner is illustrated in (Figure 4). The percentage inhibition of ethanolic seed extract was found to be 77% at 100 µg/ml concentration when compared with standard antioxidant ascorbic acid (70%). The IC_{50} value of plant extract and the standard was found to be 52µg/ml and 30µg/ml. Thus the ethanolic extract of the *P. ligularis* was capable of scavenging H_2O_2 in a dose dependent manner.

Nitric oxide scavenging activity

Nitric oxide is a very unstable species, so under aerobic condition it can react with O_2 to produce its stable products such as nitrate and nitrite through intermediates NO_2, N_2O_4. In the presence of a scavenging test compound, the amount of nitrous acid will decrease and can be measured at 546nm [53].The nitric oxide radical scavenging activities of *Passiflora ligularis* extract were shown in Figure 5. The percentage inhibition of ethanolic extract was found to be 76% at 100 µg/ml concentration when compared with standard antioxidant ascorbic acid (82%). The IC_{50} value of the stem ethanolic extract was found to be 71 µg/mL and standard ascorbic acid 61 µg/mL respectively.

Figure 4: Hydrogen peroxide radical scavenging activity of *P. ligularis*

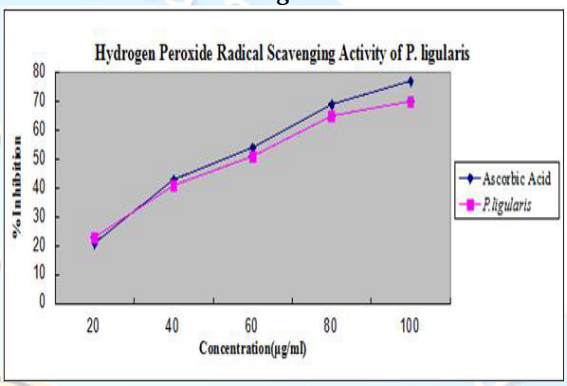
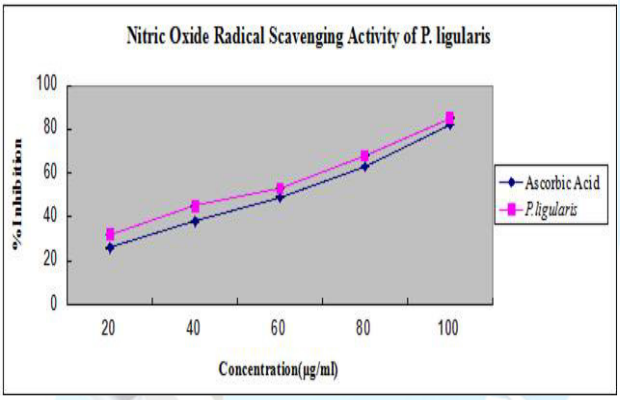


Figure 5: Nitric oxide scavenging activity of *P. ligularis*



Superoxide radical scavenging activity

In biochemical systems, superoxide radical can be converted into hydrogen peroxide by the action of superoxide dismutase and the H_2O_2 can subsequently generate extremely reactive hydroxyl radicals in the presence of certain transition metal ions on by UV photolysis. Hydroxyl radicals can attack DNA molecules to cause strand scission [54]. The extract showed maximum scavenging activity of 80% at a concentration of 100µg/ml and the standard reported 76% scavenging activity at a concentration of 100µg/ml. The IC_{50} value of stem ethanolic extract of *Passiflora ligularis* and ascorbic acid is 66 µg/mL and 70 µg/mL (Figure 6) respectively.

Figure 6: Superoxide radical scavenging activity of *P. ligularis*

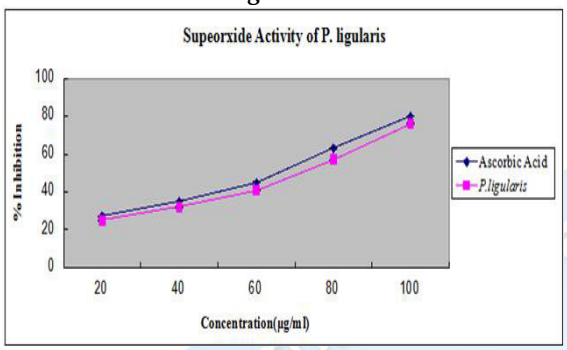
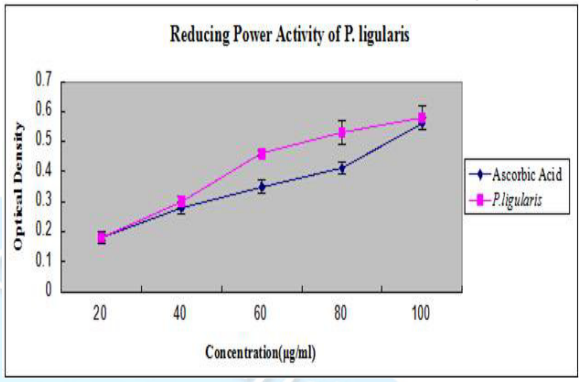


Figure 8: Reducing power assay of *P. ligularis*



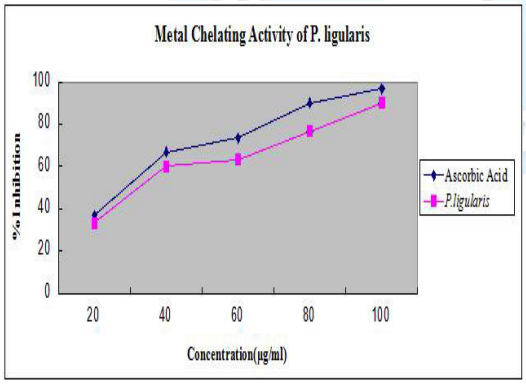
Metal ion chelating activity

Transition metal ions, especially iron can stimulate lipid peroxidation by Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} OH^- + OH^-$) and can also raise the speed of lipid peroxidation by decomposing lipid hydro peroxides into peroxyl and alkoxyl radicals that can perpetuate the chain reaction. Metal ion chelating capability is significant since it reduces the concentration of the transition metal that catalyzes lipid peroxidation [55]. Metal chelating activity was given in figure 7. At the concentration of 100 µg/ml, the ethanolic extract exhibited 96% inhibition whereas with standard antioxidant showed 90% inhibition respectively. The % inhibition of stem ethanolic extract of *Passiflora ligularis* and ascorbic acid were established to be 32 µg/mL and 29 µg/mL.

Reducing power assay

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants [56]. The Figure 8 represents the reductive capabilities of and stems ethanolic extract of *Passiflora ligularis*. In the concentration range investigated, all the extracts demonstrated reducing power that increased linearly with concentration.

Figure 7: Metal ion chelating activity of *P. ligularis*



Ferric reducing antioxidant power (FRAP) assay

The Ferric Reducing Antioxidant Power (FRAP assay) is widely used in the assessment of the antioxidant component in dietary polyphenols. The FRAP scavenging capacity of the ethanolic seed extracts of *Passiflora ligularis* at five different concentrations (20-100 µg/ml) exhibited optical density like 0.24, 0.46, 0.61, 0.79 and 0.97 respectively at 595nm which is depicted in figure 9. Since FRAP assay is easily reproducible and linearly related to molar concentration of the antioxidant present, it can be reported that ethanolic extract may act as free radical scavenger, capable of transforming reactive free radical species into stable non radical products [57].

Figure 9: Ferric reducing antioxidant power (FRAP) assay of *P. ligularis*

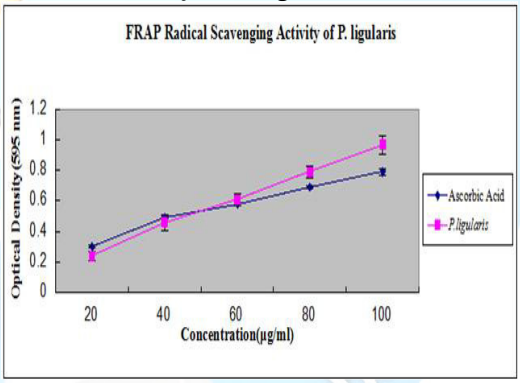


Table 2: Indicates the IC_{50} value of *P. ligularis* and the standard ascorbic acid

Radical scavenging assays	IC_{50} Values	
	Ascorbic acid (standard)	*P. ligularis*
DPPH	36 ± 0.45	27 ± 0.66
ABTS	50 ± 0.51	58 ± 0.74
Hydroxyl	61 ± 0.42	73 ± 0.57
Hydrogen per oxide	52 ± 0.63	58 ± 0.86
Nitric oxide	61 ± 0.71	71 ± 0.74
Superoxide	66 ± 0.65	70 ± 0.54
Metal chelating	29 ± 0.47	32 ± 0.51

Based on the IC_{50} values compared with the standard, the ethanolic extract of *P. ligularis* exerts better free radical scavenging ability. The plant extract play a central role in fascinating and neutralizing the free radicals which is due to the presence of secondary metabolites like alkaloids, flavanoids, phenols and saponins in it.

Quantitative analysis

Alkaloids, flavonoids, glycosides and phenols have been reported to exert numerous biological effects like anti-inflammatory, anti allergic, antioxidant, antidiabetic, antiviral and anti cancer activities. Saponins prevent the excessive intestinal absorption of cholesterol and thus lessen the risk of cardiovascular diseases such as hypertension[58]. Flavanoids are important secondary metabolites of plant which modulates lipid peroxidation complicated in atherogenesis, thrombosis and carcinogenesis[59]. Phenolics are vital plant secondary metabolites with antioxidant activity due to their redox potential, quenching singlet and triplet oxygen, or decomposing peroxides[60]. Based upon the quantitative determination of phytoconstituents carried out for the powdered plant material by various standard methods found that alkaloid 3.64 g%, total phenol 480 mg/g, tannin 6.33 mg/g, flavonoids 10.6 g% and saponin 9.2 g% were present in the aerial parts of this plant(table 3).

Table 3: Quantitative analysis of phytoconstituents of *P. Ligularis*

Constituents	% W/W
Alkaloid	3.64 g%
Flavonoid	10.6 g%
Phenol	480 mg/g
Saponin	9.2 g%
Tannin	6.33 mg/g

CONCLUSION

The present study results indicate that the the seed ethanolic extract of *Passiflora ligularis* was found to possess more secondary metabolites and so it exhibits better radical scavenging activities. In future, this plant extract is a significant source of natural antioxidant, which may be helpful in preventing the progress of various oxidative stresses and as a possible food supplement.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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