PHYTOCHEMICAL AND ANTIOXIDANT STUDY OF PHYLLANTHUS DEBILIS KLEINEX WILD

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ABSTRACT: ***This investigation aims to study the antioxidant activity of petroleum ether, chloroform, acetone, ethanol, and aqueous extract of root and shoot of Phyllanthus debilis and their total phenol, tannin, and flavonoid content. The extract was screened for possible antioxidant activities by free radical scavenging activity (DPPH) and FRAP assay. The results showed that all the plant parts possessed antioxidant properties including radical scavenging and FRAP assay. The antioxidative activities were correlated with the total phenol. The leaf extract of L. Camara was more effective than other parts. This study suggests that DPPH shows that ethanol, acetone, and aqueous extracts of root and shoot of P. debilis possess significant free radical scavenging activity. When ethanol extracts of these samples were further subjected to FRAP assay, the shoot extract of P. debilis activity shows maximum free radical scavenging activity and may be useful for their nutritional and medicinal functions.***

Keywords: Phyllanthus debilis, Antioxidant, DPPH, FRAP, Flavonoid, Tannin

I. INTRODUCTION

Oxidative stress is an important risk factor in the pathogenesis of numerous chronic diseases. Free radicals and other reactive oxygen species are recognized as agents involved in the pathogenesis of sicknesses such as asthma, inflammatory arthropathies, diabetes, Parkinson's and Alzheimer's diseases, cancers as well as atherosclerosis. Reactive oxygen species are also said to be responsible for human aging[1,2].

An antioxidant can be broadly defined as any substance that delays or inhibits oxidative damage to a target molecule[3]. The main characteristic of an antioxidant is its ability to trap free radicals. Antioxidant compounds like phenolic acids, polyphenols, and flavonoids scavenge free radicals such as peroxide, hydroperoxide, or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases[4]. Herbal plants considered good antioxidants since ancient times.

Phyllanthus debilis *Klein* ex Willd (Euphorbiaceae) is a s Monoecious erect annual herb. Globally distributed in Tropical Africa, India, Bhutan, Sri Lanka, and New Guinea. Within India, it is found in North West India, Sikkim, Bihar, Assam, and Peninsular India on plateaus above an altitude of 1000 m. the whole plant is used in Sri Lanka to treat diabetes mellitus[5]. Furthermore, various parts of PD are being used as a remedy for jaundice, sickle-cell anemia, diarrhea, wounds, inflammation, intestinal worms, scabies, ringworm, gall stones, and kidney stones[6-11]. Hence, the current study was designed to evaluate the antioxidant activity of extracts of root and shoot of Phyllanthus debilis by using DPPH scavenging assay, FRAP assay, and determination of total phenolic content tannin and flavonoid content.

II.MATERIALS AND METHODS

Collection of plant specimens

Phyllanthus debilis was collected from Ranga Reddy, Telangana. The specimens were identified by Dr. Madhava Chetty, Associate professor, Sri Venkateshwara University, Tirupati The reference number for *Phyllanthus debilis* Klein ex Willd is NISCAIR/RHMD/Consult/2021/2532/111-1.

Preparation of extracts

About 25g of dried powder of root and shoot of *P. debilis* was taken in a conical flask and extracted with 100ml of water and organic solvents such as ethanol, acetone, chloroform, and, petroleum ether in a mechanical shaker with temperature control (of 28 ± 2^{o}C) at the constant stirring rate at 200 rpm. After 24 h it was filtered through Whatman No.1 filter paper in a Buchner funnel. The filtered solution was evaporated under vacuum in a rota-evator at 40^{o}C to a constant weight and preserved at 10^{o}C in an airtight container for future use.

$$\%\ of\ yield \frac{Weight\ of\ crude\ extract}{Weight\ of\ dried\ plant\ materials} \times 100$$

III.PHYTOCHEMICAL ANALYSIS

Qualitative phytochemical analysis:

Analysis of phytochemical constituents was carried out by using the standard methods[12-15] and the results were recorded.

Quantification of Phytochemicals

Total Phenol content[16]

The total phenol content of the sample was determined by the Folin-Ciocalteu reagent method. Ethanolic solution of the extract in the concentration of 1mg/ml was used in the analysis. A hundred µl of the extracts were taken in a test tube and mixed with 0.5ml of Folin Ciocalteu reagent (1/10) dilution and 1.5ml Na_2CO_3 (2% w/v). The diluted standard solution of Gallic acid (0.5ml) of different concentrations of 20, 40, 60, 80, and 100µg/ml was prepared and the absorbance of various Gallic acid concentrations was plotted in a standard graph. A blank was prepared with the distilled water, Folin Ciocalteu reagent, and Na_2CO_3. The blend of standard, blank, and test samples was incubated in the dark at room temperature for 15 minutes. The blend was incubated in the dark at room temperature for 15 min. The absorbance of the blue-colored solution of all samples was measured at 765nm using a UV-visible spectrophotometer. The results were expressed in mg of gallic acid equivalent (GAE) per gram dry weight of plant powder.

Estimation of Tannin[17]

The tannin content of the sample was estimated. One ml of the extract was mixed with Folin Ciocalteu reagent (0.5ml), followed by the addition of saturated Na_2CO_3 solution (1ml) and distilled water (8ml). The reaction mixture was allowed to stand for 30min at room temperature. The supernatant was obtained by centrifugation and absorbance was recorded at 725 nm using a UV-Visible spectrophotometer. Different concentrations of standard tannic acid were prepared and the absorbance of various tannic acid concentrations was plotted on a standard graph. The tannin content was expressed as mg tannic acid equivalent (TAE) per gram of the sample.

Estimation of Flavonoid[18]

Aluminum chloride colorimetric method

The aluminum chloride colorimetric method was modified from the procedure reported by Woisky and Salatino (1998). Quercetin was used to make the calibration curve. 10mg of Quercetin was dissolved in 80% ethanol and then diluted to 25, 50, and 100µg/ml. The diluted standard solutions (0.5ml) were separately mixed in the 1.5ml of 95% ethanol, 0.1ml of 10% Aluminium chloride, 0.1ml of 1M potassium acetate, and 2.8ml of distilled water. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415nm with a spectrophotometer. The amount of 10% Aluminium chloride was substituted by the same amount of distilled water in the blank. Similarly, 0.5ml of ethanol extracts or 15 flavonoid standard solutions (100ppm) was reacted with Aluminium chloride to determine flavonoid content as described above.

Antioxidant Activity

DPPH (1, 1-Diphenyl-2-picryl-hydrazyl) radical scavenging assay

(A) Qualitative analysis[19]

An aliquot of 50µL of the sample was taken in the microtiter plate. Methanolic DPPH (100µL of 0.1%) was added to the samples and incubated for 30 minutes in dark conditions. The samples were then observed for discoloration from purple to yellow and pale pink was considered as strong and weak positive respectively.

(B) Quantitative analysis[20]

The antioxidant activity was determined using DPPH, (Sigma-Aldrich) as a free radical. Sample extracts of 100µl were mixed with 2.7ml of methanol and 200µl of 0.1 % methanolic DPPH. The suspension was incubated for 30 minutes in a dark condition. Blank without the sample containing the same amount of methanol and DPPH solution was prepared and used as a control. Subsequently, at every 5 min interval, the absorption of the solution was measured using a UV double beam spectra scan (Chemito, India) at 517nm. The antioxidant activity of the sample was compared with the known synthetic standard of (0.16%) Butylated Hydroxy Toluene (BHT). The experiment was carried out in triplicate; free radical scavenging activity was calculated by the following formula

$$Inhibition\,\% \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

FRAP (Ferric Reducing Antioxidant Power) assay[21]

The ability to reduce ferric ions was measured using this method. The FRAP reagent was generated by mixing 300mM sodium acetate buffer (pH 3.6), 10.0 mM (tripyridyltriazine) TPTZ solution and 20.0 mM $FeCl_3.6H_2O$ solution in a ratio of 10:1:1in volume. Test samples of 100 µl were then added to 3ml of FRAP reagent and the reaction mixture was incubated at 37°C for 30 min. The increase in absorbance at 593nm was measured. Fresh

working solutions of $FeSO_4$ were used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of the sample was calculated from the linear calibration and expressed as mMol $FeSO_4$ equivalents per gram of sample (DW).

IV.RESULTS

Phytochemical Analysis

The dry powder of root and shoot of *P. debilis* and *P. virgatus* was subjected to a cold maceration extraction method with solvents viz., petroleum ether, chloroform, acetone, ethanol, and aqueous. The total yield of respective extracts of *P. debilis* was calculated (Table 1).

Table 1: Total yield of the crude extracts of root and shoot of *P. debilis*

Sl. No.	Root		Shoot	
	Extraction solvent	% Yield	Extraction solvent	% Yield
1	Pet. Ether	0.70	Pet. Ether	1.48
2	Chloroform	1.69	Chloroform	2.60
3	Acetone	2.38	Acetone	5
4	Ethanol	8.37	Ethanol	5.85
5	Aqueous	4.19	Aqueous	15.87

This shows that polar solvents procure more yield compared to nonpolar solvents. The amount of yield was more in shoot extracts than root extracts.

Qualitative Phytochemical Analysis

Extracts of petroleum ether, chloroform, acetone, ethanol, and aqueous of shoot and root of *P. debilis* were tested for different groups of constituents using standard methods, and results were noted (Table 2). The phytochemical screening revealed the presence of bioactive constituents such as tannins, saponins, flavonoids, quinines, cardio glycosides, terpenoids, phenols, coumarins, steroids, alkaloids, and betacyanin in all 4 samples. Glycosides and Anthocyanin were absent.

The number of phytochemicals varied with the different plant extracts. Solvents play a major role in the extraction of phytochemicals. Ethanol extract of shoot and root of *P. virgatus* showed the presence of a maximum number of phytochemicals under study except for anthocyanin. Aqueous and acetone extracts of the plant extracts showed the presence of a moderate number of phytochemicals. Very a smaller number of phytochemicals was found in the chloroform extracts. Petroleum ether extracts showed the presence of a minimum number of phytochemicals.

Table 2: Phytochemical content of shoot and root extract of *Phyllanthus debilis*

Sl.No	Phytochemicals	Shoot					Root				
		Pet. ether	Chl	Ac	Et	Aq	Pet. ether	Chl	Ac	Et	Aq
1	Tannin	-	-	+	++	+	-	-	+	+	-
2	Saponin	-	+	-	+	+	-	+	-	-	++
3	Flavonoids	-	-	-	+	-	-	-	+	+	-
4	Quinone	+	-	-	+	+	-	-	++	+	+
5	Glycoside	-	-	-	-	-	-	-	-	-	-
6	Cardioglycoside	+	-	+	+	-	-	+	++	+	-
7	Terpenoid	-	-	+	+	+	+	+	++	+	+
8	Phenol	-	-	+	+	++	-	+	+	+	+
9	Coumarin	-	-	+	-	-	-	-	+	+	-
10	Steroid	-	-	+	+	+	+	+	++	+	+
11	Alkaloid	-	-	+	+	-	-	-	+	+	-
12	Anthocyanin	-	-	-	-	-	-	-	-	-	-
13	Betacyanin	-	-	-	-	-	-	-	+	+	-

[++ = strong positive; + = positive; - = negative, Pet. Ether – Petroleum ether, Chl – Chloroform, Ac-Acetone, Et-Ethanol, Aq – Aqueous]

Tannin, saponin, flavonoids, quinine, cardio glycoside, terpenoid, phenol coumarin, steroid, and alkaloid were the

phytochemicals present in the shoot and root extracts of *P. debilis* (Table 3). Glycoside and anthocyanin were absent in the extracts of *P. debilis*. Betacyanin is present only in the root extract of *P. debilis*.

Quantitative Phytochemical Analysis

The total phenol, tannin, and flavonoid content were estimated for the ethanol extract of root and shoot of *P. debilis* (Table 3). The phenol content in terms of gallic acid equivalent (GAE) is given for the following samples, the amount of phenol present in the shoot extract of *P. debilis* is found to be 91mg GAE/g of dry sample and the root extract of *P. debilis* is 66.4mg GAE/g of dry sample.

Table 3: Quantification of Phytochemicals

Sl.No.	Sample Name	Total Phenol Content mg GAE/g	Total Tannin Content mg TAE/g	Total Flavonoid content mg QE/g
1	*P. debilis* (Shoot)	91	36.8	11.63
2	*P. debilis* (Root)	66.4	18.4	9.38

The tannin content in terms of tannic acid equivalent (TAE) for shoot extract of *P. debilis* is found to be 36.8mg TAE/g of dry sample and root extract of *P. debilis* is 18.4 mg TAE/g of dry sample. The flavonoid content in terms of quercetin equivalent (QE) in the shoot extract of *P. debilis* is 11.63mg QE/g and the root extract of *P. debilis* is 9.38mg QW/g.

Antioxidant Activity

DPPH (1, 1-Diphenyl-2-picryl-hydrazyl) Assay

Root and shoot extracts of *P. debilis* were subjected to qualitative antioxidant activity by DPPH (1, 1-Diphenyl-2-picryl-hydrazyl) radical scavenging assay. An experiment conducted on the different extraction of acetone, ethanol (75%), petroleum ether, chloroform, and aqueous extract showed the presence of antioxidants. Hundred µl of extracts were estimated for free radical scavenging activity using the DPPH assay. The samples were observed for the color change from purple to yellow and pale pink was considered as strong positive and positive respectively (Table 4).

Table 4: Qualitative analysis of the antioxidant activity of root and shoot extracts of *P. debilis*

Sl. No.	Sample	*P. debilis* (shoot)	*P. debilis* (root)
1	Control	-	-
2	Standard (BHT)	++	++
3	Ethanol	++	++
4	Aqueous	+	T
5	Acetone	+	+
6	Petroleum ether	-	-
7	Chloroform	-	-

[++ = strong positive; + = positive; - = negative, T = Trace]

When compared to the activity of standard BHT, ethanolic extract of shoot and root of *P. debilis* showed strongly positive antioxidative activity and acetone extract showed positive antioxidative activity, aqueous extract of shoot of *P. debilis* showed positive antioxidant activity. The root of *P. debilis* and root showed trace (minimum) antioxidant activity. The activity exhibited by Petroleum ether and chloroform extract of shoot and root of *P. debilis* is insignificant.

The quantitative antioxidant activity of aqueous, ethanol, acetone, chloroform, and petroleum ether extracts of root and shoot of *P. debilis* were evaluated by their capacity to scavenge the DPPH as free radicals. The absorbance of DPPH was measured at 517 nm using a spectrophotometer and the OD values were noted for 30 min with 5 min time intervals and the results were compared with the standard BHT. The OD values were then converted to free radical scavenging effect (%) using the prescribed formula and graphs were plotted for the root and shoot of *P. debilis*.

The activity of plant extracts was compared with the percentage of activity recorded with BHT (standard) 98.2%. The shoot of *P. debilis* shows maximum activity with the ethanol extract of 92.1% followed by the aqueous extract of 88.9% and acetone extract of 85%. The minimum activity was recorded with chloroform extract at 27.5% (Fig. 1). The root of *P. debilis* shows maximum activity with acetone extract at 92.1% followed by ethanol extract at 90.5% and aqueous extract at 55.1%. The minimum activity of this sample was recorded with petroleum ether

extract measuring 25.1% (Fig.2).

Table 5: Quantitative analysis of antioxidant activity from shoot extracts of *P. debilis*

Time	Antioxidant activity %					
	P.ether	Chloroform	Acetone	Aqueous	Ethanol	BHT
0	25	23	82	85	86	84
5	30	25	84	86	90	91
10	33	29	86	88	92	94
15	35	30	88	89	94	95
20	35	31	89	91	95	99
25	37	33	91	93	96	99
30	39	35	93	95	97	99

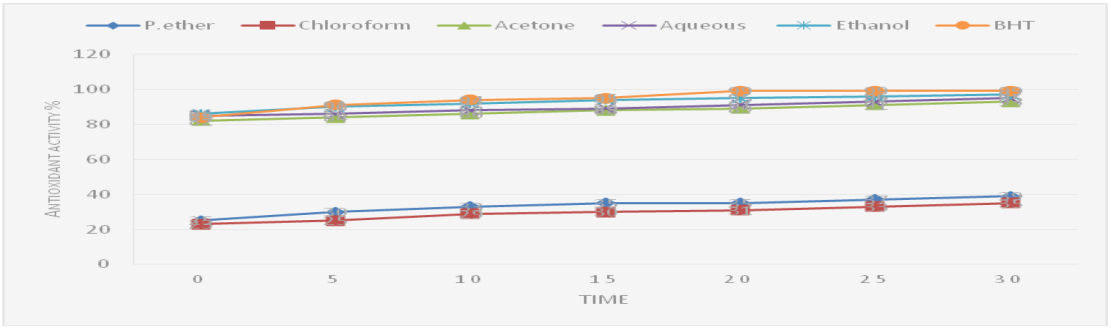

Fig.1. Quantitative analysis of antioxidant activity from shoot extracts of *P. debilis*

Table 6: Quantitative analysis of antioxidant activity from root extracts of *P. debilis*

Time	Antioxidant activity %					
	P.ether	Chloroform	Acetone	Aqueous	Ethanol	BHT
0	22	26	82	41	79	84
5	23	30	84	45	81	91
10	25	31	85	49	82	94
15	29	33	89	51	88	95
20	31	35	90	53	88	99
25	33	37	90	56	88	99
30	35	39	90	58	88	99

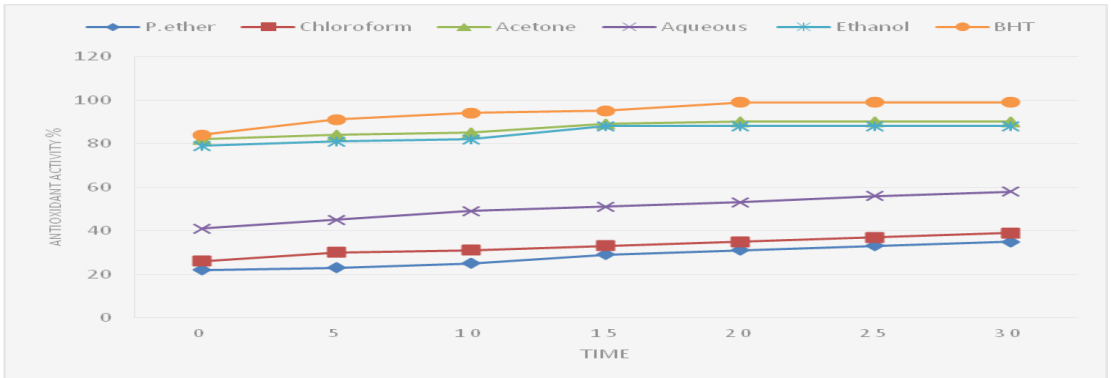

Fig.2. Quantitative analysis of antioxidant activity from root extracts of *P. debilis*

FRAP Assay

In the present study, the tendency of ferric ion reducing activities of the ethanolic extract of root and shoot of *P.*

debilis was measured (Table 7). The absorbance of the test samples is the result obtained due to the formation of the Fe^{2+}-TPTZ complex. Among the 4 test samples, shoot extract of *P. debilis* exhibited the maximum capacity to reduce ferric ions of the sample which is measured as 100 mMole Fe (II)/g, and root extract of *P. debilis* measuring 25 mMole Fe(II)/g). The shoot extracts of *P. debilis* exhibited more reducing power compared to the root extracts of the plant.

Table 7: Antioxidant activity of shoot and root extracts of *P. debilis* FRAP assay

S.No.	Test samples	mMole Fe(II)/g
1	*P. debilis* (Shoot)	100
2	*P. debilis* (Root)	25

IV.DISCUSSION

Plant products have been a part of phytomedicines since time immemorial. This can be derived from barks, leaves, flowers, roots, fruits, seeds etc[22]. Each part of a plant is composed of a specific composition of phytochemical constituents and hence specific part of a plant has been used for the ailment of a certain disease.

In that way, the root and shoot of *P. debilis* were separately tested for their phytochemicals. The sample of root and shoot was extracted with 5 different solvents *viz* aqueous, ethanol, acetone, chloroform, and Petroleum ether (2 polar protic, 1 polar aprotic, and 2 non-polar solvents respectively).

The percentage yield of the extracts was calculated. Maximum yield was recorded with ethanol extracts followed by aqueous and acetone. And minimum yield was recorded with chloroform extracts and petroleum ether. This scale of variation in the extraction may be due to the polarity of the solvents.

The extracts of root and shoot of *P. debilis* when subjected to quantitative analysis of phytochemical content revealed the presence of tannins, saponins, flavonoids, quinines, cardio glycosides, terpenoids, phenols, coumarins, steroids, alkaloids and betacyanin in all the samples. These plant constituents are known to be biologically active, eliciting a variety of pharmacological actions[23]. Glycosides are known to lower blood pressure according to many reports[24]. Coumarins are potent antioxidants and are also known to act against Gram-positive bacteria and it is produced in carrots in response to a fungal infection which could be attributed to their antimicrobial activity[25-26]. Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity[27].

Tannins and Flavonoids, commonly found in plants have been reported to have significant antioxidant activity[28]. Masturah *et al.*, (2006)[29] state that the major components of *Phyllanthus* sp. are active hydrolyzable tannins that can be extracted using an ethanol-water mixture which are semipolar compounds such as ellagitannins and gallotannins. Saponins possess antimicrobial property[30]. The triterpenes include steroids, sterols, and cardiac glycosides with anti-inflammatory, sedative, insecticidal, and cytotoxic activity[31]. Plant steroids (or steroid glycosides) also referred to as 'cardiac glycosides' is one of the most naturally occurring plant phytoconstituents that have found therapeutic applications for arrow poisons or cardiac drugs[32].

It was observed that both the root and shoot of *P. debilis* are comparatively rich in phytochemicals. Ethanol extracts yield a greater number of phytochemicals followed by aqueous, acetone, chloroform, and petroleum. This result lies parallel to the extraction yield of the plant extracts. Glycoside and Anthocyanin were absent in *P. debilis*.

In the previous literature, it was reported that phenol was absent in the shoot and extract of P. Debilis[33]. In the present study, phenol is found in the acetone, aqueous, and ethanol extract of the shoot of *P. debilis* and chloroform, acetone, ethanol, and aqueous extracts of the root of *P. debilis.*

The quantification of phytochemicals of ethanolic extract of shoot and root of *P. debilis* was done to estimate the total amount of tannin, phenol, and flavonoid present. The result shows that shoot extracts contain a greater number of tested phytochemicals than the root extracts. The shoot of *P. debilis* contains more phenol content compared with the root. The tannin content is found to be more in a shoot in *P. debilis* than root. The flavonoid content is found to be more in the shoot of *P. debilis* than root.

The primary step to measure any curative potential stands on the evaluation of their antioxidative property. In the aspect of safety concerns, the plant antioxidants before administering to humans or animals are being tested using various antioxidant assays. The most common spectrophotometric assay method applied is the DPPH radical scavenging system in which the hydrogen or electrons donation ability of plant extracts is measured from bleaching of purple methanol solution of 2, 2`-diphenyl-1- picrylhydrazyl (DPPH) free radical[34]. Phenolic content and reducing the power of extracts are often determined using the Folin-Ciocalteu method[35]. For determination of the reducing power of plant extracts, the ferric reducing/ antioxidant power (FRAP) assay method was applied.

DPPH offers a convenient and accurate method for titrating the oxidizable groups of natural or synthetic antioxidants[36]. The various extracts (ethanol, aqueous, acetone, petroleum ether, and chloroform) of the shoot and root of *P. debilis* were subjected to DPPH assay. Butylated Hydroxy Toluene (BHT) is used as the reference standard. Both qualitative and quantitative DPPH assay was performed. In the qualitative antioxidant assay, the ethanolic extracts show the color of the solution changed from purple to yellow which shows that the DPPH radicals were scavenged and thus it is recorded as strongly positive. In the case of the acetone extract, the color changed from purple to pale pink which showed positive antioxidant activity. The aqueous extract shows a positive result with the shoot of *P. debilis* and semi-positive/Trace activity with the other extracts. The chloroform and petroleum ether extracts show no such color change which means that the activity of these extracts was negligible.

The same extracts of root and shoot of *P. debilis* were subjected to a quantitative antioxidant assay. Analysis of the two plants with 5 different solvents showed that ethanol extract of *P. debilis* shoot and acetone extract root recorded the maximum antioxidant activity values being close to synthetic antioxidant BHT (positive control).

Aqueous extract of shoot of *P. debilis was* found to possess moderate radical scavenging activity.

The ethanol and acetone extracts recorded a higher percentage of free radical scavenging followed by the aqueous extract. The root and shoot extracts show variation in their inhibition percentage with different solvents. There is a gradual increase in the rate of scavenging activity with an increase in time.

To test the reducing power of plant extracts an advanced antioxidant assay, the ferric reducing/ antioxidant power (FRAP) assay was done. Since the results of the DPPH assay of antioxidant activity reveals that ethanolic extracts show maximum activity, the ethanol extracts of shoot and root of *P. debilis* were subjected to FRAP assay. This assay is based on the ability of the plant extracts to reduce ferric ions to ferrous ions. The shoot extract of *P. debilis* shows the maximum free radical reducing potential and the minimum activity was recorded with the root extract of *P. debilis*. The shoot extracts of *P. debilis* show more reducing power than the root extracts.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity and generally correlates with the presence of reductions, which have been shown to exert antioxidant activity by breaking the free radical chain and donating a hydrogen atom. Phenolic compounds have been said to account for most of the antioxidant activities of plant extracts[37]. Thus, the antioxidant activity of the extracts would be attributed to these phenolic compounds. The result of the present study shows a linear correlation between the DPPH, FRAP antioxidant assays, and the total phenol content. This indicates that the antioxidant activity of these plant extracts is mainly due to their phenolic content.

V.CONCLUSION

The qualitative and quantitative phytochemical studies were carried out. The quantification of total phenol, flavonoid, and tannin was estimated. These phytochemicals possess specific pharmacological values.

The antioxidant assays of both DPPH show that ethanol, acetone, and aqueous extracts of root and shoot of *P. debilis* possess significant free radical scavenging activity. When ethanol extracts of these samples were further subjected to FRAP assay, the shoot extract of P. debilis activity shows maximum free radical scavenging activity.

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