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Research Article

PHYTOCHEMICAL INVESTIGATION AND IN VITRO ANTIOXIDANT ACTIVITY OF *PANCHAVALKALA* BARK EXTRACTS

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ABSTRACT

Natural antioxidants have an important role in the prevention of many age-related diseases and promotion of health. Among natural antioxidants from plants, flavonoids and other phenolic compounds are potent antioxidants and chelating agents. *Panchavalkala* the barks of five trees i.e. *Nyagrodha* (*Ficus benghalensis* L.), *Udumbara* (*Ficus racemosa* L.), *Ashwatha* (*Ficus religiosa* L.), *Plaksha* (*Ficus virens* Aiton) and *Parisha* (*Thespesia populnea* (L.)Sol.ex Correa) are also known as *Pancha Ksheeri Vrikshas* in use since Vedic period. Barks of these trees are dried in shade and are used for different formulations (*Pancha Kashaya Kalpanas*), in different pathological conditions, especially as wound healing, gynecological disorders and etc. The plant samples were extracted using ethanol and water, and subjected for the phytochemical analysis. It was confirmed that samples contain many biologically active compounds like flavonoids, polyphenols, tannins, alkaloids, glycosides and terpinoids etc. The marker compound of each trial drug and the quantitative analysis has been carried out by high performance liquid chromatography. The antioxidant study was done by using in vitro method 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay. The marker compounds caffeic acid and gallic acid were quantified in each extract for their quality and efficacy. PVK barks showed high free radical scavenging activity as evidenced by the low IC_{50} values in DPPH (EE PVK- 20.46µg/ml, AE PVK-37.79µg/ml, EE *T.poulenea*-22µg/ml, AE *T. poulenia*- 23.31µg/ml AE *F. benghalensis*- 25.53µg/ml, EE *F. benghalensis*- 26.23µg/ml, EE *F. religiosa* - 34µg/ml). Quercetin- IC_{50} value 4.026µg/ml is used as standard.

The results of the study demonstrated that PVK barks possess phyto-constituent's viz. tannins, flavonoids, polyphenols etc. and has potential antioxidant activity. Thus these barks have good therapeutic potential as natural antioxidant and might be used in life style related conditions like hyperlipidemia, diabetes, obesity, cardiovascular disorders and etc.

INTRODUCTION

Ayurveda is one among the most ancient medical systems of the world. Grouping of drugs having similar activity under a common heading and using that particular combination is a popular practice in Indian system of medicine [1]. *Panchavalkala* is a combination of five bark drugs indicated in wide range of therapeutics in Ayurveda. These are the barks of five trees *Nyagrodha* (*Ficus benghalensis* L.), *Udumbara* (*Ficus racemosa* L.), *Ashwatha* (*Ficus religiosa* L.), *Plaksha* (*Ficus virens* Aiton), *Parisha (Thespesia populnea* (L.) Sol.ex Correa). Barks of these trees are dried in shade and are used for different formulations (*Pancha Kashaya Kalpanas*), in different pathological conditions, especially as wound healing, gynecological disorders and etc. [2,3]. Tannin, Polyphenols, flavonoids, alkaloids, beta sitosterol and stegmasterol are some common phytochemical constituents found to be present in all these drugs [4] and *Kashaya* (astringent) is main taste found commonly in all these barks [5].

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Though having certain similarity each drug is having its own specificity related to their medicinal property.

Plants contain phytochemicals with various bioactivities including antioxidant, anti-inflammatory, anti-hyperlipidemic, anti-diabetic etc. activities. Currently about 25% of the active components were identified from plants that are used as prescribed medicine. [6,11]

An antioxidant can be broadly defined as any substance that delays or inhibits oxidative damage to a target molecule[7]. Antioxidants are a group of substances which, when present at low concentrations, in relation to oxidiziable substrates, significantly inhibit or delay oxidative processes, while often being oxidized themselves. Antioxidants may exert their effect on biological systems by different mechanisms including electron donation (as reducing agents), metal ion chelation (thereby eliminating potential free radicals), sparing of antioxidants (co-antioxidants) [8,9].

Antioxidants lower the burden of free radicals and they have the ability to take up the free radicals and reduce the free radical and make it stable. The main characteristic of an antioxidant is its ability to trap free radicals[7]. 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 [10].

Reactive Oxygen Species (ROS), contribute to cellular aging, mutagenesis, carcinogenesis, and coronary heart disease, likely through destabilization of membranes, DNA and protein damage, and oxidation of low-density lipoprotein (LDL)[12]. Mechanism of action of antioxidants includes the suppression of ROS formation, the inhibition of enzymes or chelating of elements involved in free-radical production. Furthermore, antioxidants scavenge reactive species, and upregulate antioxidant defenses [13].

Plants are rich sources of natural antioxidants, the best known are tocopherols, carotenoids, vitamin C, flavonoids, and different other phenolic compounds [14]. Recently, among natural antioxidants, flavonoids and polyphenols have received increasing attention as compared with vitamin C and E [15]. The exogenous antioxidants are necessary to maintain the proper physiological function for offsetting this oxidative stress (Shumin liv et. al. 2014).

Flavonoids are known to be highly effective antioxidants by scavenging oxygen radicals, by having interesting anti-cancer, hypo-lipidemic, anti-ageing, and anti-inflammatory activities [16]. Moreover, the protective effects of flavonoids in biological systems are attributed to their capacity to scavenge free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce alpha-tocopherol radicals, and inhibit oxidases [12]. Furthermore, phenolic compounds have phenolic hydroxyl groups which can dissociate to negatively charged phenolates. Dissociated phenolics can form hydrogen and ionic bonds with various proteins, which lead to a disturbance of their 3D-structures and in consequence to a change in their bioactivity [17].

There are few studies done on individual drugs for their antioxidant activity which come under PVK [18,19,20].

Nevertheless, it is still unknown whether *Panchavalkalas* has antioxidant activity on DPPH free radical scavenging although used extensively for wound healing, anti-microbial properties and indicated in various gynecological disorders. As Ficus species are rich source of polyphenolic compounds, flavanoids which are responsible for strong antioxidant properties that help in prevention and therapy of various oxidative stress related diseases, present study was undertaken to evaluate antioxidant activity of PVK bark both ethanolic and aqueous extracts together and separately on DPPH assay.

In addition, the phytochemical screening and quantification of marker compounds like caffeic acid and gallic acid were carried out to ensure the quality and efficacy of PVK.

MATERIALS AND METHODS

Plant Material

The barks of *Panchavalkala* trees i.e. *Nyagrodha (Ficus benghalensis* L.*), Udumbara (Ficus racemosa* L.*), Ashwatha (Ficus religiosa* L.*), Plaksha (Ficus virens* Aiton.*), Parisha (Thespesia populnea* (L.) Sol.ex Correa were collected in *Sharad Rutu* i.e., late autumn season (November to January) from their natural habitat, Dhanvantari Vana Bangalore University Campus and Reserve Forest, Jaraka Bande Kaval, Bangalore North. The specimens were authenticated by Dr.Ravikumar K. Taxonomist and Senior Botanist's from FRLHT TDU, Yelahanka, Bangalore. Quantity sufficient matured barks of *Panchavalkalas* were cut in to small pieces separately and were dried under mild sun light covering thin cloth for 2 days and later shade dried for 10 days. Then the dried barks were powdered using pulverizer at Sanjeevini Pharma Kengeri, Bangalore. The phytochemical analysis, quantification of marker compounds and in vitro study is carried out at Skanda Life Sciences Pvt. Ltd., Bangalore-560091.

Preparation of the Extract

The bark powders of PVK *Dravyas* were extracted using ethanol and water as solvents individually and together. Weighed 20g of dried Sample powder and dissolved in 100ml water or ethanol for aqueous and ethanolic extraction respectively in 500ml beaker with aluminium foil

covered on it. Then the beaker was kept on hot water bath at 50°C for 4 hours. After incubation period the extract was filtered with Whatmann filter paper and the filtrate was collected in 50ml beaker. Residue present over the filter paper was discarded and filtrate was taken for further use. Then the filtrate was kept at 70°C for few hours until the extract got completely dried and turned into semisolid form. This semi solid sample was weighed and the yield was noted.

Phytochemical Analysis

Phytochemical examinations were carried out for all the extracts as per the standard methods [21,22,23].

Test for Alkaloids

Dragendoff's test: 0.2ml of sample was taken and 0.2ml of HCl was added. To this 2-3 drops of Dragendoff's reagent was added and the appearance of orange or red precipitate and turbid solution indicates the presence of alkaloids.

Test for Carbohydrates

Molisch's test: 0.2ml of sample was mixed with few drops of Molisch's reagent (α- napthol dissolved in alcohol). 0.2ml of sulphuric acid was added along the sides of the test tube and observed for the appearance of a purple colour ring for positive test.

Test for Tannins

Braymer's test: 0.2ml of plant extract was mixed with 2ml water and heated on water bath for 10 minutes. The mixture was filtered and ferric chloride was added to the filtrate and observed for dark green solution which indicates the presence of tannin.

Test for Terpenoids

Salkowki's test: 0.2ml of plant extract was taken in a test tube with 0.2ml of chloroform. To this, concentrated sulphuric acid was added carefully to form a layer. Presence of reddish brown colour at the interface would show would show the presence of terpenoids.

Test for Glycosides

0.2ml of sample was mixed with 0.2ml of chloroform. 0.2ml of acetic acid was added to this solution and the mixture was cooled on ice. Sulphuric acid was added carefully and the colour change from violet to blue to green indicates the presence of steroidal nucleus (A glycone portion of glycoside).

Test for Steroids

Lieberman Burchardt tests: 0.2ml of sample was mixed with 0.2ml of chloroform. To this 0.2ml of concentrated sulphuric acid was added. The appearance of red colour in the lower layer of chloroform indicates the presence of steroids.

Test for Saponins

Test for Saponins (Foam test): To 0.2ml of extract was added 0.6ml of water in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam that confirms the presence of saponins.

Test for Flavonoids

Alkaline reagent test: 0.2ml of plant extract was taken in a test tube and mixed with dilute sodium hydroxide solution. To this diluted hydrochloric acid was added. Observation of yellow solution that turn colourless later would indicate the presence of flavonoids.

Myllon's Test

0.1ml of extract was treated with few drops of sulphuric acid. The appearance of red ring indicates the presence of proteins.

Starch test

0.1ml of extract was treated with 0.1ml of saturated potassium iodide solution. The appearance of blue color indicates the presence of starch.

Resin Test

0.1ml of extract was treated with 0.1ml of 1% ferric chloride solution. The appearance of greenish blue color indicates the presence of starch.

Carboxylic acid Test

0.1ml of extract was treated with 5% sodium hydrogen carbonate solution. Brisk appearance of CO_2 effervescence indicates the presence of carboxylic acid.

Test for phenols

To 0.2ml of extract 0.4ml of distilled water followed by a few drops of 10% aqueous ferric chloride solution were added. Formation of blue or green color indicated the presence of phenols.

Study of marker compounds Caffeic acid and Gallic acid through HPLC analysis

A. Caffeic acid standard (Sigma Aldrich, 99% w/v) stock solution (1mg/ml) was prepared in MEOH. Caffeic acid was quantified using the peak area after HPLC analysis. Chromatography analysis for the quantification of the chemical marker Caffeic acid in *Ficus virens* Ait., *Ficus religiosa* L., *Ficus benghalensis* L. and *Thespasia populnea* L. was conducted using a instrument (Shimadzu LC-MS Prominence 20AT) at ambient temperature (40°C) on an C18 column (250×4.6mm, 5.0µm particle size). The mobile phase linear comprised a mixture of Acetonitrile and HPLC grade water (60:40 v/v). The flow rate was 0.1ml/min. The detector wavelength was set to 325 nm, and the injection volume was 10µl [24].

B. Gallic acid standard (Sigma Aldrich, 99% W/v) stock solution (1mg/ml) was prepared in MEOH. Gallic acid was quantified using the peak area after HPLC analysis. Chromatography analysis for the quantification of the chemical marker Gallic acid in Thespasia populnea L. and Ficus racemose L. was conducted using an instrument (Shimadzu LC-MS Prominence 20AT) at ambient temperature (40°C)

on an C18 column (250×4.6mm, 5.0µm particle size). The mobile phase linear comprised a mixture of Acetonitrile and HPLC grade water (60:40 v/v). The flow rate was 1ml/min. The detector wavelength was set to 271nm, and the injection volume was 10µl. The peaks were detected at 271nm and were identified by comparing the retention time with standard gallic acid [25].

DPPH ASSAY: Preparation of Working Solutions and Procedure

DPPH (EEC No. 217-591-8, Sigma, USA, stored at less than 0°C)

1mg is dissolved in 6ml HPLC grade methanol (Ranbaxy Chemicals). Inhibitor (Reference standard) *Quercetin* 1mg is dissolved in 1ml methanol. DPPH assay is carried out as per the method of Rajakumar *et al*. 1994 [26]. In brief, 80µl of DPPH solution; various concentration of test solution and quantity sufficient to 240µl with HPLC grade methanol. The different concentrations tested for reference standard are 0.3125, 0.625, 1.25, 2.5, 5, 10µg/ml. The different concentrations tested for test samples are 3.1, 6.3, 12.5, 25, 50 and 100µg/ml. The reaction mixture is mixed and incubated at 25°C for 15 minutes. The absorbance is measured at 510nm using semi-auto analyzer. A control reaction is carried out without the test sample [27].

DPPH radical scavenging effect (%) = [A0 (control) - A1 (test)/ A0 (control)] ×100

Where, A0 is the absorbance of the control and A1 is the absorbance of samples of PVK.

The antioxidant activity of each sample was expressed in terms of IC_{50} (micro molar concentration required to inhibit DPPH radical formation by 50%), calculated from the graph after plotting inhibition percentage against extract concentration.

Statistical Evaluation

Half maximal Inhibitory concentration (IC_{50}) is the concentration of the substance required to inhibit a biological process such as an enzyme, cell, cell receptor or microorganism by half. IC_{50} value is calculated using Graph Prism software version 5.0 by nonlinear regression analysis of % inhibition recorded for different concentrations of test substances/standard. For compounds showing <50% inhibition, IC_{50} value is not calculated. The relative activity of the sample can be determined by comparing the IC_{50} value of sample with standard. Higher the IC_{50} value, lower will be the relative activity in comparison to standard & vice-versa.

RESULTS

Phytochemical Screening

Preliminary phytochemical screening of the aqueous and Ethanolic extracts of PVK together and separately revealed the presence of different Phytoconstituents which are represented in Table 1 and Table 2.

Table 1: Results of Phytochemical analysis of AE of *Panchavalkala*

	Aqueous extract					
	F.religiosa	***T. populenea***	***F.virens***	***F.benghalensis***	***F.racemosa***	***PVK***
Alkaloid	+	+	+	+	+	+
Carbohydrate	+	+	-	+	+	+
Tannin	+	+	+	+	+	+
Terpenoid	+	+	+	-	+	+
Glycoside	-	-	+	-	+	+
Steroid	+	+	+	+	+	+
Saponin	-	+	-	+	+	+
Flavonoid	+	+	-	+	+	+
Myllon's test	+	+	+	+	+	+
Starch test	-	-	-	-	-	-
Resin test	+	+	+	+	+	+
Carboxylic acid test	-	+	-	+	-	-
Phenol test curcumoid	+	+	+	+	+	+

Note: 1. +: Indicates presence of chemical constituents

2. - : Indicate the absence of chemical constituents

Table 2: Results of Phytochemical analysis of EE of *Panchavalkala*

Types of tests	Ethanolic extract					
	F.religiosa	***T. populenea***	***F.virens***	***F.benghalensis***	***F.racemosa***	***PVK***
Alkaloid	+	+	+	+	+	+
Carbohydrate	+	+	-	-	+	+
Tannin	-	+	+	+	+	+
Terpenoid	+	-	+	+	+	+
Glycoside	-	+	+	-	+	+
Steroid	+	+	+	+	+	+
Saponin	-	+	+	+	+	+
Flavanoid	+	-	+	+	+	+
Myllon's test	+	+	+	-	+	+
Starch test	-	-	-	-	-	-
Resin test	+	+	+	+	+	+
Carboxylic acid test	-	+	-	-	-	-
Phenol test curcumoid	+	+	+	+	+	+

Note: 1. +: Indicates presence of chemical constituents

2. - : Indicate the absence of chemical constituent

HPLC Analysis of the Marker Compounds

Caffeic acid and Gallic acid was quantified using the Retention Time (RT) and the peak area after HPLC analysis of given samples in comparison to the standard sample. The results are summarized in Table-3 and 4.

Table 3: Caffeic Acid Content of Test Sample

Sample	**Stock**	**R.T**	**Area (mv*S)**	**Caffeic acid content µg /ml of sample**	**Dilution factor**	**Caffeic acid content µg /g of sample**
Caffeic acid	100µg/ml	1.8	629.00	-	-	-
Ficus virens Ait.	10g/50ml	1.9	11.46	1.82	1	0.182
Ficus religiosa L.	10g/50ml	1.7	291.00	46.26	1	4.626
Ficus benghalensis L.	10g/50ml	1.8	207.00	32.91	1	3.291
Thespasia populnea L.	10g/50ml	1.953	248.00	39.43	1	3.943

Table 4: Gallic Acid Content of Test Sample

Sample	**Stock**	**R.T**	**Area (mv*S)**	**Gallic acid content µg/ml of sample**	**Dilution factor**	**Gallic acid content µg/g of sample**
Gallic acid	100µg/ml	2.4	482.00	-		-
Ficus racemose L.	10g/50ml	2.1	10.49	1.67	1	0.167
Thespasia populnea L.	10g/50ml	2.653	1.13	0.18	1	0.018

DPPH ASSAY

DPPH [1, 1-diphenyl-2-picryl hydrazyl] is a stable free radical with purple colour. Antioxidants reduces DPPH to 1, 1-diphenyl-2-picryl hydrazine, colorless compound which is measured at an absorbance of 510nm.

1, 1-diphenyl-2-picryl hydrazyl **(Purple colored)**

1, 1-diphenyl-2-picryl hydrazine **(Colorless)**

In the DPPH test the ability of the compound to act as a donor for hydrogen atoms or electrons was measured spectrophotometrically. *Panchavalkala* both in aqueous and ethanolic extracts individually and together were able to reduce the stable radical DPPH to the yellow colored Diphyenyl picryl hydrazine. The strongest effect was measured for the EE PVK with an IC_{50} of 20.46 followed by AE PVK with an IC_{50} of 37.79. The results are shown in table 5&6 and graph 13 & 7. Individually T. populenia shows IC_{50} of 22 and 23.31 in EE and AE respectively followed by AE of *F.benghalensis* with an IC_{50} of 25.53 and AE *F. religiosa* 26.06. The results are shown in Table-5 and 6 & Graph no. 12, 6, 3 & 2 respectively.

Table 5: DPPH activity of Aqueous & Ethonolic extracts of PVK

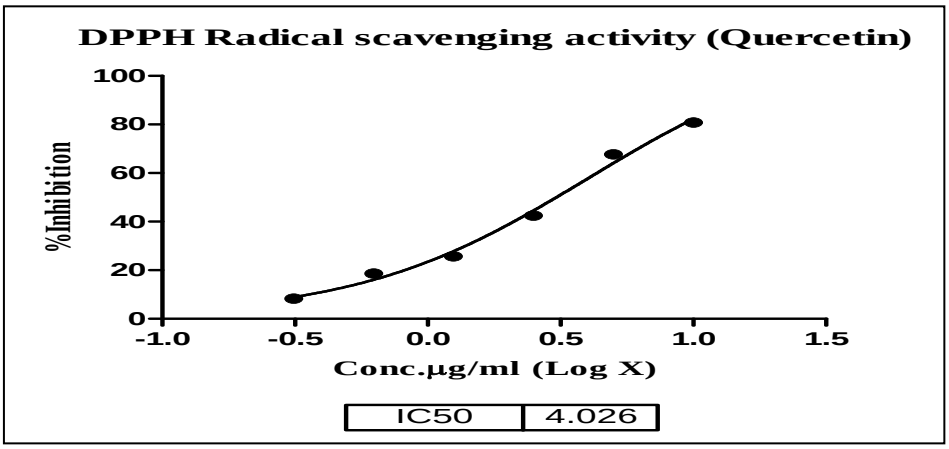
Activity	**AE**	**EE**
Sample	**IC_{50} µg/ml**	**IC_{50} µg/ml**
Quercetin	4.026	4.026
F. recimosa	35	35
F. benghalensis	25.53	26.23
F. religiosa	26.06	34
F. virens	54.17	50
T. populenia	23.31	22
PVK	37.79	20.46

Table 6: DPPH Activity of Bark Samples at Different Concentrations

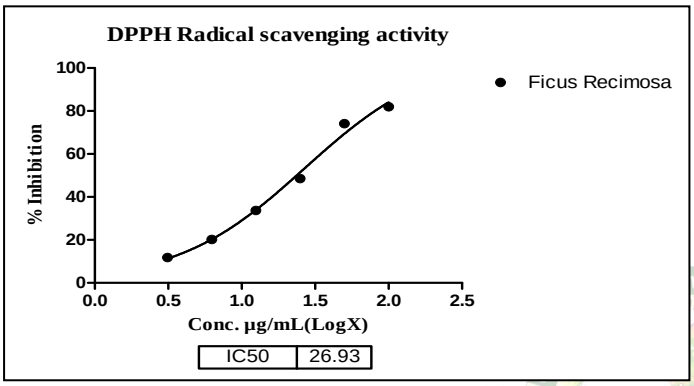
Activity	**Aqueous extracts**				**Ethanolic extracts**			
Sample	**Conc. (µg/ml)**	**Absorbance (517nm)**	**% Inhibition**	**IC50 µg/ml**	**Conc. (µg/ml)**	**Absorbance (517nm)**	**% Inhibition**	**IC_{50} µg/ml**
Quercetin	0	0.7075	0	4.026	0	0.7075	0	4.026
	0.35	0.6635	6.219081		0.35	0.6635	6.219081	
	0.613	0.5758	18.61484		0.613	0.5758	18.61484	
	1.25	0.5255	25.72438		1.25	0.5255	25.72438	
	2.5	0.4072	42.44523		2.5	0.4072	42.44523	
	5	0.2282	67.74558		5	0.2282	67.74558	
	10	0.0864	87.78799		10	0.0864	87.78799	
F.recimosa	0.0	0.820	0.000	26.9	0	0.868	0.00	35
	3.1	0.724	11.768		3.125	0.699	19.48	
	6.3	0.654	20.220		6.25	0.655	24.59	
	12.5	0.543	33.744		12.5	0.524	39.59	
	25.0	0.422	48.524		25	0.472	45.62	
	50.0	0.212	74.122		50	0.399	54.01	

	100.0	0.179	81..829		100	0.235	72.91	
F. benghalensis	0.0	0.870	0.000	25.53	0	0.870	0.00	26.23
	3.1	0.753	11.768		3.125	0.667	23.34	
	6.3	0.678	20.220		6.25	0.600	31.05	
	12.5	0.546	33.744		12.5	0.502	42.30	
	25.0	0.425	48.524		25	0.421	51.58	
	50.0	0.391	74.122		50	0.324	62.74	
	100.0	0.205	76.829		100	0.219	74.88	
F. religiosa	0.0	0.867	0.000	26.06	0	0.867	0.00	34
	3.1	0.701	19.156		3.125	0.759	12.49	
	6.3	0.682	21.405		6.25	0.669	22.84	
	12.5	0.489	43.628		12.5	0.530	38.85	
	25.0	0.425	50.998		25	0.463	46.56	
	50.0	0.284	67.293		50	0.349	59.78	
	100.0	0.120	82.138		100	0.155	82.11	
F. virens	0.0	0.86	0.00	54.17	0	0.808	0.00	50
	3.1	0.81	6.65		3.125	0.701	6.82	
	6.3	0.75	12.71		6.25	0.328	18.89	
	12.5	0.62	27.73		12.5	0.246	35.70	
	25.0	0.51	41.36		25	0.091	61.09	
	50.0	0.45	48.25		50	0.037	78.59	
	100.0	0.34	60.65		100	0.009	89.63	
T. Populenia	0.0	0.86	0.00	23.31	0	0.8079	0	22
	3.1	0.71	17.81		3.125	0.72856	9.82	
	6.3	0.64	26.01		6.25	0.65525	18.89	
	12.5	0.55	36.30		12.5	0.55984	30.70	
	25.0	0.31	64.27		25	0.46789	42.08	
	50.0	0.23	73.85		50	0.29418	63.58	
	100.0	0.14	84.24		100	0.25346	68.62	
PVK	0.0	0.85	0.00	37.79	0	0.887	0.00	20.46
	3.1	0.74	13.33		3.125	0.772	12.92	
	6.3	0.69	18.46		6.25	0.661	25.43	
	12.5	0.51	40.23		12.5	0.586	33.88	
	25.0	0.46	45.97		25	0.406	54.18	
	50.0	0.34	59.78		50	0.360	59.45	
	100.0	0.21	75.58		100	0.201	77.36	

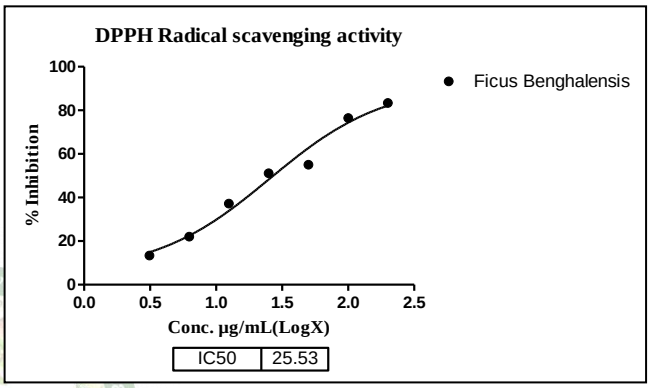
Graph No.1 DPPH activity of Quercetin



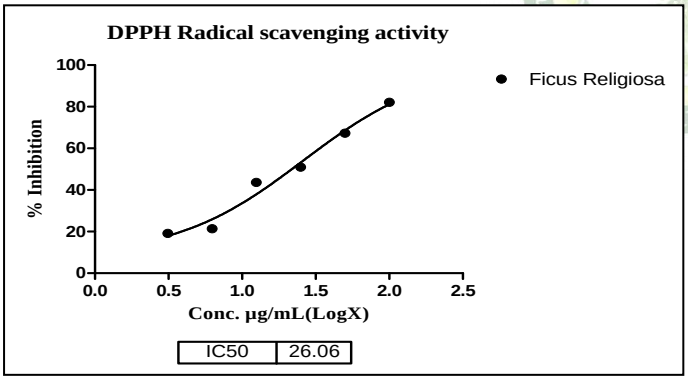
Graph No.2 DPPH activity AE *F.recimosa*



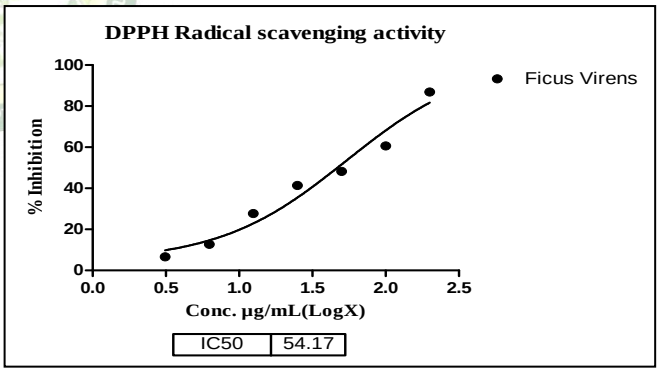
Graph No.3 DPPH activity AE *F.benghalensis*



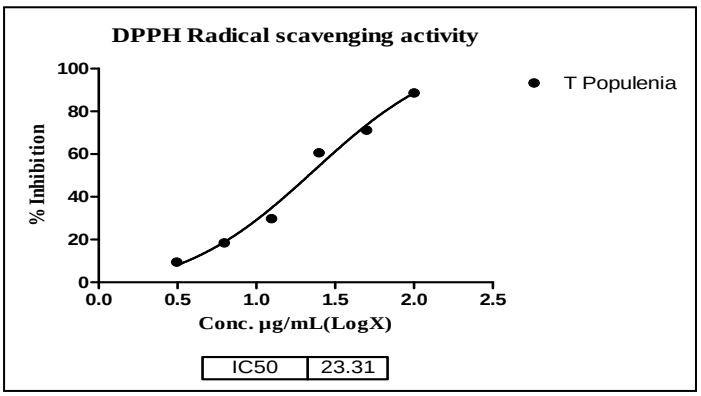
Graph No.4 DPPH activity AE *F. religiosa*



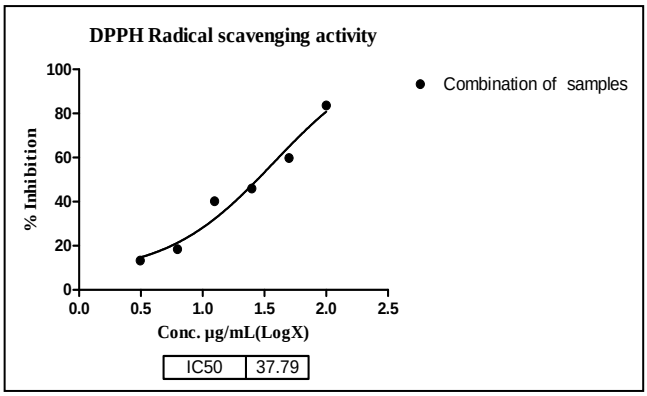
Graph No.5 DPPH activity *AE F.virens*



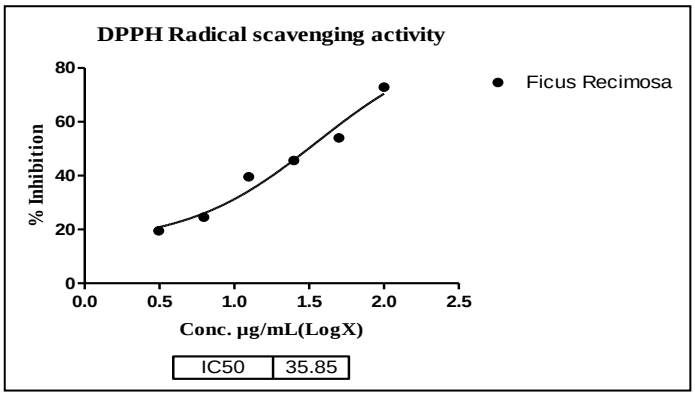
Graph No.6 DPPH activity AE *T. populenia*



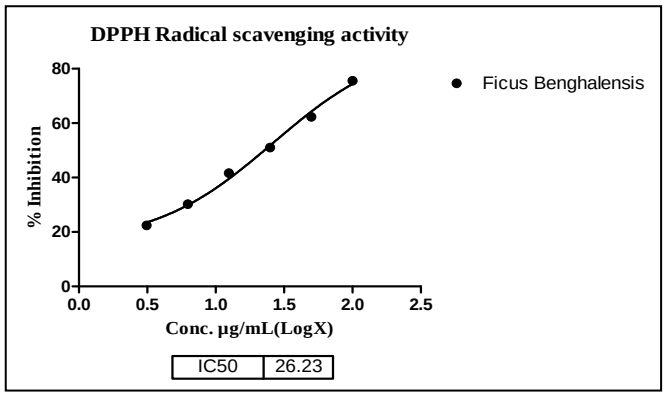
Graph No.7 DPPH activity AE PVK



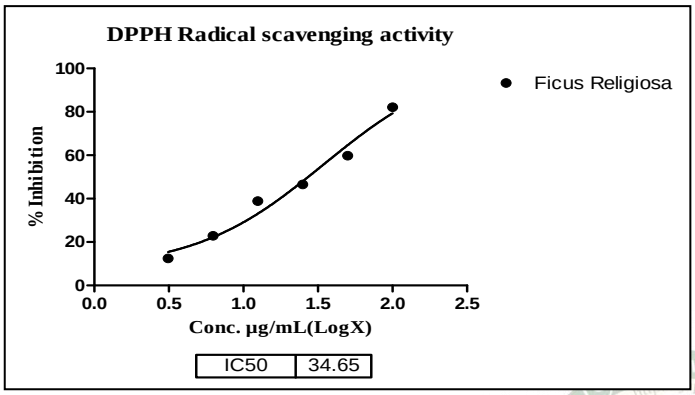
Graph No.8 DPPH activity EE *F.recimosa*



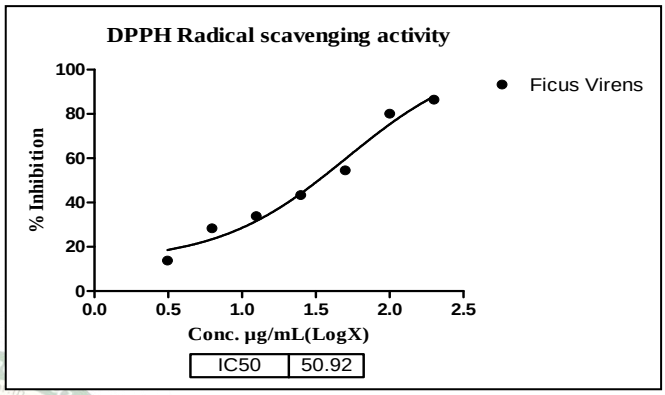
Graph No.9 DPPH activity EE *F. benghalensis*



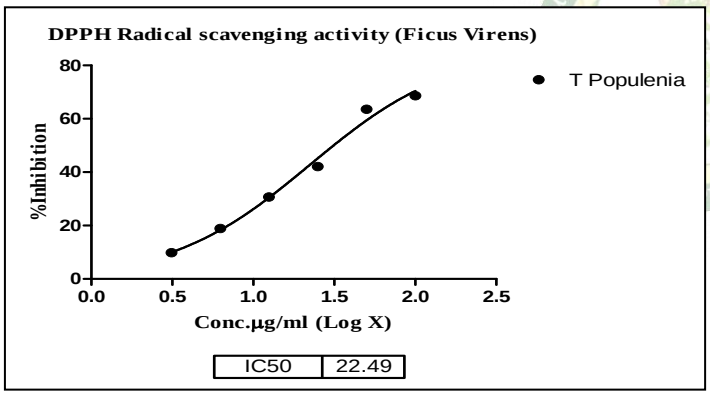
Graph No.10 DPPH activity EE *F.religiosa*



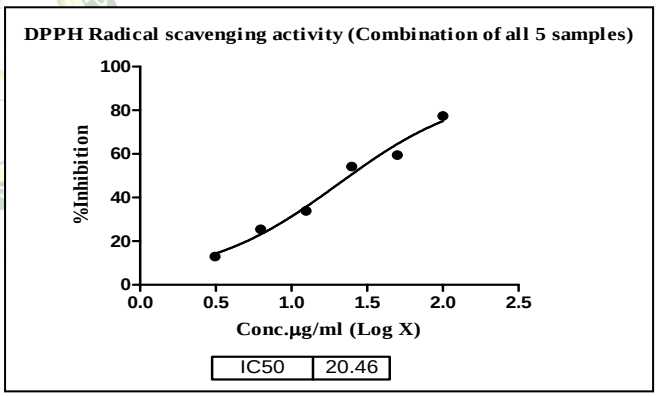
Graph No.11 DPPH activity EE *F.virens*



Graph No.12 DPPH activity EE *F. populenia*



Graph No.13 DPPH activity EE PVK



DISCUSSION

Plants possess antioxidant principles. Various classes of phytochemicals have been shown to have antioxidant property which is due to the presence of substituted groups such as carbonyl, phenolic, phenyl side chain, electron withdrawing group, electron donating group etc. Phenolic antioxidants donate hydrogen to the radical and convert it to stable non-radical product (Tripathi 1998).

Antioxidants are a group of substances which, when present at low concentrations, in relation to oxidizable substrates, significantly inhibit or delay oxidative processes, while often being oxidized themselves.

Free radicals are generally very reactive molecules possessing an unpaired electron which are produced continuously in cells either as by-products of metabolism or by leakage from mitochondrial respiration (De Zwartt et.al 1999) [28]. The free radicals produced in-vivo include the active oxygen species such as super-oxide radical O_2^-, hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl).

During metabolism, oxygen consumption involves the constant generation of free radicals and reactive oxygen species (ROS). H_2O_2 and O_2 can interact in the presence of certain transition metal ions to yield a highly- reactive oxidizing species, the hydroxyl radical (OH) [29]. The hydroxyl radical, one of the ROS, is an extremely reactive free radical formed in biological systems and reacts rapidly with molecules found in living cells, for example, sugars, lipids, DNA bases, amino acids [30].

Oxygen free radicals have been shown to be responsible for many pathological conditions [31]. Free radicals and Reactive Oxygen Species (ROS) cause DNA

damage, lipid per-oxidation, protein damage. They are known to be involved in the pathogenesis of a wide variety of clinical disorders as cancer, cardiovascular diseases, inflammatory diseases, asthma and aging (Vaniet.al 1997; Slater 1984) [32,33]. Free radicals like the hydroxyl radical, hydrogen peroxide, superoxide anion mediate components of the inflammatory response, with production of migratory factors, cyclic nucleotides and eicosanoids. Superoxide radicals amplify the inflammation process, increasing vascular permeability, adhesion of polymorphonuclear leucocytes to the endothelium and stimulation of platelet aggregation [31].

Plants have long been a very important source of drug and many plants have been screened if they contain compounds with therapeutic activity [34]. Therefore, it is vital to evaluate the antioxidant activity of *Panchavalkala* barks. DPPH is a stable, nitrogen centered free radical which produces deep purple colour in methanol solution. The principle of this assay is based on the reduction of purple coloured methanolic DPPH solution in the presence of hydrogen donating antioxidants by the formation of yellow coloured diphenyl-picryl hydrazine. As the absorbance decreases the more efficient, the antioxidant activity of the extract in terms of hydrogen atom donating capacity. The more antioxidant present in the extract, the more DPPH reduction will occur.

Plants are important source of potential compounds for the development of new therapeutic agents. Plant phenolics are widely distributed in the tissues of plants as well as play a vital role in the highly effective free radical scavengers and antioxidant activity. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers[35]. Typical phenolics that possess antioxidant activity are known to be mainly phenolic acid and flavonoids. These compounds possess diverse biological activities, such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic activities. These activities might be related to their antioxidant activity [36]. The antioxidant activity of flavonoids is due to their ability to reduce free radical formation and to scavenge free radicals. Phenolic compounds are important plant antioxidants which exhibited considerable scavenging activity against radicals. Thus the antioxidant potential of the bark extracts of PVK may be possibly attributed to the presence of high phenolic compounds in it.

Our results suggested PVK together and individually both in aqueous and ethanolic extract at different concentration have different activities and maximum inhibitory activity was observed in EE PVK at IC_{50} 20.46µg/mL, AE PVK at IC_{50} 37.79µg/mL, EE T.populenea at IC_{50} 22µg/mL, EE F.benghalensis at IC_{50} 26.23µg/mL and AE F.religiosa at IC_{50} 26.06µg/mL (as shown in table 5 & 6, graph 13, 7, 12, 9 & 4). The observed antioxidant activity of extracts may be due to the neutralization of free radicals (DPPH), either transfer of hydrogen atom or by transfer of an electron [37]. The scavenging effect can be attributed to the presence of active Phytoconstituents in them. The presence of marker phenolic compounds Caffeic acid is estimated by HPLC-Mass spectroscopy shows its highest concentration in F.religiosa- 4.626µg/g, T.populenea– 3.943µg/g and F.benghalensis-3.291 µg/g (Table-3). Similarly Gallic acid is estimated at 0.167µg/g in F.recemose (Table-4).

In the present investigation, bark extracts of PVK exhibited outstanding scavenging effects on DPPH radicals. In addition, phytochemical screening and estimation of phenolic marker compounds like caffeic acid and gallic acid of PVK drugs were evaluated. It was observed that the ethanolic extracts of barks of *Panchavalkala* drugs contained high level of phenolic content that might have accounted for the strong activity observed against the free radicals. Phytochemical study results revealed that the barks of PVK have many phytochemical constituents like flavonoids, tannins, alkaloids, saponin (table- 1 & 2), Phenolic substances like caffeic acid, gallic acid, oxalic acid, ricinolic acid, gossypol, populneol might have accounted for the strong activity observed against the free radicals.

CONCLUSION

The results of this study indicate that the PVK-*Panchavalkala* group barks together exhibited outstanding scavenging effects on DPPH radicals which can be attributed to the presence of high levels of phenolics like caffeic acid, gallic acid, chlorogenic acid, flavonoids, moderate levels of tannins and saponins. Since this investigation is a preliminary study, a detailed study of the antioxidant mechanisms of specific phenolic component is an absolute necessity. This rationalizes effectiveness of PVK in the treatment of dyslipidemia, hyperlipidemia, obesity, diabetes, CAD and etc. life style diseases. Being a plant sourced natural drug PVK extract could be a possible new source of natural antioxidant's in the pharmaceutical and cosmetic industry.

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