Original Article

Phytochemical Screening and Evaluation of *In vitro* Antioxidant Activity of *Viburnum punctatum* buch-ham. Ex d.don

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ABSTRACT

The present study was to estimate the preliminary phytochemical screening and *in vitro* anti-oxidant activity of aerial part extracts of *Viburnum punctatum* by using solvents like Petroleum ether, Chloroform, Methanol and Water. Preliminary phytochemical analysis reveals the presence of alkaloids, glycosides, flavonoids, phenolic compounds, proteins, phytosterols and saponins. The chloroform and methanol extract were screened for its potential anti-oxidant activity by DPPH radical scavenging activity, Hydroxyl radical scavenging activity, Nitric oxide scavenging activity, Reducing power activity and Superoxide free radical scavenging activity using Ascorbic acid as a standard. The results of these five methods anti-oxidant study indicate ta concentration dependent anti-oxidant activity of various concentrations of 50µg/ml, 100µg/ml and 200µg/ml of the extract respectively. The DPPH radical scavenging activity and other radical scavenging activities of the extract was increased with increasing concentration. The methanol extract exhibited better anti-oxidant activity than chloroform extract for all the five *in vitro* anti-oxidant activity screened.

Keywords: *Viburnum punctatum*, Anti-oxidant, Free radicals, DPPH, Nitric oxide, Reducing power.

INTRODUCTION

To protect cells and organs from the oxidative stress induced by ROS, living organisms have evolved with and extremely efficient and highly sophisticated protective system, the so called “antioxidant defensive System”. It involves Variety of components, both endogenous and exogenous in origin. These components function interactively and synergistically to neutralize free radicals[1]. Antioxidant means any substance when present at low concentrations compared to those of oxidizable substrates, significantly

delays or prevents oxidation of those substrates. Oxidative stress occurs is increase in the metabolism of oxidation, antioxidants can play an important role conferring beneficial healthy effects[2].

The present study was taken up on the medicinal plant namely *Viburnum punctatum* belongs to the family Caprifoliaceae. It is shrubs or small trees, evergreen, to 9mm tall. It belongs to monotypic genus Viburnum, native to India, Indonesia, Bhutan, Cambodia, Nepal, Thailand, Vietnam and China. This species is not originally from North America, Asian Viburnum features dainty lymes of creamy white flower at the ends of the branches form early to mid-spring. It has dark green foliage throughout screen. The red fruits are held in abundance in spectacular clusters in mid-summer, expected to live for 40 years or more[3,6].

The leaves were traditionally used for the treatment for fever, stomach disorder and mentioned to possess antiperiodic effect. The preliminary phytochemical investigation shows presence of flavonoids, alkaloids, glycosides, phenolic compounds, phytosterols and saponins[7,8]. The present work reports the anti-oxidant activity of aerial parts of *Viburnum punctatum* by DPPH radical scavenging activity, Hydroxyl radical scavenging activity, Nitric oxide scavenging activity, Reducing power activity and Superoxide free radical scavenging activity using Ascorbic acid as a standard.

MATERIALS AND METHODS

Plant Material

Aerial parts of Viburnum punctatum were collected from Kalakkad-Mundenthurai, Thirunelveli in the month of June 2009. The plant was authenticated by Prof. V. Chellathurai, Former Professor, Govt. Siddha Medical College, Thirunelveli. A voucher specimen of Viburnum punctatum (DVCP/11/09) was deposited in the department of Pharmacognosy in The Dale View College of Pharmacy, Trivandrum for future reference. The plant material was dried at room temperature, pulverized by a mechanical grinder, sieved through 40 mesh and stored in an air tight and light resistant container for further use.

Preparation of Extract

The coarsely powdered plant material was first defatted with Petroleum ether using soxhlet apparatus. The extract was concentrated using rotary evaporator to get solid residue. The marc from the central compartment was removed, dried and successively extracted with a series of solvents of increasing polarity with soxhlet extractor was done. Solvents used with increasing polarity were Chloroform, Methanol and Water[4,5].

Preliminary phytochemical screening

The prepared extracts were subjected to routine phytochemical analysis to identify the presence of various phytochemicals such as alkaloids, flavonoids, saponins, glycosides and phytosterols[5,9] .These results are depicted in Table 4.1.

Reagents and Chemicals

DPPH Solution, plant extracts, Ascorbic acid (AA), Butylated hydroxy toluene(BHT), Ferric chloride, EDTA, Hydrogen peroxide, Trichloroacetic acid (TCA), Tetradecyl amine (TDA), Sodium Nitroprusside, Griess reagent, phosphoric acid (2%), phosphate buffer, Riboflavin Solution, Nitroblue tetrazolium (NBT), Dimethylsulfoxide (DMSO), Deoxyribose, KH_2PO_4-KOH buffer, Thiobarbituric acid.

In vitro Anti-oxidant Activity

DPPH Radical Scavenging Activity

The RSA activities of petether, chloroform, methanol and water extracts were determined using DPPH assay[10,11]. The decrease in the absorption at 517 nm of the DPPH solution after addition of the plant extract was measured in a cuvette containing 2960µl of 0.1mM ethanolic DPPH solution and 40µl of 20-200µg/ml of plant extract. Blank containing 0.1mM ethanolic DPPH solutions without plant extract. The resulting mixtures were vortexed thoroughly; the setup was left at dark at room temperature. The absorption was monitored after 20min. Ascorbic acid (AA) and Butylated hydroxy toluene (BHT) were used as reference standards.

Hydroxyl Radical Scavenging Activity

The chloroform and methanolic extract were subjected to hydroxyl radical scavenging activity[14]. The reaction mixture included deoxy-2-ribose (2.8mM) KH_2PO_4-KOH buffer (20mM pH 7.4), $FeCl_3$ (100µm), H_2O_2 (1.0mM), ascorbic acid (100µm) and various concentrations (50µl, 100µl and 200µl) of the test sample. After incubation for 1 hour at 37^0C, 0.5 ml of the reaction mixture was added to 1ml of 2.8% TCA, then 1ml aqueous TDA was added and the mixture was incubated at 90^0C for 15minutes to develop the color. After cooling the absorbance was measured at 532nm against an appropriate blank solution and the percentage inhibition was calculated.

Nitric Oxide Scavenging Activity

The chloroform and methanol extracts were subjected to nitric oxide scavenging activity[12]. This was measured spectrophotometrically. Sodium nitro prusside (5mmoIL-1) in phosphate buffered saline pH 7.4, was mixed with different concentration of the extract (50µl, 100µl and 200µl) prepared in methanol and incubated at 25^0C for 30minutes. A control without the test compound, but an equivalent amount of methanol was taken. After 30 minutes, 1.5ml of the incubated solution was removed and diluted with 1.5mL of Griess reagent (1% Sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthyl ethylene diaminedihydrochloride). Absorbance of the chromophore formed during diazotization of the nitrate with Sulphanilamide and subsequent coupling with N-l-naphathyl ethylene diaminedihydrochloride was measured at 546nm and the percentage scavenging activity was measured with reference to the standard.

Reducing Power Activity

The chloroform and methanol extracts were subjected to reducing power activity[13]. Weighed the different concentrations of extract (50µl, 100µl and 200µl) to it added 2.5ml of phosphate buffer (pH 6.6) and 2.5ml of 1% potassium ferric cyanide was added and boiled it for 20minutes at 50^0C. To it added 2.5 ml of TCA and centrifuged for 10minutes at 2000rpm. Collected the supernatant and added 1ml of distilled water, 250µ1 of 0.1% ferric chloride and the absorbance was read at 700nm.

Superoxide Free Radical Scavenging Activity

The chloroform and methanol extracts were subjected to superoxide free radical scavenging activity[14]. To different concentrations (50µl, 100µl and 200µl) of extracts, 0.05ml of Riboflavin solution (0.12mM), 0.2ml of EDTA solution [0.1M] and 0.1ml NBT (Nitro-blue tetrazolium) solution [1.5mM] were mixed in test tube and reaction mixture was diluted up to 2.64ml with phosphate buffer [0.067M]. The absorbance of resulting solution was measured at 560nm using DMSO as blank

after illumination for 5minutes and difference in absorbance was determined after 30 minutes incubation in fluorescent light. Absorbance was again measured after illumination for 30 minutes at 560nm using UV Visible spectrophotometer.

The Percentage inhibitions were calculated by using following formula:

$$\% \text{ Inhibition} = \text{Absorbance of Control} - \frac{\text{Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

Statistical Analysis

The data are expressed as mean ± SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnet's t test. At 95% confidence interval, p values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

A preliminary phytochemical testing was done for all the four extract prepared. It was observed that petroleum ether extract found to contains mucilage, gums, fixed oils, fats and phytosterols. Chloroform extract shown the presence of alkaloids, flavonoids and phytosterols. Methanolic extract gave positive test for alkaloids, flavonoids, glycosides and saponins and water extract shown the presence of alkaloids, flavonoids, glycosides and amino acids[7].

The Radical Scavenging Activity activity of petether, chloroform, methanol and water extracts were determined using DPPH assay and these extracts absorbance were compared with the standard antioxidant Ascorbic acid as given in Table 7.1. The absorbance of methanolic extract was found to be potent as that of could be related standard and hence they may possess anti-oxidant property[11].

The hydroxyl radical scavenging activity of the chloroform and methanolic extracts were assessed using Fenton reaction and then their results of each from the chloroform extract and that of the methanol extract was compared with that of the standard ascorbic acid[15]. The methanolic extract showed potential activity when compared to chloroform extract as shown in Table 7.2.

The nitric oxide scavenging activity was checked by using Sodium nitroprusside solution and both chloroform and methanol extract was compared to the standard. Their percentage activity was assessed and the activity was not remarkable with methanolic extract which showed to have more nitric oxide scavenging activity than chloroform extract as seen in Table 7.3.

The reducing power activity of the chloroform and methanol extracts was assessed and its results were compared with that of the standard ascorbic acid. Both the extracts do not show any difference when compared with the standard Ascorbic acid (Table 7.4).

The superoxide free radical scavenging activity of the chloroform and methanol extracts were assessed using by PMS – NADH System[16]. These extracts results were compared with the standard antioxidant Ascorbic acid as seen in Table 7.5. The methanolic extract showed good activity as when compared to chloroform extract. This may be due to the presence of many phytochemicals compared to chloroform extract.

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CONCLUSION

Among the chloroform and methanolic extracts being subjected to antioxidant activity by five different methods which includes estimation of Radical scavenging activity (RSA) using DPPH Assay, hydroxyl radical scavenging activity, nitric oxide scavenging activity, reducing

power activity, superoxide free radical scavenging activity it was observed that the methanolic extract exhibited better antioxidant activity than chloroform extract. This suggests the presence of phytochemical constituents with better antioxidant activity in methanol extract when compared with chloroform extract. So the methanolic extract can be subjected to further isolation to identify the potent phytochemical constituent responsible for exhibiting marked antioxidant activity.

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Table 4.1. Preliminary phytochemical screening of pet ether, chloroform, methanol and water extracts

Test	Pet ether Extracts	Chloroform Extract	Methanol Extract	Aqueous Extract
Alkaloids	-	+	+	+
Glycosides	-	-	+	+
Flavonoid	-	+	+	+
Fixed oil and fats	+	-	-	-
Phenolic compounds, Tannin	-	+	-	-
Proteins and Amino acids	-	-	-	+
Gums and Mucilage	+	-	-	-
Phytosterol	+	+	-	-
Saponins	-	-	+	-

Table 7.1. Estimation of radical scavenging activity (rsa) using dpph assay

Concentration (µg)	Standard	Pet ether	Chloroform	Methanol	Water
20	0.298	0.758	0.745	0.621	0.749
40	0.237	0.748	0.736	0.541	0.716
60	0.192	0.734	0.719	0.462	0.687
80	0.182	0.720	0.701	0.416	0.650
100	0.164	0.709	0.688	0.374	0.626
120	0.149	0.699	0.676	0.335	0.591
140	0.123	0.683	0.659	0.316	0.548
160	0.120	0.674	0.641	0.228	0.522
180	0.096	0.661	0.608	0.189	0.496
200	0.059	0.648	0594	0.178	0.459
Control	0.362	0.782	0.782	0.782	0.782

Table 7.2. Effect of CEVP and MEVP on hydroxyl radical scavenging activity

Concentration (µg/ml)		Absorbance (at 532nm)	Percentage Inhibition
Control		0.85	--
Standard	50	0.41	51.76±1.19
	100	0.38	55.29±0.95
	200	0.31	63.52±0.65
Chloroform Extract	50	0.54	36.47±0.96
	100	0.51	40.00±1.11**
	200	0.46	45.88±0.92
Methanol Extract	50	0.44	48.23±1.21*
	100	0.41	51.76±2.21
	200	0.36	57.64±1.11**

Statistical significance was determined by one-way ANOVA followed by Dunnet's t test. Values are mean ± SEM expressed as (n=3) p*<0.05, **<0.01, ***<0.001; as compared with control.

Table 7.3. Effect of CEVP and MEVP on nitric oxide scavenging activity

Concentration (µg/ml)		Absorbance (at 546nm)	Percentage Inhibition
Control		0.152	--
Standard	50	0.033	78.28±0.96
	100	0.022	85.52±0.98
	200	0.019	87.50±0.96
Chloroform Extract	50	0.120	21.05±1.57
	100	0.093	38.81±1.76**
	200	0.059	61.18±1.34*
Methanol Extract	50	0.082	46.05±2.17
	100	0.069	54.60±2.88**
	200	0.046	69.73±2.45*

Statistical significance was determined by one-way ANOVA followed by Dunnet's t test. Values are mean ± SEM expressed as (n=3) p*<0.05, **<0.01, ***<0.001; as compared with control.

Table 7.4. Effect of CEVP and MEVP on reducing power activity

Concentration (µg/ml)		Absorbance (At 700nm)
Control		0.432
Standard	50	0.077
	100	0.108
	200	0.153
Chloroform Extract	50	0.432
	100	0.455
	200	0.655
Methanol Extract	50	0.277
	100	0.549
	200	0.609

Table 7.5. Effect of CEVP and MEVP on superoxide free radical scavenging activity

Concentration (µg/ml)		Absorbance (At 560nm)	Percentage Inhibition
Control		0.163	--
Standard	50	0.027	83.43±1.09
	100	0.021	87.11±1.11
	200	0.017	89.57±1.87
Chloroform Extract	50	0.042	74.23±2.24**
	100	0.031	80.98±2.98*
	200	0.024	85.27±1.23**
Methanol Extract	50	0.048	73.61±2.68
	100	0.029	82.20±2.18**
	200	0.022	86.50±2.13

Data were assessed by one-way ANOVA followed by Dunnet's t test. P value <0.05 considered significant *p<0.05, **p<0.01, ±SEM calculated by comparing extracts Vs control, n=3