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Original Article

ANTIOXIDANT ACTIVITY OF *PTEROLOBIUM HEXAPETALUM* (ROTH) SANT. AND WAGH.

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ABSTRACT

Objective: *Pterolobium hexapetalum* (Caesalpiniaceae) is a spiny straggler of dry deciduous forests having a wide range of medicinal uses. The plant parts like leaf, stem bark, flower and fruits resulted with flavonoids, phenols, glycosides, steroids, tannins and alkaloids as the main constituents. Antimicrobial activity against gram +ve and gram –ve pathogenic bacteria and fungal strains resulted very effective inhibition at 10mg/well with hot water and methanol extracts to that of the standard drugs *Gentamycin* and *Nystatin* with MIC ranges from 0.312-0.625 mg against bacterial strains and 0.156 and 0.625mg towards fungal strains. Hence the present work is aimed to estimate the quantitative studies of phenols and flavonoids and to assess *in-vitro* antioxidant activity of methanol and aqueous extracts of all parts to prove the efficacy of the medicinal herb as used by the Chenchu tribes of Nallamalai hills.

Materials: Quantitative analysis of phenols was carried out by Folin-ciocalteu method and flavonoids by calorimetric aluminium chloride method in leaf, stem bark, flower and fruit aqueous and methanol extracts. Antioxidant activity *in-vitro* DPPH free radical scavenging assay and reducing power was compared with ascorbic acid as control.

Results: Fruit aqueous extracts yielded highest amounts of phenols and flavonoids as 135.71 µg/mg and 67.80 µg/mg than other parts. *In vitro* antioxidant activity with flower and fruit aqueous extracts at 50 µg/ml showed 74.70 and 73.06% respectively with IC_{50} values 22.12 and 26.34 µg/ml showed effective free radical scavenging activity compared to control 17.87µg/ml. The reducing ability showed at 1.352 and 1.326 absorbance with EC_{50} values 18 µg/ml in both flower and fruit aqueous extracts to that of with19 µg/ml the standard ascorbic acid.

Conclusion: *P. hexapetalum* phenols and flavonoids quantitative analysis of flower and fruit aqueous extracts resulted highest amounts when compared to *Cassia fistula* seed methanol extracts. Antioxidant activity also closely related with *Bauhinia rufescens*, *Cassia tora*, *Caesalpinia pulcherrima*, *C.bunducella*, *Delonix regia*, *Parkinsonia aculeata* and *Tamarindus indica*. Hence aqueous extracts of flower and fruit at 50 µg/ml may be recommended as the drug for curing various disorders like diarrhoea, jaundice, ulcers, skin infections, wound healing, venereal diseases, constipation, piles, cold and cough as used by the tribals. It is also recommended for the isolation of bioactive compounds from phenols and flavonoids to develop drugs.

Keywords: Phenols, Flavonoids, Quantitative, IC_{50}, EC_{50}, free radical scavenging, Reducing ability.

INTRODUCTION

Traditional herbal medicine *P.hexapetalum* is having a wide range of applications as the stem bark against fever, cold and cough, tooth ache, chest pain, dog bite, nausea, heat boils and wound healing; Flowers against veneral diseases; Fruit and seed against diarrhoea, constipation and piles, ulcers; Leaves against delivery pains [1-4].It is also observed that majority of the herbal medicines of *Cesalpiniaceae* shows similar medicinal uses against diarrhoea and ulcerous activities to that of *P.hexapetalum*. *B. variegata* as astringent, carminative, anthelmintic, antidote to snake venom, laxative against dysentery, diarrhoea, skin diseases, ulcers, piles and leprosy [5]. *B.purpurea* bark and roots for dysentery, flowers against piles; *B.racemosa* leaf decoction to treat malaria; root bark extracts against diarrhoea; Stem bark extract with goat milk to cure epilepsy and acts as antidiabetic [6]. *C.pulcherrima* as abortifacient, febrifuge, purgative, emmenagogue, stimulant tonic for asthma, bronchitis, malarial fevers [7].

B.vahili leaf and bark juice applied externally to check excessive bleeding of wounds and root extracts for dysentery. *C.bonduc* root ointment for treating hydrocele, dysentery and stomachache. *C.sappan* astringent to control diarrhoea, acts as emmenagogue and anti-inflammatory. *C.auriculata* used as astringent, anthelmintic, urinary complaints, skin infections, diabetes and ophthalmic. *C.sophara* used as diuretic, purgative and antidote to snake bite, ring worms and bronchitis. *C.fistula* fruit pulp with *Piper longum* for dystopia. *C.occidentalis* roots used for diarrhea. *C.tora* leaves fried in castor oil applied on ulcers; root rubbed with lime juice applied on ring worm infection [8]. *C. senna*, *C.angustifolia* seeds as cathartic, *C.fistula* seed pulp as laxative, root bark rich with tannins acts as strong purgative. *C.senna* and *C.tora* leaves and flowers; *C. auriculata* seeds as antidiabetic [9]. *C.angustifolia, C.senna, B.variegata* seeds to cure wounds and tumors.[10]. *Saraca indica* in Unani system used to cure gynecological disorders [11]. *Sophora subprostrata* as antipyretic, antidote, analgesic and antitumor activity [12]. Seeds of *S. flavescens*, *S.tonkinensis, S.alopecuroides* are used in the treatment of eczema, colitis, acute pharyngolaryngeal infection, sore throat, acute dysentery and gastrointestinal hemorrhage, anti ulcerative, asthma [13-15]. *S.flavescens* root extracts as anti proliferative [16]. *S.vicifolia* used as remedy for heart stroke, odema [17]; antioxidant, anti inflammatory [18]. *S.moorcroftiana* an endemic shrub is used in the treatment of detoxification, emetic and verminosis [19]. *S.subprostrata* roots were used as antipyretic, antidote and analgesic [20]. The present study is aimed to quantify the phenols, flavonoids in all parts which are mainly responsible for the antioxidant activity and also further screened out the flower and fruit extracts against DPPH scavenging activity and reducing ability to prove the herbal uses of *P.hexapetalum* crude drugs.

MATERIALS AND METHODS

Collection and identification of plant material

P.hexapetalum leaves, stem bark, flowers and fruits were collected from the Nallamali forests of Mahanadi area (one of the Navanandis) during July – Dec 2011 and preserved as per the standard method **[21].** The taxonomic identity was confirmed by Prof. N. Yasodamma and the voucher specimens B.K:7, B.K:12 were preserved in the Herbarium, (SVUTY) Department of Botany, S.V.University, Tirupati. Plant materials were thoroughly washed and then dried under shade for one week. The dried parts were ground in a mixer grinder and sieved. The powders were stored in air sealed polythene bags at room temperature until further use.

Preparation of crude extracts

Dried leaves, stem bark, flower and fruit powders 70 g each were soaked and extracted with water after 72 hrs and the filtrates was

dried on water bath. Also the dried leaves, stem bark, flower and fruit powders each 40 g were extracted in a soxhlet apparatus using 200 ml of solvent methanol. The filtrates were concentrated on rotavapour and dried. All extracts were stored at 4^0C in refrigerator until further use.

Quantitative Analysis

Determination of Total phenolic content

Total phenolic contents was determined by the Folin-ciocalteau method **[22-23]**. Leaf, stem bark, flower and fruit extracts each 0.5ml (1mg/ml) was mixed with Folin ciocalteu reagent (5ml, 1:10 diluted with distilled water) for five minutes and aqueous Na_2Co_3 (4ml, 1M) were then added. The mixture was allowed to stand for 15 min and the phenols were determined by calorimetric method at 765nm. The standard curve was prepared 5 to 25µg/ml solutions of Gallic acid in methanol: water (50:50, v/v). Total phenol values are expressed in terms of Gallic acid equivalent (mg/g of dry mass), which is a common reference compound.

Determination of Total flavonoid content

Calorimetric aluminium chloride method was used for flavonoid determination [23, 24]. Leaf, stem bark, flower and fruit extracts each 0.5ml was separately mixed with 1.5 ml of methanol, 0.1ml of 10% aluminium chloride, 0.1ml of 1M potassium acetate, and 2.8ml of distilled water, and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415nm with a UV visible spectrophotometer. Total flavonoid content was calculated as Quercetin equivalent from a calibration curve preparing Quercetin solution 20 to 100µg/ml in methanol as the standard reference for flavonoid compounds.

Anti oxidant activity

The antioxidant activity of plant extracts were determined by *in-vitro* DPPH free radical scavenging assay and Reducing power methods. All the assays were carried out in triplicates and average values were considered.

DPPH free radical scavenging activity [25-26]

The free radical scavenging capacity of the aqueous, methanol, extracts of leaf, stem bark, flower and fruit against 2, 2-Diphenyl-1-picryl hydroxyls radical were determined by UV- visible spectrophotometer at 517 nm. Radical scavenging activity was measured by a slightly modified method previously described. Each extract 1ml of 10, 20, 30, 40 and 50µg / ml was added with 3 ml of methanol followed by 0.5ml of 1 Mm DPPH. Control sample was prepared containing the same volume without any extract with reference ascorbic acid. A blank solution was prepared containing the same amount of methanol and DPPH. The absorbance of the final reaction mixture of three parallel experiments was expressed as mean with standard error. Decreased absorbance of the reaction mixture indicates effective free radical scavenging ability IC_{50} values (the concentration required to inhibit radical formation by 50%) of the extracts were calculated from linear regression of the individual graphs. The radical scavenging activity was calculated using the following formula:

$$\% \text{ inhibition} = \{[A_b - A_a]/A_b\} \times 100$$

Where A_b is the absorption of the blank sample and A_a is the absorption of the extract.

Assay of reducing ability

Reducing ability of the drug was carried out by using the standard method [27]. Leaf, stem bark, flower and fruit extract each 1ml was taken ranging from 10 - 50µg / l was mixed with 2.5ml of phosphate buffer (0.2M, P^H 6.6), and 2.5ml of potassium ferricyanide [$K_3Fe(CN_6)$] (10g/l), then the mixture was incubated at 50^0 C for 20 minutes. After that 2.5ml of trichloroacetic acid (100mg / l) was added to the mixture and then centrifuged at 3000 rpm for 10 min. Finally, 2.5ml of the supernatant was mixed with 2.5ml of distilled water and 0.5ml $Fecl_3$ (1g/l) and absorbance was measured at 700nm in UV-visible spectrophotometer (Systronics). Ascorbic acid was used as standard and phosphate buffer used as blank solution. The absorbance of the final reaction mixture of three parallel experiments was expressed as mean with standard error. Increased absorbance of the reaction mixture indicates stronger reducing ability. The extract concentration providing 0.5 of absorbance EC_{50} - The concentration at which 50% of the effect is achieved was calculated from the graph of absorbance at 700nm against extract concentration. Statistical analysis was done using one way Anova analysis of variance followed by Dunnett's test.

RESULTS

Quantitative Analysis

Total Phenolics and Flavonoids: (Table -1; Figure -1, 2)

Total phenolics were determined according to Folin – ciocalteu procedure as Gallic acid equivalent contents to standard curve Y=0.007×+0.024, R^2=0.987 (Fig.1). The total phenolic content of leaf, stem bark, flower and fruit aqueous extracts are 117.14, 95.71, 118.57 and 135.71µg/mg; in methanol extracts100.0, 94.28, 108.57 and 111.42µg/mg respectively. The results showed that the aqueous extract of fruit shows highest amount of phenolic compounds followed by flower, leaf, and stem bark. Total flavonoids were determined according to Aluminum chloride procedure as Quercetin equivalent contents to the standard curve Y=0. 009×+0.069, R^2=0.999 (Fig.2). The results showed that the total flavonoids in the aqueous extracts of leaf stem bark, flower, and fruit are 69.0, 41.20, 72.38 and 76.0µg/mg; in methanol extracts 61.22, 37.80, 63.40 and 67.80µg/mg respectively. These results imply that aqueous extract of fruit showed highest quantity of flavonoids followed by flower, leaf and stem bark extracts.

Table 1: Total phenols and flavonoid compounds (µg/mg)

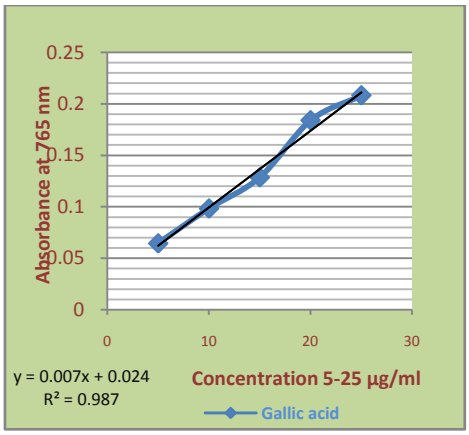
S. No	Parts	Extracts	Phenols	Flavonoids
1	Leaf	Aqueous	117.14	69.0
		Methanol	100	61.22
2	Stem bark	Aqueous	95.71	41.20
		Methanol	94.28	37.80
3	Flower	Aqueous	118.57	72.30
		Methanol	108.57	63.40
4	Fruit	Aqueous	135.71	76.0
		Methanol	111.42	67.80

Antioxidant activity

DPPH free radical scavenging activity: (Table -2; 3, Fig: 3) The antioxidant activity of leaf, stem bark, flower and fruit with aqueous and methanol extracts showed equally effective action to that of the standard drug in concentration dependent manner. Flower aqueous extract at 50µg/ml showed effective inhibition with 74.70% of free radical scavenging activity approximately equal to that of standard ascorbic acid 86.53%. The IC_{50} values (The conc. required to inhibit radical formation by 50%) ranges between 22.12 - 32.59µg/ml respectively.

Whereas standard ascorbic acid IC_{50} value is 17.87µg/ml which is closely related to the flower aqueous extract 22.12µg/ml, followed by fruit aqueous extracts at 26.34 µg/ml. The results are proved that ** P<0.01 more accurate.

Gallic acid for Phenolics



Quercetin for Flavonoids

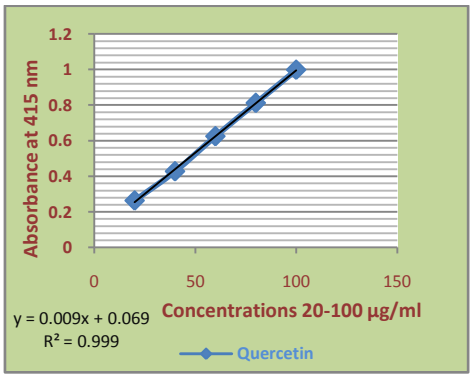


Fig. 1&2: Standard graphs

Assay of reducing ability: (Table -4; Figure -3)

Flower and fruit aqueous extracts showed very high reducing ability when compared to methanol extract. The EC_{50} values (The concentration at which 50% of the effect is achieved) with leaf, stem bark, flower and fruit aqueous and methanol extracts ranges between 18-24µg/ml. Ascorbic acid EC_{50}19µg/ml is also equal with fruit and flower aqueous extracts as 18µg/ml. The results are proved that ** P<0.01 more accurate.

DISCUSSION

Phenolics and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of single oxygen and on free radicals [28].

The present investigation quantitative analysis of *P.hexapetalum* revealed that fruits, flowers, leaf and stem bark contain significant amounts of phenols and flavonoids. Aqueous extracts of fruit showed high phenolics and flavonoids as 135.71µg/mg and 76.0µg/mg, when compared with the other species of the family as *Cassia fistula* seed methanol extracts with phenol content 474.25ug GAE/mg and flavonoids 70.86ug CE/mg respectively [29]. The antioxidant activity of *P.hexapetalum* flower and fruit aqueous extracts at 10 - 50µg/ml showed 73.06 - 74.70% of free radical inhibition. It is also closely related when compared with *B. rufescens* leaf methanol extracts between 10-50µg/ml showed 56.21-74.65%. The reducing capacity serve as indicator of potential antioxidant property. The higher absorbance indicates the strong reducing power of the extract. [30].

The maximum absorbance of *P. hexapetalum* aqueous flower and fruit extracts at 50µg/ml showed 1.352, 1.326 to that of ascorbic acid 1.398 is very nearer to *B. rufescens* leaf methanol extracts between 10-250 µg/ml, ranges 56.2-83.75 % of inhibition [31]. *C. pulcherrima* wood methanol and aqueous extracts between 5 to 25µg/ml ranges 48.58-94.33% and 40.57- 89.76% compared with gallic acid 77.81-91.62% scavenging activity [32].

Table: 2: Absorbance with DPPH free radical assay

Conc. (µg/ml)	Absorbance at (517 nm)				Control Ascorbic acid
	Leaf		Stem bark		
	Aqueous	Methanol	Aqueous	Methanol	
10	0.613±0.000**	0.610±0.000**	0.702±0.000**	0.702±0.000**	0.510±0.000
20	0.573±0.001**	0.531±0.002**	0.621±0.001**	0.621±0.000**	0.413±0.000
30	0.460±0.000**	0.426±0.000**	0.561±0.003**	0.531±0.000**	0.258±0.001
40	0.326±0.004**	0.334±0.001**	0.428±0.000**	0.412±0.002**	0.192±0.000
50	0.280±0.000**	0.278±0.000**	0.368±0.000**	0.327±0.001**	0.115±0.000
	Flower		Fruit		
10	0.523±0.000**	0.590±0.000**	0.513±0.001	0.641±0.002**	0.510±0.000
20	0.430±0.000**	0.520±0.000**	0.497±0.003**	0.546±0.001**	0.413±0.000
30	0.373±0.001**	0.457±0.002**	0.413±0.000**	0.451±0.000**	0.258±0.001
40	0.298±0.000**	0.324±0.001**	0.350±0.002**	0.313±0.002**	0.192±0.000
50	0.216±0.002**	0.234±0.003**	0.230±0.000**	0.252±0.001**	0.115±0.000

All the data are expressed as mean ± SEM: ** p<0.01 as compared to control ascorbic acid with One –way ANOVA followed by Dunnett·s test

Table: 3: % of DPPH free radical scavenging activity: IC_{50} :Values µg/ml.

Conc. (µg/ml)	Leaf		Stem bark		Flower		Fruit		Control Ascorbic acid
	Aq	Me	Aq	Me	Aq	Me	Aq	Me	
10	28.22	28.57	28.22	28.57	38.75	30.91	39.92	24.94	40.28
20	32.90	37.82	32.90	37.82	49.64	39.11	41.80	36.06	51.63
30	46.13	50.11	46.13	50.11	56.32	46.48	51.63	47.18	69.78
40	61.82	60.88	61.82	60.88	65.10	62.06	59.01	63.34	77.51
50	67.21	67.44	67.21	67.44	74.70	72.59	73.06	70.49	86.53
IC_{50}	32.59	31.03	32.59	31.03	22.12	29.79	26.34	31.32	17.87

Table 4: Reducing ability and EC_{50} µg/ml

Conc. (µg/ml)	Absorbance at (700 nm)				
	Leaf		Stem bark		Control
	Aqueous	Methanol	Aqueous	Methanol	Ascorbic acid
10	0.451±0.000**	0.552±0.001**	0.391±0.002**	0.302±0.001**	0.688±0.001
20	0.692±0.001**	0.702±0.002**	0.501±0.001**	0.451±0.000**	0.822±0.000
30	0.831±0.002**	0.982±0.001**	0.632±0.003**	0.697±0.004**	0.104±0.006
40	0.991±0.001**	1.010±0.002**	0.864±0.000**	0.816±0.003**	1.196±0.000
50	1.191±0.002**	1.324±0.001**	0.901±0.005**	0.962±0.003**	1.398±0.000
EC_{50}	22	18	24	24	19
	Flower		**Fruit**		
10	0.652±0.000**	0.522±0.000**	0.521±0.003**	0.522±0.000**	0.688±0.001
20	0.901±0.004**	0.786±0.001**	0.701±0.003**	0.786±0.000**	0.822±0.000
30	1.022±0.001**	0.912±0.000**	0.921±0.000**	0.912±0.006**	0.104±0.006
40	1.084±0.000**	1.067±0.000**	1.180±0.002**	1.067±0.000**	1.196±0.000
50	1.352±0.002**	1.296±0.000**	1.326±0.004**	1.296±0.000**	1.398±0.000
EC_{50}	18	20	18	20	19

All the data are expressed as mean ± SEM: **p<0.01 as compared to control ascorbic acid with One –way ANOVA followed by Dunnett's test

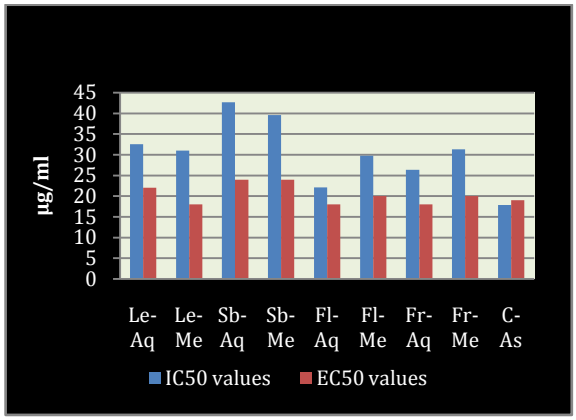


Fig. 3: µg $IC_{50}$and EC_{50} values /ml

Le- Aq: Leaf Aqueous; **Le- Me:** Leaf methanol; **Sb- Aq:** Stem bark Aqueous; **Sb- Me:** Stem bark Methanol;

Fl- Aq: Aqueous; **Fl- Me:** Flower methanol; **F- Aq:** Fruit Aqueous; **Fr-** Fruit methanol; **C- As:** Control -Ascorbic acid.

D. regia leaf methanol extracts showed 80% activity with IC_{50} value 8.89ug/ml and reducing power at 1.87ug/ml **[33]**; *Kingiodendrom pinnatum* leaf methanol extract showed 36, 52, 93, 95% inhibition between 2 to 8ug/ml **[34]**. *Parkinsonia aculeata* leaf chloroform extracts 71.7%; ethyl alcohol 86.7%; aqueous 70.81%; ethyl acetate 75.29%; to that of the standard Gallic acid 83.5% scavenging activity and IC_{50} reported as 0.14, 0.29, 0.33, 0.38mg/ml with each extract respectively **[35]**. *Tamarindus indica* seed coat extracts showed 70% scavenging activity with IC_{50} 1.791 ppm with EtoAc fraction followed by EtoH 3.002; aqueous 3.024 and CH_2Cl_2 with 5.122 ppm respectively **[36]**.

CONCLUSION

P. hexapetalum phenols and flavonoids quantitative analysis of flower and fruit aqueous extracts resulted highest amounts when compared to *Cassia fistula* seed methanol extracts. Antioxidant activity also closely related with *B. rufescens*, *C. tora*, *C.pulcherrima*, *C.bunducella*, *D. regia*, *P. aculeata* and *T. indica*. Hence aqueous extracts of flower and fruit at 50 µg/ml may be recommended as the drug for curing disorders like diarrhoea, jaundice, ulcers, skin infections, wound healing, venereal diseases, constipation and piles and also cold and cough as used by the tribals. And also recommended for the isolation of bioactive compounds from phenols and flavonoids to develop drugs against various disorders.

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