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In Vitro Assessment of Antioxidant Activity, Total Phenolic and Flavonoid Content for Various Extracts of *Caesalpinia pulcherrima* (L.) Sw.

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Abstract

The leaves and stem parts of *Caesalpinia pulcherrima* (L.) Sw (Fabaceae) shown in many traditional medicinal uses including folklore curative for various biological disorders. The present study is focused in determination of *in-vitro* antioxidant activity by DPPH free radical scavenging method, Total Phenolic Content by Folin's ciocalteu method and Total Flavonoid Content by quantitative aluminum chloride method (using Quercetin and Catechin standards) for various extracts of *C. pulcherrima*. From the study it was inferred that the highest antioxidant activity was found to exhibited by leaf methanolic extract (with 92. 85% inhibition rates on free radicals at 50 µg/ mL), highest phenolic content found to be in leaf Ethyl acetate extract (70.55 mg GAE / gm extract) and highest flavonoid content found to be in leaf & stem Ethyl acetate extracts (82.3 & 73.01 mg QE & CEs / gm extract).

Keywords
Ascorbic acid, Catechin, DPPH, Gallic acid, Quercetin.

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1. INTRODUCTION

Medicinal plants possess curative nature due to presence of various complex chemical substances of different or varied composition, which are called as plant secondary metabolites in the plant organs like roots, stems, leaves and flowers which have medicinal properties for curative of diseases and may further acts as precursors for producing semi synthetic derivatives [1].

Caesalpinia pulcherrima is an evergreen, fernlike, fast growing; upright shrub or small tree, grows up to 3-4 meters tall, continuously blooming and belongs to family Fabaceae. It is classified under *Caesalpinia* genus commonly known as peacock flower and called as Ratnagandhi in local regions of Andhra Pradesh and Telangana states of India. It is distributed in Southeast Asia, tropical America, Brazil, Indonesia, Africa, Canada and India. In India it founds in the states like Kerala, Tamilnadu, West Bengal and

Andhra Pradesh. It was used to cure various ailments since ancient times as a folklore curative. It is considered to be medicinal because of their diverse medicinal activities like diuretic, astringent, sedative, hepatoprotective, expectorant, demulcent, antitussive, anti-hypercholestermic, aromatic, treats dysentery and diarrhea. [2-8]

This plant is rich in diverse chemical constituents like phellandrene, ocimene, phenolic compounds like Brazilin, Brazilide, homoflavanoids, di, tri and sesique terpenoids, Chalcones like pulicherrimachalcone, pulicherrimain along with some sugars [9, 10], essential aminoacids and fatty acids. Some special constituents that are reported were methoxy Bonducellin [11], α-terpenene [12], homoisoflavanoids [13], Cassene diterpenes [14], methylene dioxyflavonones [15], furanoid diterpenes [16], diterpene esters [17], Myricitroside [18], Isoflavones [19], spathulenol, Caesaldekarin and Neocaesalpins.

C. Pulcherrima reported to exhibit antibacterial [20], antidiabetic [21], Larvicidal [22], anti-inflammatory [23], antiulcer [24], antioxidant [25], antiviral [26], antitubercular [27], and molluscidal [28] activities in various articles.

Based on the above information with respect to its diverse chemical constituents and various pharmacological activities that it possesses, the plant *C. pulcherrima* was selected for further, different studies like antioxidant, total Phenolic and Flavonoid content in order to estimate the amount of antioxidant, phenolic and flavonoid principles present in its extracts.

2. MATERIALS AND METHODS

2.1. Plant collection:

Caesalpinia pulcherrima plant was collected (for its leaves & stems) from CSIR-IICT field station, Mallapur, Hyderabad, Telangana, India. The plant was taxonomically identified and authenticated by Dr.A.Sabhitha Rani, Assistant Professor, Department of Botany, Osmania University, Hyderabad, India. A voucher specimen (CIMAP-CP/18) was stored at CIMAP Research Centre, Hyderabad; shade dried, milled to a coarse powder by Cutter mill.

2.2. Extraction:

The leaf and stem powder (100 gms each) extracted using various solvents like hexane, ethyl acetate, methanol by ultrasonication for 30 mins (30 min x 4 times) and 30% aq. Methanol by overnight maceration, filtered and concentrated under reduced pressure by Rota evaporator. These extracts were used further for *in-vitro* antioxidant, total Phenolic and Flavonoid content estimation studies.

2.3. *In-Vitro* Anti-Oxidant activity Determination by DPPH assay method

The extracts ability to scavenge DPPH radical was determined by using DPPH radical scavenging method. Various concentrations like 50, 25, 20, 15, 10 and 5 µg/ml in methanol were prepared from the sample stock solutions (1.0 mg/mL). 1 mL of 0.3 mM DPPH methanol solution was added to 2.5 ml solution of the extract or standard and allowed to react at room temperature for 30 min. The absorbance of the sample mixture was measured at 517 nm and compared with the absorbance values of ascorbic acid standard. 1 mL of 0.3 mM DPPH and methanol (2.5 ml) was used as a blank and the results were shown in (Table 1 Figure 1). From the obtained absorbance values the percentage antioxidant activity was calculated by following formula [29].

$$\text{Percentage of Inhibition (\%)} = \frac{\text{Blank absorbance - Sample absorbance}}{\text{Blank absorbance}} \times 100$$

General inference:

If the sample's absorbance was less than blank's absorbance, then it is an indication of higher antioxidant activity (i.e. inversely proportional) since blank has deep purple colour due to lack of sample or phenolic group or any antioxidant compound shows higher absorbance than sample (which may contain low or high amount of phenolic groups) and gives lower absorbance values due to reduction of dark purple colored free radical DPPH to pale yellow colour after neutralization.

2.4. Estimation of Total Phenolic Content

The total phenolic content of *C. pulcherrima* leaf and stem extracts were determined using the Folin-Ciocalteu assay. An aliquot (1 mL) from each prepared extract samples & prepared standard solutions of Gallic acid in concentrations (50, 100, 150, 200 and 250 mg/ lit) were added to 25 mL volumetric flask, already containing 9 mL of distilled deionized water. 1 mL of Folin-Ciocalteu phenol reagent was added after 5 min, 10 mL of 7% Na_2CO_3 solution was added to the mixture. The solution was diluted to volume (25 mL) with deionized water and shaked well. After incubation for 90 min at room

temperature, the blue colour developed was checked for the absorbance against prepared reagent blank by determining at 750 nm with an UV-Visible spectrophotometer [30] (Table 3).

Blank preparation: To 25 mL volumetric flask, 9 ml distilled water, 1 mL Folin-Ciocalteu reagent was added after 5 min and 10 mL of 7% sodium carbonate solution was added to the mixture. The final volume was made upto (25 mL) with distilled deionized water and shaked and incubated for 90 min in dark condition at room temperature. The phenolic content expressed in mg Gallic acid equivalents (GEA)/ g fresh weight. Total phenolic content was calculated by

$$\text{Total Phenolic Content} = \text{Concentration} \times \frac{\text{Vol. of the Sample}}{\text{Weight of the Sample}}$$

2.5. Estimation of Total Flavonoid content

The total Flavonoid content of different extract of *C. pulcherrima* leaf and stem were determined by Aluminum chloride colorimetric assay using Quercetin and Catechin standards.

***Principle*:** A quantitative colorimetric analysis which involves an indirect estimation of Total Flavonoid content in the extract samples. The principle involves the formation of strong complex (acid soluble) between C4 ketone group of Flavonone and C3 and C5 hydroxyl group of Flavonols with aluminum and the complex producing dark yellow colour whose absorbance is measured at 510nm in UV and is compared with the Quercetin and Catechin standard by establishing standard calibration curve and is calculated as follows

$$\text{Total Flavonoid content} = \text{Concentration} \times \frac{\text{Vol. of the Sample}}{\text{Weight of the Sample}}$$

The darker the colour complex, higher is the complexation with aluminum and indicates the presence of more number of flavonoid principles.

a. Colorimetric assay (Quercetin)

Total flavonoid content was measured by the aluminum chloride colorimetric assay. 1 mL prepared extracts or standard solution of Quercetin in various concentrations (100, 200, 300, 400 and 500 mg/lit) was added to 10 mL volumetric flask, containing 4 ml of distilled deionized water and added 0.3 ml of 5 % sodium nitrite ($NaNO_2$). After 5 min, 0.3 ml of 10 % aluminum chloride was added. At 6th min, 2 ml of 1 M NaOH was added and total volume was made up to 10 ml with distilled deionized water. The solution was mixed well and the absorbance (in UV-VIS spectrophotometer) was measured against prepared reagent blank at 510 nm. Flavonoid content expressed as mg Quercetin equivalents (QE) / g fresh mass (Table 5).

b. Colorimetric assay (Catechin)

Total Flavonoid content was measured by the aluminum chloride colorimetric assay. 1 mL of prepared extracts and standard solution of Catechin (100, 200, 300, 400 and 500 mg/lit) was added separately to 10 mL volumetric flask, containing 4 ml of distilled deionized water and added 0.3 mL of 5 % sodium nitrite ($NaNO_2$). After 5 min, 0.3 ml of 10 % $AlCl_3$ was added. At 6th min, 2 ml of 1 M NaOH was added and total volume was made up to 10 mL with distilled deionized water. The solution was mixed well and the absorbance (in UV-VIS spectrophotometer) was measured against prepared reagent blank at 510 nm. Flavonoid content expressed as mg Catechin equivalents (CE)/g fresh mass [31] (Table 5).

***Blank preparation*:**

To the 10 mL capacity volumetric flask, water (10 mL), 5% $NaNO_2$ and 10% $AlCl_3$ (0.3 mL each) were added one after the other with a gap of 5mins time interval. 1 M NaOH (2 mL) was added after 5 mins and final volume was made up to 10 mL with deionized water.

3.0. RESULTS AND DISCUSSION

3.1. *In Vitro* Antioxidant Activity (DPPH radical scavenging activity)

The leaf and stem (hexane, ethyl acetate, methanol and aq.methanol) extracts of *C. pulcherrima* determined for *in-vitro* antioxidant assay by DPPH method showed dose dependent inhibition of DPPH radicals. Percentage scavenging of DPPH radical examined at different concentrations (by leaf and stem hexane, ethyl acetate, methanol and aq. methanol extracts) was depicted in (Table-1, Fig-1).

S. No	Concentration	Ascorbic Acid	% Inhibition by Leaf Extracts				% Inhibition by Stem Extracts			
			Hexane	Ethyl acetate	Methanol	Aq. Methanol	Hexane	Ethyl acetate	Methanol	Aq. Methanol
1	5	38.23	08.52	26.30	**39.40**	11.33	3.38	19.91	11.45	12.01
2	10	58.56	11.08	22.68	**26.18**	18.48	1.84	27.00	27.32	18.02
3	15	74.58	05.03	20.73	**37.30**	28.79	2.15	34.49	41.60	31.75
4	20	93.92	06.16	32.64	**60.99**	41.04	2.71	41.27	56.57	42.97
5	25	95.02	07.80	41.58	**75.73**	51.70	0.28	44.35	72.44	51.58
6	50	97.23	07.18	72.58	**92.85**	93.08	5.54	83.26	93.19	88.77

Table 1: Effect of various extracts of *C. pulcherrima* on DPPH radicals.

The *In-Vitro* Anti-oxidant activity using DPPH method was observed for various extracts and they were to be (39.40, 26.18, 37.30, 60.99, 75.73 & 92.85% at 5, 10, 15, 20, 25 and 50 µg/ mL). From the obtained above results it was showed that the % inhibition rate by antioxidant principles on DPPH free radicals found to be more in leaf methanol extract with 92.85% at 50 µg/ mL concentration when compared to other extracts of *C. pulcherrima* with respect to ascorbic acid reference standard whose percentage inhibition was (97.23% at 50 µg/ mL). The Order of *in-vitro* anti-oxidant activity for *C. pulcherrima* plant extracts by DPPH method is

Leaf Methanol > Stem Methanol > Stem Aq. Methanol > Leaf Aq. Methanol > Stem Ethyl acetate > Leaf Ethyl acetate > Leaf Hexane > Stem Hexane extracts.

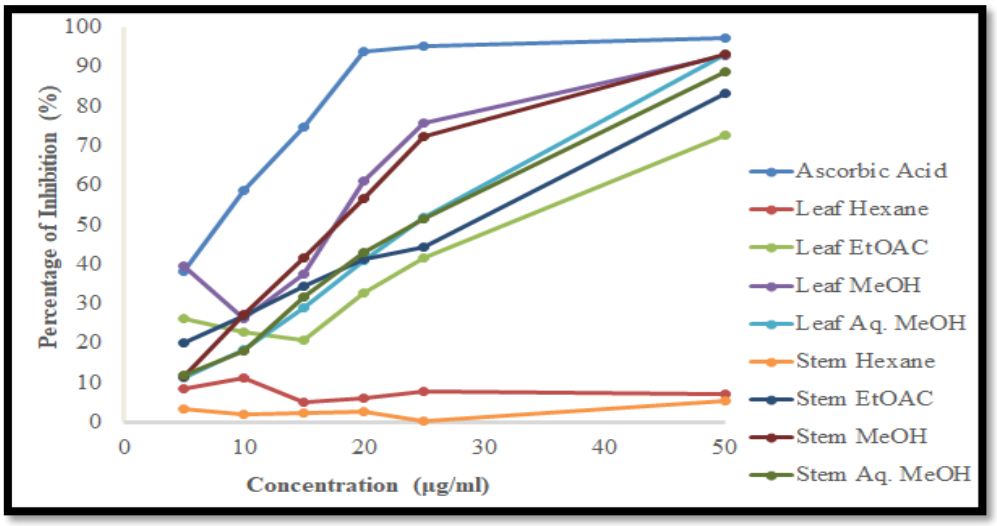

Figure 1: Graph of Extracts by DPPH method

3.2. Total Phenolic Content

The total phenolic content of *C. pulcherrima* leaf and stem extracts were estimated by Folin-Ciocalteu assay method. The leaf and stem (hexane, ethyl acetate, methanol and 30% aq. methanolic extracts) the total phenolic content found to be 7.77, 54.61, 60.24, 58.34, 7.27, 54.12, 56.28 and 41.56 mg respectively as Gallic acid equivalents/gram of extract and is depicted in (Table-3). Standard graph of Gallic acid was depicted in (Table 2, Fig2).

S. No	Concentration (µg/ml)	Absorbance (nm)
1	0	0
2	50	0.258
3	100	0.519
4	150	0.761
5	200	1.034
6	250	1.310

Table 2: Absorbance values of Gallic acid standard

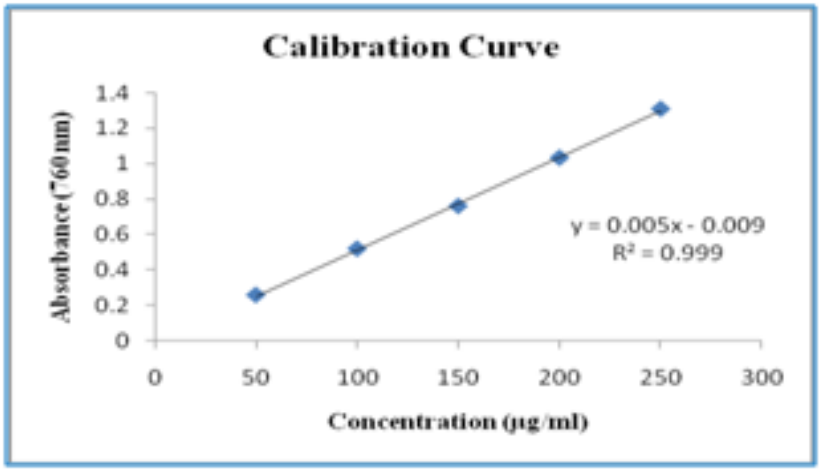

Figure 2: Standard graph of Gallic acid.

S. No	Plant Part	(Extracts)	Unknown Concentration (µg/mL)	mg Gallic acid equivalents / gm extract
1	Leaf	Hexane	247.87	24.78
2		Ethyl acetate	**705.52**	**70.55**
3		Methanol	549.64	54.96
4		Aq. Methanol	113.49	11.34
5	Stem	Hexane	188.16	18.81
6		Ethyl acetate	704.90	70.49
7		Methanol	381.67	38.16
8		Aq. Methanol	361.90	36.19

Table 3: mg/gm Gallic acid equivalents (GAEs) of different extracts of *C. pulcherrima*

Based on the results obtained, the total Phenolic content showed the presence of highest phenolic content in leaf ethyl acetate extract with (70.55 mg GAE/gr. Ext.) compared to other extracts. The Order of Phenolic content of plant *C. pulcherrima* leaf and stem extracts **Leaf Ethyl acetate > Stem Ethyl acetate > Leaf Methanol > Stem Methanol > Stem Aq. Methanol > Leaf Hexane > Stem Hexane > Leaf Aq. Methanolic extracts**

3.3. Total Flavonoid Content

Total Flavonoid content of *C. pulcherrima* leaf and stem extracts (Hexane, Ethyl acetate, Methanol and aq. Methanol) were found to be 12.59, 82.36, 11.11, 58.88, 71.36, 25.56, 74.97 and 40.66 mg QE/gm extract respectively and was depicted in (Table-5). Standard graph of Quercetin is depicted in (Table-4, Fig-3).

S. No	Concentration (µg/mL)	Absorbance	
		Quercetin	Catechin
1	0	0	0
2	100	0.200	0.336
3	200	0.380	0.407
4	300	0.509	0.509
5	400	0.622	0.600
6	500	0.814	0.814
7	600	0.972	1.071

Table 4: Absorbance values of Flavonoid content using Quercetin and Catechin as standards.

From the obtained overall results, it was shown that the total Flavonoid content were found to be more in leaf ethyl acetate extract of *C. pulcherrima* with 82.3 mg QEs/ gm extract followed by **Stem Methanol > Stem Hexane > Leaf Aq. Methanol > Stem Aq. Methanol > Stem Ethyl acetate > Leaf Hexane > Leaf Methanolic extracts**

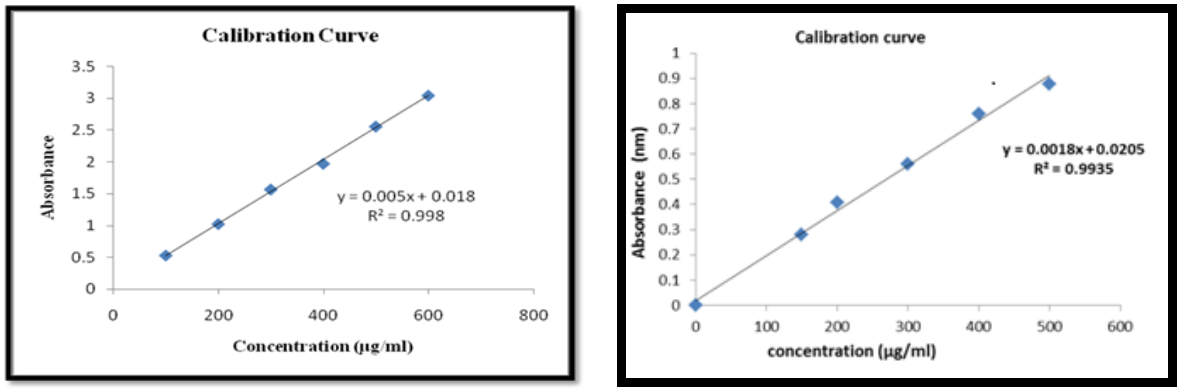

Figure 3: Standards graph of Quercetin and Catechin

Total flavonoid content of *C. pulcherrima* leaf and stem extracts (Hexane, Ethyl acetate, Methanol and aq. Methanol) were found to be 44.83, 72.50, 46.06, 3.66, 27.08, 73.01, 13.25 and 14.34 mg CE/gram extract respectively and was depicted in (Table-5). Standard graph of Catechin depicted in (Table-4, Fig-3).

From the obtained overall results which were depicted in (Table-5 & Fig-3) it was inferred that the total Flavonoid content was found to be more in stem ethyl acetate extract of *C. pulcherrima* with 73.01 mg CE/ gm extract.

The Order of Flavonoid content of plant extracts using Catechin as standard.

Stem Ethyl acetate > Leaf Ethyl acetate > Leaf Methanol > Leaf Hexane > Stem Hexane > Stem Aq. Methanol > Stem Methanol > Leaf Aq. Methanolic extracts

Plant Part	Extract	Quercetin		Catechin	
		Unknown Concentration (µg/ml)	Flavonoid Content mg QEs/ gr. Extract	Unknown Concentration (µg/ml)	Flavonoid Content mg CEs/ gr. Extract
Leaf	Hexane	125.92	12.59	448.38	44.83
	Ethyl acetate	823.63	**82.36**	725.01	72.50
	Methanol	111.14	11.11	460.60	46.06
	Aq. Methanol	588.87	58.88	36.650	3.660
Stem	Hexane	713.63	71.36	270.82	27.08
	Ethyl acetate	255.61	25.56	730.16	**73.01**
	Methanol	749.75	74.97	132.50	13.25
	Aq. Methanol	406.64	40.66	143.44	14.34

Table 5: mg/gm Quercetin and Catechin equivalents (QEs & CEs) of Hexane, Ethyl acetate, Methanol and 30% Aq. Methanol extracts of *C. pulcherrima* leaf and stem

3.4. SUMMARY AND CONCLUSION

From the overall studies conducted on various extracts of *C. pulcherrima*, the antioxidant activity performed using DPPH method showed leaf methanolic extract as the most active one with percentage inhibition rate of 92.85% at 50µg/mL concentration indicating the presence of more number of electron rich chemical constituents. Similarly, the total phenolic content performed by Folin-Ciocalteu assay method using Gallic acid as a standard showed that the leaf ethyl acetate extract contains high phenolic content of about 70.55 mg GAE/ gr. extract. Likewise, the total Flavonoidal content performed by aluminum chloride colorimetric assay method using Quercetin and Catechin as a standard visualized that the leaf ethyl acetate extract contains high amount of Flavonoidal content in Quercetin standard method (with 82.3 mg

QE/ gr Extract) where as in Catechin standard method, the stem ethyl acetate extract showed high amount of Flavonoid content with (73.01 mg CE/ Extract).

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