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In-vitro study of TLC profile and antioxidant activity of *Caesalpinia pulcherrima* extracts

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Abstract

The present work was aimed to determine thin layer chromatographic screening and antioxidant properties of leaves and flower extracts of *Caesalpinia pulcherrima*. Ethyl acetate and acetone extracts were screened for identification of phenolic and flavonoids. Thin layer chromatography (TLC) was performed first to confirm the qualitative characterization of phytochemical screening. Antioxidant activity of the extracts was tested qualitatively with Superoxide radical scavenging assay. Phytochemical screening test showed that both ethyl acetate and acetone fractions from the plant materials contain phenolic and flavonoids and exhibited higher antioxidant activity in comparison of Standard Ascorbic acid. Thin Layer chromatography of the ethyl acetate and acetone were showed colour spots with different RF values under UV light which confirmed the presence of phytochemicals and antioxidants. IC₅₀ values of the plant material extracts revealed the potential of antioxidant activity of this plant. Thus these findings concluded that, this can be further investigated for isolation and identification of active secondary metabolites of medicinal utilities.

Keywords: Ethyl acetate, *Caesalpinia pulcherrima*, superoxide radical, phytochemicals, TLC, antioxidants.

1. Introduction

India has one of the oldest, richest and most diverse cultural traditions associated with the use of medicinal plants. As per the concern to the health of an individuals and communities in general, medicinal plants are great importance. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. Alkaloids, tannins, flavonoids and phenolic compounds are the most important bioactive constituents of plants ^[1]. *Caesalpinia pulcherrima* is known as peacock flower is the type of genus Fabaceae sub family Caesalpinieae. It is an ever green shrub growing to 3 m tall. It is a striking ornamental plant, widely grown in tropical gardens. *Caesalpinia pulcherrima* is used for a various purpose of herbal medicine. It is used as emmenagogue, purgative, stimulant, and abortifacient also used in bronchitis, asthma, malarial fever, and used against kidney stone. Leaves used as antipyretic, antimicrobial, antibacterial, antioxidant ^[2].



Caesalpinia pulcherrima leaves



Caesalpinia pulcherrima flowers

Chromatography is the separation of two or more compounds or ions by the distribution between two phases, one which is moving and the other which is stationary. TLC is a solid-liquid form of chromatography where the stationary phase is normally a polar absorbent and the mobile phase can be a single solvent or combination of solvents.

TLC is one of the simplest, fastest, easiest and least expensive of several chromatographic techniques used in qualitative and quantitative analysis to separate organic compounds and to test the purity of compounds.

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Thin-layer chromatography (TLC) is a technique used to separate non-volatile mixtures and monitor the progress of a reaction, identify compounds present in a given mixture, and determine the purity of a substance.

Antioxidants in biological systems have multiple functions, including depending against oxidative damage and in the major signaling pathways of cells. The major action of antioxidants in cells is to prevent damage caused by the action of reactive oxygen species (ROS). ROS such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radical (OH^{\cdot}), peroxide radical (ROO^{\cdot}) and nitric acid radical are generated in living organisms during excessive metabolism and involved in extensive oxidative damage to the cells that leads to age related degenerative diseases, cancer and wide range of other human diseases [3].

Superoxide is biologically toxic and is deployed by the immune system to kill invading microorganisms. In phagocytes, superoxide is produced in large quantities by the enzyme NADPH oxidase for use in oxygen-dependent killing mechanisms of invading pathogens. Mutations in the gene coding for the NADPH oxidase cause an immunodeficiency syndrome called chronic granulomatous disease, characterized by extreme susceptibility to infection, especially catalase-positive organisms. In turn, microorganisms genetically engineered to lack superoxide dismutase (SOD) lose virulence.

The half maximal inhibitory concentration (IC_{50}) is a measure of the potency of a substance in inhibiting a specific biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor or microorganism) by half. The values are typically expressed as molar concentration. It is commonly used as a measure of antagonist drug potency in pharmacological research. According to the FDA, IC_{50} represents the concentration of a drug that is required for 50% inhibition *in vitro*.

Based on the traditional knowledge of medicinal system, The present study were carried out to screening of thin layer chromatography and antioxidant activity of ethyl acetate and acetone solvent extracts of leaves and flowers of *Caesalpinia pulcherrima*.

2. Material and Methods

2.1 Collection and Extraction of leaves and flowers

Caesalpinia pulcherrima leaves and flowers were collected from nearby local area of Shegaon of Buldhana district. Plant materials were washed and air dried to complete removal of soil from it and ground into a uniform powder using a grinder and stored in plastic bottles at $4^{\circ}C$ for future use in experiment. Extracts prepared with non-polar and polar solvents such as Ethyl acetate and acetone by Soxhlet apparatus as per respective boiling point until the extract turned to colorless. Dried extracts were used for analysis.

2.2 Thin Layer Chromatography

Cylindrical glass chamber (TLC Tank) with airtight lid was used for the development of chromatoplates. With increasing polarity, Toluene, Ethyl acetate and Acetic acid solvents were used to prepare solvent systems at different ratio, such as 5:4:1, 4:4:2, 3:6:1, 2:7:1 as mobile phase. The tank was then made airtight and kept for few minutes to saturate the internal atmosphere with the solvent vapor. A small spot of the sample was applied on the activated silica plate (Aluchrosep Silica

gel 60 TLC plate) with capillary tube just 1 cm above the lower edge of the plate. The spot was air dried and straight line was drawn 2cm below the upper edge of the activated plate, which marks the upper limit of the solvent flow. The spotted plate was then placed in the tank in such a way as to keep the applied spot above the surface of the solvent system and lid was placed again. The plate was left for development. When the solvent front reached up to the given mark, the plate was taken out and air dried. The properly developed plates were kept in the UV chamber to see the bands. All detected spots were noted according to their R_f values. Gallic acid and Quercetin were used as Standard. The movement of the active compound was expressed by its Retention Factor (RF) values were calculated for different samples [4].

$$R_f = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent front TLC plates}}$$

2.3 Estimation of Total Phenolic Content

The total phenolic content in the extract was determined with Folin-Ciocalteu reagent by using UV- Spectrophotometer technique. 0.2 ml sample extract was mixed with 1.0 ml of 10% (v/v) Folin-Ciocalteu reagent and was vortex for 3 min followed by addition of 0.8 ml of 7.5% (w/v) Sodium carbonate. This reaction mixture was incubated for 30 min at room temperature. The absorbance was measured at 765 nm. Same procedure was carried out for Gallic acid standard curve and results were expressed as mg Gallic acid equivalent/ ml of extract [5].

2.4 Estimation of Total Flavonoid Content

The amount of total flavonoid in the extract was measured spectrophotometric ally. Briefly, 500 μ l of each extract was mixed with 1.50 ml of 95% ethanol, 0.10 ml of 10% aluminium chloride ($AlCl_3 \cdot 6H_2O$), 0.10 ml of sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) (1M) and 2.80 ml of distilled water. After incubation for 40 min, absorbance was measured at 415 nm using a spectrophotometer. To calculate the concentration of flavonoid, a calibration curve was prepared using Quercetin as standard. The flavonoid concentration is expressed as Quercetin equivalents in mg per ml of extract. All assays were carried out in triplicate [6].

2.5 Superoxide radical assay

The scavenging activity of the extract on superoxide radicals was determined by the pyrogalllic acid method. Volumes of 4.5 ml Tris-HCl buffer (0.05 mol, pH 8.2), 1 ml of sample solution, and 0.4 ml pyrogalllic acid (3.0 mM) were added together; the solution was incubated at $25^{\circ}C$ for 15 minutes, 0.5 ml of hydrochloric acid was added for termination the reaction, and it was determined at 525 nm. Ascorbic acid was used as the positive control.

Superoxide radical Scavenging activity (%) = $\{(A_o - A_i)/A_o\} \times 100$

Where, A_o is the absorbance without sample and A_i the absorbance with sample [7].

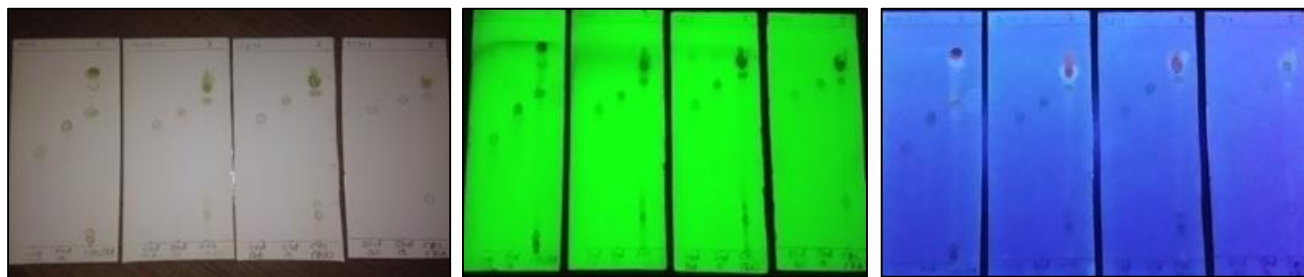
The half maximal inhibitory concentration (IC_{50}) values were calculated using linear trendline, R-squared equation in excel where the abscissa represented the concentration of tested plant extracts and the ordinate the average percent of scavenging capacity.

3. Result and Discussion

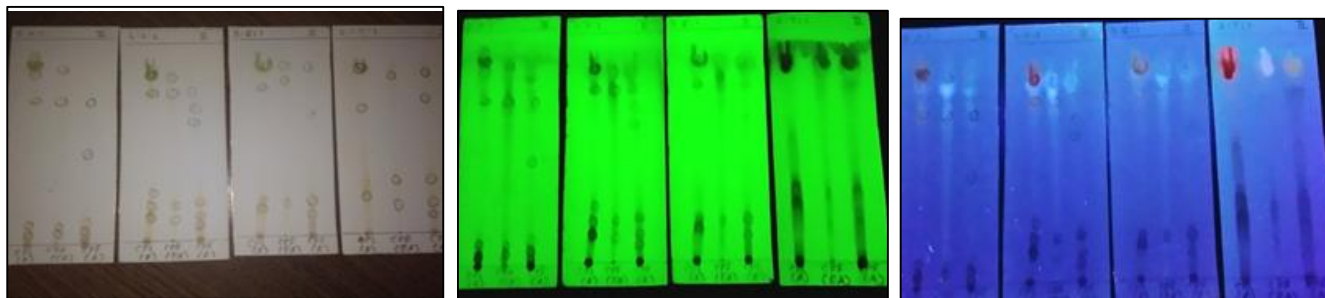
Caesalpinia pulcherrima leaves and flowers were used for the

study of TLC profile and antioxidant properties. Thin layer chromatography had been done using different solvent

systems such as 5:4:1, 4:4:2, 3:6:1, 2:7:1 (Toluene: Ethyl acetate: Acetic acid).



3.1 Spot observed at visual light, UV₂₅₄ light and UV₃₆₆ light (Standard Gallic acid and Quercetin, CPL EA)



3.2 Spot observed at visual light, UV₂₅₄ light and UV₃₆₆ light (CPL A, CPF EA, CPF A)

Thin layer chromatographic studies for Standard Gallic acid (Phenolic contents) observed 1 spot for each solvent system with different R_f values such as in Solvent system I = 0.48, II = 0.6, III = 0.74 and IV = 0.69.

Thin layer chromatographic studies for Standard Quercetin (Flavonoid contents) observed 1 spot for each solvent system with different R_f values such as in Solvent System I = 0.61, II = 0.67, III = 0.64 and IV = 0.75.

Thin layer chromatographic studies of the ethyl acetate extract for *Caesalpinia pulcherrima* leaves (CPL EA) observed 5 spots in Solvent system I (R_f values 0.04, 0.08, 0.66, 0.8, 0.88), 4 spots in solvent system II (R_f values 0.15, 0.21, 0.74, 0.8), 4 spots in solvent system III (R_f values 0.15, 0.2, 0.77, 0.84), 3 spots in solvent system IV (R_f values 0.22, 0.75, 0.82).

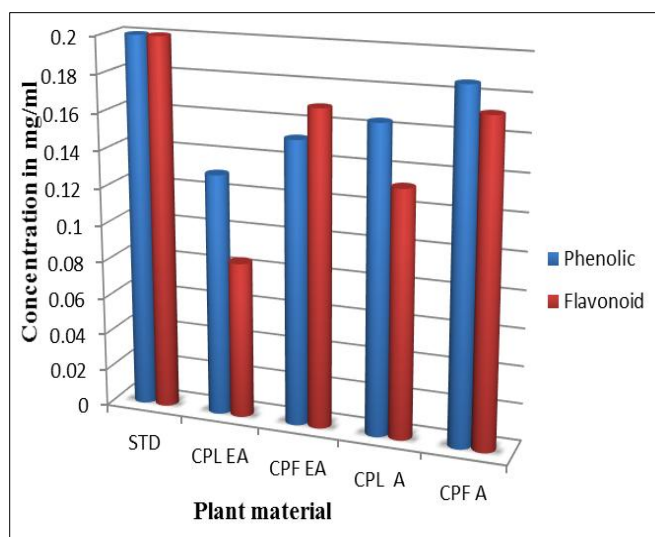
Thin layer chromatographic studies of the acetone extract for *Caesalpinia pulcherrima* leaves (CPL A) observed 5 spots in Solvent system I (R_f values 0.07, 0.1, 0.69, 0.83, 0.89), 5 spots in solvent system II (R_f values 0.13, 0.19, 0.25, 0.75, 0.84), 4 spots in solvent system III (R_f values 0.14, 0.19, 0.76, 0.88), 3 spots in solvent system IV (R_f values 0.21, 0.73, 0.96).

Thin layer chromatographic studies of the ethyl acetate extract for *Caesalpinia pulcherrima* flower (CPF EA) observed 4 spots in Solvent system I (R_f values 0.07, 0.1, 0.74, 0.85), 4 spots in solvent system II (R_f values 0.11, 0.19, 0.75, 0.84), 3 spots in solvent system III (R_f values 0.17, 0.79, 0.87), 3 spots in solvent system IV (R_f values 0.17, 0.31, 0.92).

Thin layer chromatographic studies of the acetone extract for *Caesalpinia pulcherrima* flower (CPF A) observed 4 spots in Solvent system I (R_f values 0.07, 0.1, 0.74, 0.85), 5 spots in solvent system II (R_f values 0.09, 0.13, 0.2, 0.6, 0.67), 5 spots in solvent system III (R_f values 0.07, 0.12, 0.17, 0.63, 0.86), 5 spots in solvent system IV (R_f values 0.12, 0.19, 0.32, 0.79, 0.93).

Total Phenolic and Total Flavonoid Contents of *Caesalpinia*

pulcherrima leaves and flowers were evaluated according to the Folin-Ciocalteu method and Aluminum chloride assay respectively. Table 1 showed a significant difference in total phenolics and Flavonoids were noticed between leaves extract (CPL EA = 0.13mg/ml for phenolic, 0.084mg/ml for flavonoids and CPL A = 0.164mg/ml for phenolics and 0.132mg/ml for flavonoids) and flowers extract (CPF EA = 0.152 mg/ml for phenolics, 0.169 for flavonoids and CPF A = 0.186mg/ml for phenolics and 0.172mg/ml for flavonoids) and both the standards (0.2 mg/ml).



(STD: Standard, CPL EA: *Caesalpinia pulcherrima* Leaves in Ethyl acetate, CPL A: *Caesalpinia pulcherrima* Leaves in Acetone, CPF EA: *Caesalpinia pulcherrima* Flowers in Ethyl acetate, CPF A: *Caesalpinia pulcherrima* Flowers in Acetone).

Fig 1: Graphical representation of phenolic and Flavonoids contents in *Caesalpinia pulcherrima* and Standards

Superoxide radical is the most common free radical generated *in vitro*. Pyro Gallic acid can autoxidize in slightly alkaline conditions to produce superoxide radical. The constant rate of

this autoxidation reaction is dependent on the pyro gallic acid concentration. Antioxidants can inhibit the spontaneity oxidation of pyrogallol but it has been found that the reaction is stable in 1 - 3 min. Consequently the data at 5 min was adopted to evaluate the inhibition activity [9]. The free radical scavenging activity of superoxide radicals for *Caesalpinia pulcherrima* leaves and flowers extract were detected and compared with standard Ascorbic acid. Percent scavenging activities of the Acetone extracts were found to be higher than that of Ethyl acetate extracts of leaves and flowers of the given plant material where percent scavenging activities of leaves were found to be higher than that of found to be in flowers of both solvent extracts at 10µg/ml, 60µg/ml, 400µg/ml as shown in Table 1.

Figure-2 and Table-1 illustrates that CPL EA was obtained inhibition from 16.1% - 98.8% followed by CPL A (11.5%- 97.2%), CPF EA (13.5 - 97.2%), CPF A (10.9- 95.5%) in comparison of standard ascorbic acid (12.8% - 98.1%).

Table 1: Percent inhibition of superoxide radicals in *Caesalpinia pulcherrima* extracts

Concentration in µg/ml	STD	CPL EA	CPL A	CPF EA	CPF A
10	12.8±0.12	16.1±0.26	11.5±0.20	13.5±0.28	10.9±0.19
20	42.7±0.49	35.9±0.34	34.4±0.31	35.4±0.36	35.1±0.31
40	56.5±0.62	47.8±0.56	59.6±0.75	49.6±0.66	53.1±0.67
60	68.9±0.89	59.1±0.70	65.9±0.76	58.9±0.68	60.3±0.70
80	77.1±0.95	76.5±0.77	75.9±0.77	72.9±0.74	83.7±1.12
100	84.1±1.06	84.8±1.15	81.1±1.02	82.1±1.11	90.1±1.12
200	95.2±1.23	95.9±1.28	95.4±1.12	95.4±1.13	93.3±1.16
400	98.1±1.40	98.8±1.48	97.2±1.41	97.2±1.42	95.5±1.24

(STD: Standard, CPL EA: *Caesalpinia pulcherrima* Leaves in Ethyl acetate, CPL A: *Caesalpinia pulcherrima* Leaves in Acetone, CPF EA: *Caesalpinia pulcherrima* Flowers in Ethyl acetate, CPF A: *Caesalpinia pulcherrima* Flowers in Acetone).

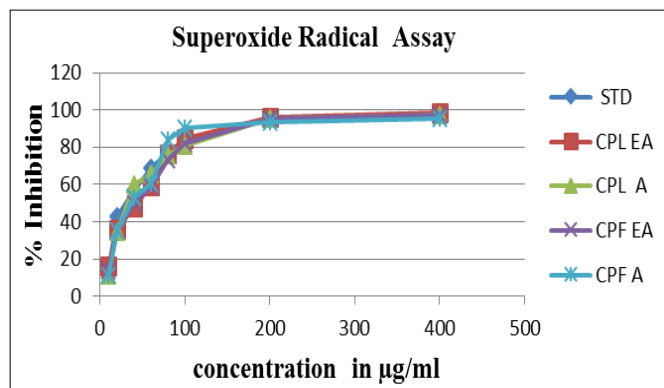


Fig 2: The activity of free scavenging superoxide radical

Table 2: Half Inhibitory Concentration of antioxidant activity

S. N.	Plant parts	IC50 (µg/ml)
	STD AA	9.93
1	CPL EA	33.05
2	CPL A	24.28
3	CPF EA	39.48
4	CPF A	22.2

(STD AA: Standard Ascorbic Acid, CPL EA: *Caesalpinia pulcherrima* Leaves in Ethyl acetate, CPL A: *Caesalpinia pulcherrima* Leaves in Acetone, CPF EA: *Caesalpinia pulcherrima* Flowers in Ethyl acetate, CPF A: *Caesalpinia pulcherrima* Flowers in Acetone).

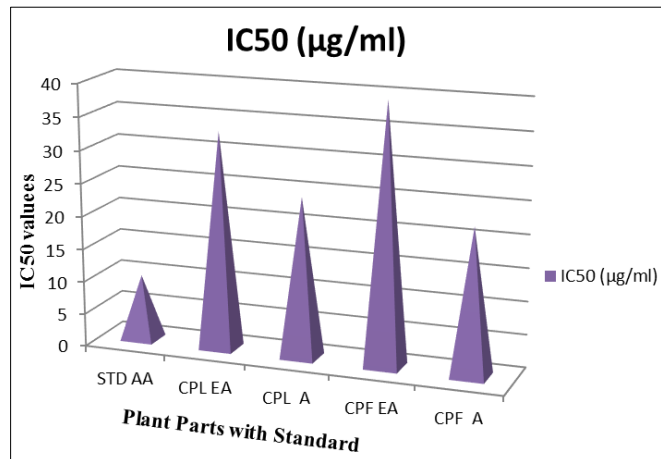


Fig 3: Inhibitory concentration at 50% of plant extracts against standard

IC50 values denote the concentration of sample required to scavenge 50% of free scavenging activity. IC50 values for superoxide assay of CPL(EA) and CPF(EA) showed significant towards standard IC50 value and CPF(A), CPL(A) showed moderate towards standard IC50 value.

Above figure revealed that *Caesalpinia pulcherrima* leaves and flowers had been highest potential of antioxidant activity as it inhibited maximum superoxide radical scavenging activity as compare to standard Ascorbic acid (AA).

4. Conclusion

The findings of study indicate that the extract of *Caesalpinia pulcherrima* contained many phytochemicals as revealed by TLC analysis. The present study found to be inhibitory effect of leaf and flower extracts of selected plant. The extracts also exhibited greater antioxidant activity which may be due to the presence of phenolic and flavonoid content. The plants can be used as potential source for the development of antioxidant agents. Thus, it is recommended to further investigate and identify these compounds, with the support of different analytical techniques for pharmacological activities and potential applications of such compounds as natural antioxidants in different food/pharmaceutical products which can be used to treat various oxidative stress-related diseases in plants.

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