IMMUNOMODULATORY AND ANTIOXIDANT ACTIVITY OF HYDRO-ALCOHOLIC EXTRACT OF CAESALPINIA PULCHERRIMA (AERIAL PARTS)

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Aim of the study: In the present study the immunomodulatory and the antioxidant activity of aerial parts of Caesalpinia pulcherrima were investigated by in vitro and in vivo methods. Materials and methods: The hydro-Alcoholic extract was screened for various phytoconstituents and tried for co-relation with biological activity. Hydro-alcoholic extract was tested for antioxidant by DPPH radicals scavenging activity and hydrogen peroxide scavenging (H₂O₂) assay. Immunomodulatory activity was evaluated by delayed-type hypersensitivity (DTH) response and percentage adhesion of neutrophils to nylon fibers compare with control group. Results: The evaluation of immunomodulatory potential of Caesalpinia pulcherrima (100, 250 and 500 mg/kg, p.o.) evoked a significant dose-dependent increase in DTH reaction induced by SRBC and at higher dose significantly (P<0.01) increased the percentage of adhesion of Neutrophils to nylon fibers when compared with the normal animals group. The results were comparable with control group. Hydro-alcoholic extract of Caesalpinia pulcherrima showed significant immunomodulatory and antioxidant activity. The hydro alcoholic extract showed significantly the highest % of inhibition of DPPH scavenging ability and H₂O₂ assay when compare to chloroform and ethanolic extract but minute less to ascorbic acid as a refer. Conclusion: The present investigation supports the use of Caesalpinia pulcherrima for their immunomodulatory and antioxidant effects. Hydro-Alcoholic extract of Caesalpinia pulcherrima contains bioactive principles, which possess immunostimulant activity.

KEYWORDS: Caesalpinia pulcherrima, Immunomodulatory, Antioxidant, DTH Activity, Neutrophil adhesion test, Hydro-Alcoholic extract.

INTRODUCTION

Caesalpinia pulcherrima (Caesalpiniceae) popularly known as Gulutre (Hindi), Peacock flower (English) and Ratna Gandhi (Sanskrit)[1] found in India, is a shrub or small tree up to 5m in height abundantly cultivated.[2] It is broadly distributed in the tropics and subtropic regions, Native of Americas, West Indies, and also cultivated as an ornamental plant in India but its exact origin is unknown due to widespread cultivation. The flowers are borne in racemes up to 20 cm long, each flower with five yellow, orange, or red petals, flowering season of this plant start from September to November and fruits from March to April and leaves are bipinnate, 20–40 cm long bearing 3-10 pairs of pinnate with 6-10 pairs of leaflets 15-25mm long broad.[3] Seeds are poisonous and grayish brown in color. The bark is grey in color. The fruit is a pod 6–12 cm long and wood is white or brownish and hard in nature.[4] The various parts of the plant of the study had been traditionally used for the treatment of multiple ailments. The leaves are utilized as a part of the treatment of pyrexia, and sometimes used as a substitute for senna. It is also given in cases of a cough and catarrh. Root part is used for curing intermittent fevers. The bark is used as emmenagogue and abortifacient. It is also reported to possess antimicrobial and antitubercular action and used in bronchitis, asthma and malarial fever. Flowers of Caesalpinia pulcherrima have been reported to posse’s antiviral and antioxidant effects Caesalpinia pulcherrima has reportedly been used as food; in Mexico and Nicaragua, green seed pods are boiled or cooked and eaten.[5-8] Two compounds such as tetra acetyl brazilin and proto C.pulcherrimain were isolated from the stem of C. pulcherrima and the phenolic compounds mainly included phenolic acids, flavonoid, tannins, coumarins, lignans, quinones, stilbenes, and curcuminoids were isolated from different traditional medicines including C. pulcherrima.[9] The seeds contain 7% protein and apart from it amino acids like alanine, cystine, glycine, isoleucine, lysine, threonine, tryptophan and valine are also present.[10] Wood is reported to contain a glycoside like B-amyrin, and sugars like lactose, galactose were
present.\[^{11,12}\] Heartwood consists of aromatic compounds like brazilin, C. Pulcherrima chalcone, Caesalpin J, Caesalpin P. proto and sitosterol with the presence of monohydroxybrazilin and benzyl dihydrobenzofuran derivatives were isolated from the part.\[^{13,14}\]

**MATERIAL AND METHODS**

Collection and identification of plant material

Fresh aerial parts of plant *Caesalpinia pulcherrima* were collected from the campus of NIET, Greater Noida, during the months of August 2017 and authenticated by Dr. Anjula Pandey (Taxonomist), National Bureau of Plant Genetic Resources (NBGR), Pusa Campus, New Delhi. A voucher specimen (Specimen No: NHCP/NBGR 2017-5) is preserved in Herbarium section of Taxonomic Deptt. of NBGR, New Delhi.

Preparation of hydroalcoholic extract

The shade dried aerial parts [flower, bark, leaves] of *Caesalpinia pulcherrima* plant mixed in equal ratio and extraction was carried out by maceration process with Hydroalcoholic solvent in equal ratio for seven days with occasionally shaking every 4 hours and placing them in dark condition so as to minimize the light entrapment and maintaining a proper temperature. After seven days filtration was carried out and the marc was re-macerated with a solvent for another day. The filtrate was concentrated over water bath by maintaining a temperature not more than 35°C with continuous stirring. The extract was concentrated to a syrupy mass and placed in desiccator for removal of moisture. The dried extract obtained was found to be 15.9% w/v with respect to an air-dried drug.

Suspension of test sample

An accurately weighed extract was calculated according to body weight of animals which were suspended in distilled water and gum acacia to form a homogenous suspension and avoiding phase separation. The prepared suspension was placed in airtight container and was administered to the animal’s orally inaccurate doses.

Preliminary phytochemical screening

The crude extract was subjected to a phytochemical screening by non-polar to polar solvent to check the presence or absence of various active constituents like alkaloid, carbohydrate, glycosides, saponins etc.\[^{15}\]

Experimental Animals

Male Wistar rats about 150 - 200 g were obtained from NIET animal house. All animals were housed in polyethylene cages under standard housing conditions (12h/ 12h light and dark cycle, temperature 22±2°C and humidity 50±10%) with standard feed pellet and free access water adlibitum. Standard hygiene conditions were maintained. The animal experiments were performed by Institutional Animal Ethics Committee (IAEC/NIET / 2017 / 01 / 05) and by the animal regulatory body of the government CPCSEA guidelines.

Antigenic material

The Sheep red blood cells (SRBCs) were used as an antigenic material. Fresh blood was collected from sheep sacrificed from the local slaughterhouse. Sheep red blood cells (SRBCs) were washed three times in large volumes of pyrogen free 0.9% normal saline and adjusted to a concentration of 0.5×10^9 cells/ml for immunization and challenge.\[^{16}\]

Toxicity assay

The toxicity study was performed according to OECD guidelines no 420. The animal was given doses as per the mentioned scheduled guidelines, and no mortality was observed up to the dose of 4000 mg/kg body weight and the extracts was administered orally.\[^{17}\]

Evaluation of immunomodulatory activity: experimental design

Neutrophil adhesion test

Wilkinson (1978) method was employed for neutrophil adhesion test. Animal was divided into 4 groups, Group I, served as control and received 10 ml/kg normal saline, whereas groups II, III and IV were pre-treated with different concentrations of hydroalcoholic extract of *Caesalpinia pulcherrima* (100,250 & 500mg/kg, oral). On day 14 of drug treatment, blood samples were collected by puncturing retro-orbital plexus into heparinized vials and analyzed for total leukocyte cell (TLC) and differential leukocyte cell (DLC) counts. After starting counts, blood samples were incubated with nylon fibers for 15min at 37°C. The incubated blood samples were again analyzed for TLC and DLC, respectively to obtain a neutrophil index of blood samples. The following formula was used for calculating calculated the percentage of neutrophil adhesion:

\[ \text{Neutrophil adhesion (\%) = } \frac{\text{NI}_a - \text{NI}_t}{\text{NI}_a} \times 100 \]

Where NI\(_a\) is the neutrophil index of untreated blood samples, and NI\(_t\) is the neutrophil index of treated blood samples.\[^{18}\]

Delayed-type hypersensitivity (DTH) response

The animal was divided into four groups. Group I, served as control and received 10 ml/kg normal saline, whereas Groups II, III and IV were challenged by injection of 0.5×10^9 cells SRBCs in right hind footpad. Foot thickness was measured after +24, and +48 h of this challenge and the extract was administered orally on day 0 and continued till day 7 of the trail. The differences obtained for pre- and post-challenge foot thicknesses were taken for the measurement of DTH and were expressed in mm.\[^{19}\]

Evaluation of antioxidant activity: experimental design

DPPH radicals scavenging activity

Estimation of the free radical scavenging activity of an extract of *Caesalpinia pulcherrima* aerial parts was carried out by decreasing the absorbance of a methanolic
solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH). A stock solution of DPPH was prepared by dissolving 4 mg DPPH in 20 ml methanol, and 3 ml of this stock solution was added to 1 ml of CPHAE solution at 60μg/ml. After 30 minutes, the absorbance was estimated at 517 nm and was compared with the standard.

The scavenging activity was calculated as the percentage of inhibition, using the following formula:

\[
\% \text{ scavenged} (H_2O_2) = \left(\frac{A_i - At}{A_i}\right) \times 100
\]

Where A_i is the absorbance of control and At is the absorbance of test.\(^{[20]}\)

**Hydrogen peroxide scavenging (H_{2}O_{2}) assay**

A solution of hydrogen peroxide (40mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by Ultraviolet at the absorption of 230 nm using a spectrophotometer. Extract 60μg/ml was added to hydrogen peroxide, and absorbance was measured at 230 nm and determined after 10 min against a clear solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging was calculated as follows:\(^{[21]}\):

Formula: % Scavenged = Absorbance of the Control - Absorbance of the test/Absorbance of Control ×100.

**Statistical analysis**

The collected data of all groups were analyzed using one way ANOVA with Dunnett’s comparison test. Data were expressed as mean ± the correspondent standard error of mean (SEM) and n=6.

**RESULTS AND DISCUSSION**

**Phytochemical screening**

The preliminary phytochemical screening of aerial parts of *Caesalpinia pulcherrima* revealed the presence of alkaloids, carbohydrate, flavonoids, phenolics, polysaccharides, saponins, and terpenoids as essential phytochemical constituents of the hydro-alcoholic extract of *Caesalpinia pulcherrima*.

**Neutrophil Adhesion Test**

The % neutrophil adhesion rate in control group animals was noted to be 2.01±0.10, whereas, in hydro-alcoholic extract-treated groups it was found with an increased pattern as compared to their respective control groups. As shown in [Table 1], a significant (**p<0.01) increase in neutrophil adhesion was observed when hydro-Alcoholic extract administered at a dose of 100mg/kg, 250mg/kg and 500mg/kg suggesting possible immunostimulant action of the herbal formulation.

**Delayed-type hypersensitivity (DTH) reactions**

DTH response assessed the cell-mediated immune response of Hydro-alcoholic extract, i.e. footpad reaction. As shown in Table 2, Hydro-alcoholic extract produced a significant (**p<0.01) dose-related increase in DTH response in Rats. Rise in DTH reaction in Rats in response to T-cell dependent antigen revealed the stimulatory effect Hydro-alcoholic extracts on T cells.

**DPPH scavenging activity**

The DPPH free radical scavenging action showed (Figure 1) by various solvents extract of *Caesalpinia pulcherrima*. The scavenging activity (% hindrance) was more in ethanol and hydroethanolic separates. Hydroethanolic extract of *Caesalpinia pulcherrima* (60μg/ml) indicated better searching movement when contrasted with other extract solvents.

**H_{2}O_{2} scavenging activity**

The Hydrogen peroxide scavenging action was displayed (Figure 2) by various solvents extract of *Caesalpinia pulcherrima*. The rate searching movement (% restraint) was more in ethanol and hydroalcoholic extract. Hydroalcoholic concentrate of *Caesalpinia pulcherrima* (60μg/ml) indicated better scavenging activity when contrasted with other extract solvents.

"Table 1: Effect of aerial parts of a Hydro-Alcoholic extract of *Caesalpinia pulcherrima* on Percent Neutrophil Adhesion test in Rats".

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose(mg/kg) (CPHAE)</th>
<th>TLC(10^4/mm3)</th>
<th>[A]</th>
<th>Neutrophil % [B]</th>
<th>Neutrophil index [AxB]</th>
<th>Netrophil adhesion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>7.4±0.1</td>
<td>7.5±0.1</td>
<td>29.7±0.4</td>
<td>30.0±0.4</td>
<td>221±6.2</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>5.5±0.03</td>
<td>4.4±0.07</td>
<td>22.2±1.2</td>
<td>17.8±0.3</td>
<td>125.3±13.9**</td>
</tr>
<tr>
<td>2</td>
<td>250</td>
<td>5.9±0.2</td>
<td>3.0±0.09</td>
<td>23.7±1.0</td>
<td>13.6±0.3</td>
<td>142±12.8**</td>
</tr>
<tr>
<td>3</td>
<td>500</td>
<td>6.01±0.4</td>
<td>2.0±0.07</td>
<td>24.0±1.8</td>
<td>8.2±0.3</td>
<td>148±21.5**</td>
</tr>
</tbody>
</table>

Value are mean ±SEM. n=6. UB: Untreated blood, NFTB: Nylon fiber treated blood **p<0.01, *p<0.01 **p<0.01

CPHAE= Caesalpinia pulcherrima hydro-alcoholic extract followed Dunnett’s Test.
"Table 2: Effect of Hydro-Alcoholic extract of *Caesalpinia pulcherrima* on DTH response using SRBCs as antigen in rats".

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>DTH response (mm) 24h</th>
<th>DTH response (mm) 48h</th>
<th>DTH response (mm) 7day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control(10mg/kg saline)</td>
<td>_</td>
<td>2.2 ±0.07</td>
<td>1.46 ±0.09</td>
<td>1.1 ±0.04</td>
</tr>
<tr>
<td>2</td>
<td>Hydro-Alcoholic extract of <em>Caesalpinia pulcherrima</em></td>
<td>100</td>
<td>6.16 ±0.03***</td>
<td>6.16 ±0.03***</td>
<td>5.66 ±0.21***</td>
</tr>
<tr>
<td>3</td>
<td>Hydro-Alcoholic extract of <em>Caesalpinia pulcherrima</em></td>
<td>250</td>
<td>6.33 ±0.33***</td>
<td>5.66 ±0.21***</td>
<td>3.56 ±0.22***</td>
</tr>
<tr>
<td>4</td>
<td>Hydro-Alcoholic extract of <em>Caesalpinia pulcherrima</em></td>
<td>500</td>
<td>6.0 ±0.36***</td>
<td>4.0 ±0.36***</td>
<td>2.16 ±0.30*</td>
</tr>
</tbody>
</table>

Value are Mean ±Sem, N=6 ***P<0.01 denotes significance with concerning the control group using ANOVA followed by Dunnett’s Test Significance.

"Fig. 1", DPPH Scavenging activity of different solvents extracts activity

"Fig. 2", Hydrogen peroxide scavenging of different solvents extracts activity
DISCUSSION
The present investigation suggests that hydro Alcoholic extract derived from aerial parts of *Caesalpinia pulcherrima* may stimulate both cellular and humoral immune responses. The extracts not only potentiate nonspecific immune response, but also improve humoral as well as cell-mediated immunity effectively. Modulation of the immune response through stimulation or suppression may help in maintaining a disease-free state. Agents that activate host defense mechanisms in the presence of an impaired immune responsiveness can provide supportive therapy to conventional chemotherapy as reported by previous authors who have conducted the study in another species. [23] The results were significant in the study and were potent immunostimulant, stimulating both the specific and non-specific immune mechanisms. The neutrophil, an end cell unable to divide and with limited capacity for protein synthesis is, nevertheless, capable of a wide range of responses, in particular chemotaxis, phagocytosis, exocytosis and both intracellular and extracellular killing. [23] Neutrophil plays an important role in enhancing immunity of the body against microbial infection. *Caesalpinia pulcherrima* 500 mg/kg p.o. significantly (P<0.01) increased the adhesion of neutrophils to nylon fibers. The results are comparable with that of normal. Further studies are required to exactly elucidate the mechanism of immunostimulatory activity. In the present study, hydroalcoholic extract of *Caesalpinia pulcherrima* evoked a significant increase in percent neutrophils. This may help in increasing immunity of body against microbial infections. [23] [Table1]. Cell-mediated immunity (CMI) involves effectors mechanisms carried out by T lymphocytes and their products (lymphokines). CMI responses are critical to defence against infectious organisms, infection of foreign genes, tumour immunity and delayed-type hypersensitivity reactions. [25] Therefore, increase in DTH reaction in a rat in response to T cell- dependent antigen revealed the stimulatory effect of a hydro-alcoholic extract of *Caesalpinia pulcherrima* on T cells. [Table2].

The reaction based with DPPH and Hydrogen peroxide can be diverted by a dismutation reaction from superoxide anion by superoxide dismutase which in turn may also give a colour reaction when treated with coloring agents placed in dark conditions. They mainly target the free radicals and try to minimize the active molecules which are responsible for degradation reactions. Many enzymes like amino acid oxidase and xanthine oxidase produce hydrogen peroxide from superoxide anion. They are highly diffusible and cross the plasma membrane easily by means of diffusion. Hydrogen peroxide is the least reactive molecule among reactive oxygen species and is stable under physiological pH and temperature in the absence of metal ions. They are also weak oxidizing and reducing agent and due to this, they are poorly reactive. Hydrogen peroxide can generate the hydroxyl radical in the presence of metal ions and superoxide anion (·O₂ + H₂O₂ → ·OH + OH + O₂). They generally produce singlet oxygen through reaction with superoxide anion or with HOCl or chloramines in a living system and thereby act on them thus antioxidant and immune systems are inter-related to each other via defense mechanism and follow the complement pathway for its reparation. [26]

CONCLUSION
In the current experimental study, it was observed that the Hydroalcoholic extract obtained from the aerial parts of *Caesalpinia pulcherrima* showed a significant activity in both immunomodulatory and antioxidant activity when they were studied against standard substances. Further exploration of the plant can be done with the necessarily active constituents responsible for it, so that more statistically and scientific exploration can be done.

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