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In-vivo and *in-vitro* Immunomodulatory potential of *Cassia auriculata* Linn

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Abstract

The present study was undertaken with an objective to investigate *in-vivo* and *in-vitro* immunomodulatory potential of *Cassia auriculata* Linn. The methanolic extracts of flowers and roots of plant were investigated for their immunomodulatory potential by using *in-vivo*, oxazolone-induced cell mediated inflammation in rat's model and various *in-vitro* immunomodulatory models. Methanolic extracts of both flowers and roots were screened scientifically through various activities models. The result of oxazolone-induced cell mediated inflammation, showed that methanolic flowers extract possesses immunomodulatory potential. While in *in-vitro* immunomodulatory models like NBT reduction assay by human PMN cells, methanolic flower extract showed dose dependent stimulation. With the same extract similar observation was recorded with phagocytosis of *Candida albicans* by human PMN. The methanolic flower extract also increased candidacidal activity in dose-dependent manner. The methanolic flower extract significantly induced human PMN cells chemotaxis and exhibited potent effects than positive control. Thus results of *in-vitro* studies clearly indicated that methanolic flower extract has immunostimulant effect, where as in *in-vivo* study of oxazolone-induced delayed type hypersensitivity, methanolic flower extract significantly reduced rat ear edema in dose dependent manner. The *in-vitro* assays, revealed that methanolic flower extract possess potent immunomodulatory activity due to its antioxidant potential. In all, results represented here indicated that *Cassia auriculata*, in addition to its reputed cytoprotected effects, also passes significant immunomodulatory activity. Thus the flavonoids rich methanolic flowers extract can be exploited in chronic inflammatory condition, such as rheumatoid arthritis usually in combination with conventional drugs to reduce the dose and dose related toxicities of later.

Keywords: Immunomodulatory, NBT assay, *Cassia auriculata,* Oxazolone

Introduction

Cassia auriculata Linn. belongs to family Leguminoseae is an evergreen shrub with large bright yellow flowers growing wild in central and western India. The extraordinary observations recorded that in extreme drought situations as well as prolonged water lodgings; plant was not only surviving but also continues with various ontogenetic events such as flowering and fruiting without any appreciable disturbances. Thus these observations clearly indicated that plant synthesize certain secondary metabolites that support the survival in such stress situations. The genus *Cassia* is known for various activities.[(1)] Ethnomedicinally *Cassia auriculata* has been used for the treatment of chronic purulent ophthalmia, conjunctivitis, gout, dysentery, cough, asthma, astringent, alexeteric.[(2)] It is useful in thirst, urinary disorder and skin diseases, recommended in nocturnal emission, gargles in sore throat, in enemas, rheumatism and eye diseases.[(3)] Its decoction is given in stomachache and fresh bark juice used in dysentery. The aqueous extract of various species exhibited hypoglycemic activity.[(4,5)] In Srilanka, herbal tea prepared from dried flower of *Cassia auriculata* most frequently consumed for its beneficial effects in diabetes mellitus, constipation, and diseases of urinary tract. Additionally, plant has been investigated in terms of modern pharmacology for its oral hypoglycemic,[(6)] antioxidant[(7)] hypolipidemic,[(8)] antibacterial, antiviral[(9)] and anti-spasmodic activities.[(10)] The ethanolic extract of root protected the rat from nephrotoxicity induced by cisplastin and gentamicin.[(11)] The aqueous extract of seeds exhibited hypoglycemic activity in experimental animals.[(12)] The plant contained promising phytochemicals like flavonoids, anthracene derivatives, triterpenoids, kaempferol and β-sitosterol and auricassidin.[(13)] Tannins, saponins, polysaccharides and flavonoids like quercetin and rutin.[(14)]

The literature survey revealed that plant is promising and shown wide spectrum of pharmacological activities, thus the objective of present study was to investigate immunomodulatory potential of methanolic flowers and roots extracts of *Cassia auriculata*.

Materials and Method

Collection and authentication of the plant material: The plant material including flowers, roots and other morphological parts of *Cassia auriculata* were collected from the dry stony hilly area of village Holnanthe, Dhule district of Maharashtra, with the help of local tribes. The specimen was prepared and authenticated by Department of Botany, S.S.V.P. S's L. K. Dr. Ghogrey Science College of Dhule, Maharashtra, India. A voucher specimen (MMR-03) has been preserved for future reference. The flowers and roots were washed, cleaned; shade dried and then powder was made and passed through a 40-mesh sieve,

and kept in a well-closed container for extraction purpose.

Preparation of standardized extracts: The coarse powder of flowers and roots, 3000 gm each was extracted out by cold maceration method by using methanol (80%) as a solvent. These extracts were concentrated in rotary vacuum evaporator (Roteva-Equitron, Mumbai) under reduced pressure and then dried by vacuum dryer. Then extracts were screened for their prime phytochemical content.

Phytochemical analysis of the methanolic extracts: The conventional phytochemical tests were carried out on methanolic extracts of flowers and roots of *Cassia auriculata* and confirmed the presence of different classes of secondary metabolites like flavonoids, phenolics, and triterpenoids.[15]

Animals: Healthy Wistar albino rats of either sex weighing about 180-200 gm were used for immunomodulatory potential. Rats were fed with standard diet, water ad libitum and were housed in polypropylene cages maintained under standard condition of 12/12 hrs. of light and dark cycles. The ethical clearance was obtained by the Institutional Animal Ethics Committee R. C. Patel college of Pharmacy, Shirpur, Dist-Dhule (Maharashtra) (Registration no.651/02/c/CPCSEA) before the experiment.

Immunomodulatory activity

***In-vivo* model: Oxazolone-induced immune type of inflammation:** The effects of methanolic (80%) extracts of flowers and roots in 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone) induced immune type inflammation was studied according to the method reported.[16] Wistar albino rats with a body weight between 180-200 gm were divided into eight groups, (n=6). The rats were sensitized by application of 300 µl of 2% oxazolone to the shaved abdomen and then a total of 60 µl of 1.6% oxazolone was applied to both sides of the ear every 3 days starting from 7 days after sensitization. Ear thickness was measured using micrometer (Mitutoyo Corporation, Japan, 0.01-10.0 mm) 72 hrs after each application of oxazolone. Betamethasone valerate was applied in total volume of 60µl to both side of the ear 30 min before and 3 hrs after each application of oxazolone. The extract treated groups were given 200 and 400 mg/kg of 80% methanolic extracts of flower and root respectively, 30 min before and 3 hrs after each application of oxazolone and study was continued for 22 days. Betamethasone valerate and oxazolone (Sigma) were dissolved in ethanol to the appropriate concentrations. Oxazolone was dissolved in ethanol to a concentration of 2% for initial sensitization or in vehicle (acetone: olive oil, 4:1) to 1.6% for subsequent application to the ear. The observations are shown in Table 1.

Table 1: In-vivo oxazolone induced inflammation (Delayed type hypersensitivity)

Sr. No.	Group	Dose mg/kg p.o.	7th day	10th day	13th day	16th day	19th day	22nd day
1	Negative control	---	0.46±0.0076	0.59±0.012	0.65±0.010	0.69±0.0079	0.73±0.014	0.78±0.011
2	Positive Control [a]	60µl	0.46±0.011	0.52±0.0095**	0.50±0.012**	0.51±0.014**	0.49±0.010**	0.48±0.012**
3	CAMF	200	0.45±0.011	0.51±0.010**	0.55±0.0060**	0.60±0.0060**	0.64±0.010**	0.68±0.011**
4	CAMF	400	0.44±0.012	0.53±0.012**	0.55±0.014**	0.58±0.014**	0.62±0.019**	0.65±0.019**
5	CAMR	200	0.45±0.0095	0.55±0.017*	0.58±0.015**	0.62±0.011**	0.66±0.013**	0.71±0.0080**
6	CAMR	400	0.45±0.0067	0.56±0.010*	0.59±0.015**	0.62±0.013**	0.66±0.019**	0.69±0.019**

Values are expressed as mean ± SEM, n = 6

Data analysed by One way ANNOVA followed by Dunnette's test ** P< 0.01, * P< 0.05

CAMF - *Cassia auriculata* methanolic flowers extract.

CAMR - *Cassia auriculata* methanolic roots extract.

a- Betamethasone valerate.

***In-vitro* immunomodulatory assays**

Nitroblue Tetrazolium (NBT) test: Fresh blood sample was withdrawn from a healthy human volunteer in anticoagulant (heparin) containing tubes. PMN cells were separated from the blood sample by dextran sedimentation method. The blood was added to sterile centrifuge tubes containing equal volume of 6% w/v dextran solution in saline. The tube was set aside for 15 minutes at room temperature. The supernatant and turbid ring above the RBCs layer was collected with an auto pipette, centrifuged and the resultant pellet was washed thrice with PBS and was finally re-suspended in MEM at a concentration of 5 X 106 cells per ml.[17]

0.3% solution of NBT in sucrose and leucocytes suspension (5 X 106 cells/ ml) was prepared in MEM. It was adjusted to concentrations of all extracts ranging from 2500µg/ml, 1250µg/ml, 625µg/ml, 250µg/ml, and 125µg/ml from stock solution (10 mg/ml). The final mixture was contained MEM, PMN cells, NBT compound and extract. In positive control instead of extract endotoxin was taken.[18] All these test tubes were incubated for 20 min at 37^0C and at room temperature for further 20 minutes. At the end of the incubation, the assay tubes were manually shaken and a 20 µl of assay mixture was taken on microscopy slides and smears were prepared. The slides were then air dried, stained with Giemsa's stain and observed under oil immersion microscope (using Labomed CXL Plus Sl. Microscope, Model No-038086) for the NBT positive cell containing blue deposits of the phagocytosed NBT dye.[19] Percentage of NBT positive cells was determined as shown in Table 2.

Table 2: Effect of *Cassia auriculata* extracts on *in vitro* stimulation of Human PMN cells for NBT reduction

Sr. No.	Concentration (µg)	CAMF	CAMR
1	2500	22 ± 0.70	47.25 ± 0.47*
2	1250	41 ± 0.40*	43.75 ± 0.75*
3	625	41.25 ± 0.47*	40.75 ± 0.47*
4	250	31.50 ± 0.64*	40.50 ± 0.64*
5	125	28.50 ± 0.64*	30.00 ± 0.40*

Values are expressed as Mean ± SEM, n=4
* p<0.01 vs negative control
Negative control 21.25 ± 0.47, positive control 48.00 ± 0.40
(CAMF*) Cassia auriculata* methanolic flowers extract
(CAMR) *Cassia auriculata* methanolic roots extract

Phagocytosis: The PMN cell suspension was prepared as stated above. The count of PMN cells was adjusted approximately to 5 X 106 cells/ ml. For preparation of *Candida albicans (C. albicans)* suspension, 12-hour old, unicellular culture of *C. albicans* was used. The candida cell suspension was prepared in PBS at a concentration of about 5 X 106 cells/ ml. It was adjusted to concentrations of all extracts ranging from 2500 µg/ml, 1250 µg/ml, 625 µg/ml, 250 µg/ml, 125 µg/ml from stock solution (10 mg/ml). The final mixture contained MEM, PMN cells, *C. albicans* cells and extract. In the positive control assay unit, instead of fruit extract, 100 µl of serum derived from the same human volunteer was taken.

The assay tubes were incubated for 30 min at 37^0C. At the end of the incubation period, the tubes were centrifuged and small amounts of the residues were taken on separate microscopy slides. Smears were prepared, air dried and stained with Giemsa's stain. Neutrophils were examined for the number of ingested and associated *C. albicans* with each cell.[20,21] Average number of *C. albicans* associated PMN cell was determined for each assay unit as shown in Table 3.

Table 3: Effect of *Cassia auriculata* extracts on phagocytosis of *candida albicans* by Human PMN *in vitro*

Sr. No.	Concentration (µg)	CAMF	CAMR
1	2500	4.5± 0.28	4.00±0.0
2	1250	4.2± 0.25	3.75±0.25
3	625	2.5±0.28*	3.5±0.28
4	250	2.2±0.25**	3.5±0.28
5	125	2.75±0.25	2.75± 0.25

Values are expressed as Mean ± SEM, n=4
* p<0.05 **p<0.01
Control 3.75 ± 0.25
(CAMF*) Cassia auriculata* methanolic flowers extract
(CAMR) *Cassia auriculata* methanolic roots extract

Candidacidal assay: Same procedure and dilutions were followed as mentioned in section B-II (Phagocytosis) and the pellets of the assay tubes were again suspended in 100 µl MEM and further incubated at 37^0C for 30 minutes. At the end of the incubation period, 0.25 ml sodium deoxycholate (2.5% in PBS) was added to each tube to lyse the leucocytes. After this, 0.25 ml 0.01% methylene blue was added to each tube and mixed well. The tubes were centrifuged, supernatant was decanted and smears were prepared on microscopic slides using resultant pellets. The percentage of dead *candida* cells (stained) in each case was determined.

The time schedule was adjusted in such a way that after addition of methylene blue to an assay tube, the readings were taken within next 5 minutes as shown in Table 4.[22]

Table 4: Effect of *Cassia auriculata* extracts on in cellular killing of *candida albicans*

Sr. No.	Concentration(µg)	CAMF	CAMR
1	2500	35.25±0.47**	33.50±0.28
2	1250	35.25±0.47**	31.25±0.47
3	625	30.25±.25**	31.75±0.25
4	250	28.00±0.40**	30.50±0.28
5	125	20.00±0.40**	21.75±0.62**

Values are expressed as Mean ± SEM, n=4
* p<0.05 ** p<0.01
Control 32.00 ± 0.40
(CAMF*) Cassia auriculata* methanolic flowers extract
(CAMR) *Cassia auriculata* methanolic roots extract

Chemotaxis

Boyden's Chamber: It was simple self-constructed apparatus in which the lower chamber is a 5 ml beaker and upper chamber is a tuberculin syringe with filter to its lower end. In upper chamber in which the cell suspension is placed, this is separated by a micropore

filter (5µm) (Millipore, Cat. No. SMWP-04700) from the lower chamber, in which the chemotactic factor was placed as shown in Fig. 1.[23]

The upper compartment of Boyden's chamber was filled with cell suspension ensuring that the fluid level in the upper chamber was same as in the lower chamber, otherwise the gradient will be disturbed (0.2ml solution was used in upper compartment of Boyden's chamber). Allowed the filters to wet from the top before putting them in the lower compartment.

When the upper compartment was placed in the lower compartment, the concentration of chemotactic factor through the filter was zero and as soon as the filter was placed in the chemotactic solution, the gradient begins to form from the bottom of filter.

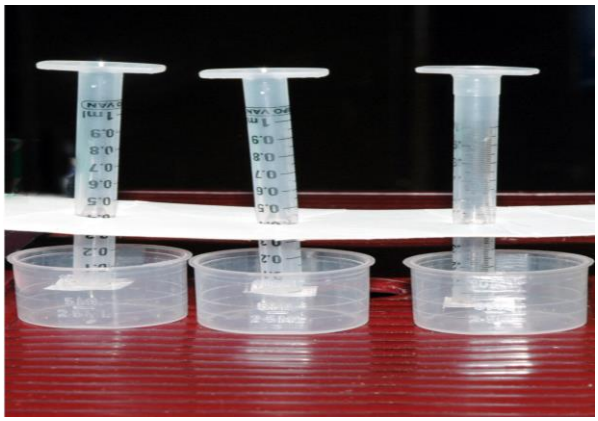

Fig. 1: Boyden's Chamber

Procedure

The upper compartments of the assay units contained PMN cell suspension in MEM at concentration of about 1 X 106 cells/ ml. The lower compartments contained 2.5 ml PBS consisting different concentrations of the methanolic flower extracts as 2500µg/ml, 1250µg/ml, 625µg/ml, 250µg/ml, 125µg/ml. In a positive control assay unit, lower compartment contained 2.5 ml PBS and 0.5 ml casein solution (5 mg/ml). The negative control assay unit contained only 3 ml of PBS in the lower compartment.[24]

All the assay units were incubated at 37^0C for 75 minutes, and the cells were allowed to migrate.

At the end of the incubation period, the filters attached to the tuberculin syringe in each assay unit were picked off gently with forceps, fixed with 70% methanol and it was stained. After a few minutes in the alcohol, the glue was melt and the filter was become loose from the bottom of the syringe barrel. While care was taken to touch only the rim and not the surface of the filter.[25]

Staining: Staining was the important step in chemotaxis.

Staining procedure was as follows:

1. Filters were transferred from methanol to distilled water then,
2. Distilled water for 1min.
3. Harris Haematoxylin for 30 sec. to 1min.
4. Distilled water for 1min.
5. Tap water for 10 min.
6. 6.70 % ethanol for 1 min.
7. Then in a mixture of:
   95 ethanol
   80 ethanol
   20 butanol for 5 min.
8. Finally in Xylol for 10 min.

And then filter was put it on slide taking care that lower surface of filter which was in lower compartment must be on upper side on the slide.

The cover slip was put on slide and examined cell migration microscopically. The count of cell on the lower surface was directly proportional to the number of cells placed on the top of the filter at the start of the experiment. The observations were recorded as shown in Table 5.

Table 5: Effect of Cassia *auriculata* extracts on human PMN cells chemotaxis.

Sr. No.	Concentration µg	CAMF	CAMR
1	2500	144.3±0.85**	68.00±0.40**
2	1250	130.8±0.47**	159.5±0.64**
3	625	131.3±0.47**	130.8±0.47**
4	250	118.8±0.47**	110.8±0.47**
5	125	110.5±0.28**	78.25±0.47**

Values are expressed as Mean ± SEM, n=4

* * $p<0.01$ vs negative control

Negative control 20.75 ± 0.47, positive control 136.00 ± 0.40

(CAMF*) Cassia auriculata* methanolic flowers extract

(CAMR) *Cassia auriculata* methanolic roots extract

Result and Discussion

The delayed type hypersensitivity reaction in the ear skin of rats which may aid in evaluating the efficacy of therapeutic modalities of newly developed anti-inflammatory drugs. In oxazolone-induced immune type of inflammation, both the methanolic extracts of flowers and roots were evaluated. The results *in-vivo* model showed methanolic flowers extract exhibited potent immunomodulatory properties in a dose dependent manner as compared to methanolic roots extract.

The NBT test has been applied widely to assist in the diagnosis of bacterial infections. Also NBT tests are carried out for the study of immunodeficiency disorders. In case of, *in-vitro* activities, as studied in NBT test, neutrophils treated with endotoxins showed an enhanced ability for reduction of Nitroblue tetrazolium (NBT) dye. These findings suggest that bacterial endotoxins modulate the functions of human blood neutrophils and lymphocytes. The main principle

in the NBT test is that only stimulated neutrophils are responsible for reduction of NBT dye and we have to measure the % of neutrophils containing blue deposits. The NBT test was performed on PMN cells from a healthy human volunteer. High spontaneous NBT reduction has been confirmed for neutrophils and assessed for monocytes. The stimulation of both neutrophils and monocytes with *E. coli* endotoxin induces a statistically significant increase of NBT positive cells. NBT reduction by neutrophils was increased after incubation and this increase was related to the concentration of extracts used. Methanolic flowers extract showed potent activity at all concentrations in NBT test than methanolic roots extract. The extent of reduction of NBT was concentration dependent. At higher concentration, methanolic flowers extract produced strong reduction of NBT compound which was, comparable to that of positive control. From these observations it is evident that methanolic flowers extract produces more stimulation of neutrophils, and consequents rise in the reduction of NBT compound.

In phagocytosis of Candida albicans by human PMN cells assay, C. albicans are recognised as foreign particle and they are engulfed by PMN cell span. In this assay, we measured the ingested and associated C. albicans with each cell. Both the extracts showed increase in PMN cell phagocytosis activity, however methanolic flowers extract was potent than methanolic roots extract.

In candidacidal assay, serum was used as standard. The mechanisms by which macrophages kill ingested microorganisms were explored using Candida albicans. The results indicate that efficient macrophage candidacidal activity depends upon the generation of oxygen metabolites by the phagocytic cell.

Methanolic flowers extract of C*assia auriculata* showed significant candidacidal activity at all concentration levels and effect was concentration dependent. At higher concentration the activity of methanolic extract was better than positive control. Methanolic roots extract also showed good candidacidal activity but less as compare to methanolic flowers extract.

Chemotaxis is concerned with, cell migration of neutrophils and macrophages into the local region of the antigenic agent and towards chemotactic substance. Bacterial endotoxin is a potent agonist for priming and stimulating leukocytes. The endotoxins (lipopolysaccharides, LPS) induced stimulation of neutrophils in suspension and the responses were dose and time dependent. Variable proportions, of the neutrophils were stimulated with different endotoxins. The methanolic flowers extract showed highest activity of chemotaxis as compared to methanolic roots extract. The maximum activity of 159.5 was recorded with methanolic roots extract at 5µg dose in comparison to 136.0 and 20.0 of positive and negative control respectively. However, the activity was appreciably reduced at higher dose 10µg. In case of methanolic flowers extract, activity shown concentration dependent increases in cell migration.

Conclusion

Since antiquity *Cassia auriculata* Linn. is promising folk medicinal plant having wide ranges of traditionally uses. In recent era, the plant has been screened through various pharmacological activities for its rationalization. The data of scientific studies supported that plant exhibited promising pharmacological activities. Importantly numbers of active phytoconstituents isolated from plant hence the plant is now achieving importance place to into pharma sector. In present study both the methanolic extracts of flowers and roots were studied scientifically by using various *in-vivo* and *in-vitro* immunomodulatory models and finally it is concluded that methanolic flowers extract exhibited potent immunomodulatory activity in comparison with methanolic roots extract. Thus, considering versatile medicinal uses of flowers there is an ample scope for flowers in future research.

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