Free Radical Scavenging and Anti-inflammatory Potential of Methanolic Extract of Flowers and Roots of *Cassia auriculata* Linn.

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ABSTRACT

Background & objectives: *The objective of present study is to find free radical scavenging and anti-inflammatory activity of methanolic flowers and roots extracts of Cassia auriculata Linn.*

Methods: *The methanolic flowers and roots extracts of Cassia auriculata Linn. were investigated for their antioxidant potential and anti-inflammatory activity by using carrageenan-induced acute inflammation and cotton pellet-induced sub-chronic inflammation in rats.*

Result: *The methanolic flowers extract of Cassia auriculata exhibited significant antioxidant and anti-inflammatory activity by reducing carrageenan and cotton pellet induced change in bio-chemical parameters. The methanolic flowers extract showed 54.20 % and 55.76% while methanolic roots extract showed 52.52% and 53.84% inhibition in carrageenan-induced, cotton pellet-induced inflammation in rats respectively. Both the extracts of flowers and roots were tested for the presence of phytochemical constituents and studies confirmed the presence of the active phytoconstituents in methanolic extract of flowers, which may interfere with free-radical formation and accounts for the significant anti-inflammatory activity.*

Interpretation & conclusions: *The present study indicates the medicinal value of Cassia auriculata in the management of inflammation.*

Keywords: *Cassia auriculata, Antioxidant, anti-inflammatory, Carageenan, cotton pellet.*

INTRODUCTION

The most of the species of genus *Cassia* are trees, shrubs or under shrubs and distributed throughout the warm part of the world. Several species of this genus are extensively used in traditional medicine in the treatment of urinary tract disorders, throat disorders and as an astringent. *Cassia auriculata* Linn. is medium-sized deciduous tree, distributed throughout the hotter parts of India, commonly known as "Tanners senna", Taravada belongs to the Leguminosae family. In India plant grows best in well-drained soil of deciduous and evergreen forest and also found abundantly in Satpuda hills of Maharashtra. It is credited with innumerable medicinal properties, since traditionally various parts of the plant are widely used as an astringent, as an anthelmintic, hypoglycemic agent, in the treatment of leprosy, ulcers, throat disorders, urinary tract disorders, skin diseases, eye diseases and also used in the treatment of asthma, rheumatism, gout and dysentery [1,2,3]. The recent studies show that flowers of plant possess anti-diabetic and hypolipidemic activity. The leaf extract can be used as hepatoprotective and antioxidant agent [4, 5]. The ethanolic extract of roots has shown nephroprotective activity in rats against cisplastin and gentamicin induced nephrotoxicity[6]. The aqueous extract of seeds exhibited hypoglycemic activity in experimental animals [7]. Phytochemically the plant is promising, contained flavonoids, anthracine derivatives triterpenoids, kaempferol and β-sitosterol and auricassidin[8]. Tannins, saponins, polysaccharides and flavonoids like quercetin and rutin [9].The plant bark contained more amount of tannins and two new triterpenoids glycosides 3β, 24s-dihydroxyurs-12-en-28-oicacid–24-O-β–D-xylopyrnoside and 3β, 24-dihydroxyurs-12-en-28-oic acid–3-O-β–D-xylopyrnoside were reportedin the bark [10]. The present study was carried out to find free radical scavenging and anti-inflammatory activity of methanolic flowers and roots extracts of Cassia *auriculata*.

MATERIALS AND METHODS

Collection of the plant material

The plant material including flowers and roots and other morphological parts of *Cassia auriculata* were collected from the dry stony hills area of village Holnanthe, Dhule district of Maharashtra, with the help of local tribes. The specimen was prepared and authenticated by Dr. D. A. Patil, Department of Botany, S.S.V.P.S's L. K. Dr. Ghogrey Science College of Dhule, Maharashtra, India. A voucher specimen (MMR-33) has been preserved for future reference. The flowers and roots were washed, cleaned, shade dried and powdered and

passed through a 40-mesh sieve, and kept in a well-closed container for further extraction.

Preparation of standardized extracts

The coarsely flowers and roots powdered of *Cassia auriculata* 3000 gm each was extracted out by cold maceration method by using methanol (80%). These extracts were concentrated in rotary vacuum evaporator (Roteva-Equitron, Mumbai) under reduced pressure and then dried by vacuum dryer. The total phenolic and flavonoid content of the extracts was determined[11]. The mean of three readings was used and the total phenol content and total flavonoid content was expressed in milligram of gallic acid equivalents/g extract and quercetin equivalents/g extract, respectively (Table 1).

Phytochemical analysis of the methanolic extract

The conventional phytochemical tests were carried out on flowers and roots extract of *Cassia auriculata* and it confirmed the presence of different classes of secondary metabolites flavones, flavonoids, phenolics, quinones, and triterpenoids in methanolic flowers extract.

Presence of catechin in *Cassia auriculata* methanolic flowers extract (CAMF) extract was confirmed by HPLC using catechin (Sigma-Aldrich Chemie, Steinheim, Germany) as the standard marker. Shimadzu HPLC system with LC-10AT, UV detector (Spectra system UV1000), Luna C18 reverse-phase column (250 x 4.6 mm, i.d. particle size 5µm) was used. Mobile phase consisted of Acetonitrile: Water (80:20) mixture with flow rate 0.3 ml/min at 25ºC and detection wavelength was set at 280 nm. Data was acquired and analyzed using chromquest version 3.0 software. Retention time of peak at 280 nm was used to identify catechin in CAMF extract.

Animals

Healthy Wistar albino rats of either sex weighing about 180-200 gm were used for the anti-inflammatory study. They were fed with standard diet, water *ad libitum*. Animals were housed in polypropylene cages maintained under standard condition of 12/12hrs.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.

***In vitro* assay**

Antioxidant potential of *Cassia auriculata* extracts were assessed with DPPH radical-scavenging[12,] ABTS radical cation scavenging[13,] phosphomolybdenum[14,] and Superoxide radical-scavenging assay (Riboflavin-Light-NBT System).

DPPH radical scavenging: Scavenging of the stable DPPH radical (Methanolic solution of 100mM) was assayed *in vitro* and the absorbance was measured at 517 nm. Percentage inhibition was calculated with respect to control. Ascorbic acid and silymarin were used as a standard compound in DPPH assay (Table 2).

Total antioxidant capacity: The total antioxidant capacity of the extracts was estimated using the phosphomolybdenum reduction assay[14]. The antioxidant capacity of the extracts was expressed as the ascorbic acid equivalent (AAE) and silymarin equivalent (SE) (Table 2).

Superoxide radical-scavenging activity by Riboflavin-Light-NBT System: The super oxide free radical scavenging activity was carried out[15]. Reaction commenced by illuminating the reaction mixture for 15 minutes using fluorescent lamp. After illumination, the absorbance was measured at 590 nm. Ascorbic acid and silymarin were used as a standard compound and percent inhibition was calculated (Table 2).

ABTS radical cation scavenging activity: ABTS radical cation scavenging activity was performed[16]. The absorbance was measured at 734 nm after 5 min of reaction. The percentage inhibition was calculated from the control (Table 2).

Acute toxicity: Acute toxicity studies of *Cassia auriculata* extracts were determined in albino mice according to OECD guidelines No. 425,[17]. The animals were fasted overnight. One group was maintained as control and was given 0.5% Tween-80. The test extracts was administered orally with an initial dose 2000 mg/kg. They were observed continuously for 1 h for any gross behavioral changes and death, if any, and then, intermittently for the next 6 h, and then again at 24 h after dosing with test extracts.

***In vivo* anti-inflammatory activity**

Carrageenan-induced rat paw edema: Wistar albino rats with a body weight between 180-200 gm were divided into six groups (n=6), the animals were starved overnight before the experiment to ensure uniform hydration; the rats received water ad libitum. The effect of methanolic flowers extract and methanolic roots extract were investigated in carrageenan-induced paw edema[18,19,20]. The edema was induced by injecting 0.1 ml of 1% carrageenan (Sigma Chemical Co.) in distilled water into the subplanetor tissue of the right hind paw of each rat[21,22]. These extracts were

administered orally, 200, 400 mg/kg, 30 min prior to carageenan administration. The paw volume was measured at 60,120,180 and 240 min using the plethysmometer (Ugo Basile, Italy). The percentage inhibition of paw volume in extracts-treated groups compared with the control group (treated with vehicle) and diclofenac sodium (5 mg/kg, p.o) was used as reference standard [23, 24,] (Table 3).

Cotton pellet granuloma test (cotton pellet method): The effect of methanolic flowers and roots extracts were studied in chronic inflammation by using cotton pellet-induced granuloma test in rats[25]. Wistar albino rats with a body weight between 180-200 gm were divided into six groups (n=6), Cotton pellets weighing 20±1 mg were autoclaved and implanted subcutaneously into both sides of the groin region of each rat. Control group received the normal saline, 5 ml/kg p.o. The methanolic flowers and roots extracts at concentration of 200, 400 mg/kg were administered orally for 7 days. Standard treated group received phenylbutazone, 150 mg/kg, p.o. for same period. On the 8th day, the animals were sacrificed and the pellets together with the granuloma tissue were carefully removed, dried in an oven at 60^0C, weighed and compared with control (Table 4).

Table 1: The extract yields and total phenolic (gallic acid equivalent) and total flavonoid (quercetin equivalent) compounds in CAMF and CAMR extracts

Plant extract	Total phenolic (mg/g)	Total flavonoids (mg/g)	Extract yield (%w/w)
CAMF	277.3 ± 3.3	5.4 ± 0.2	3.7 ± 0.6
CAMR	205.37± 7.3	3.2 ± 0.5	5.4 ± 0.8

Values are mean ± SEM, n = 3
CAMF- *Cassia auriculata* methanolic flowers extract
CAMR- *Cassia auriculata* methanolic roots extract

Table 2: Antioxidant effect (EC50) on free DPPH radicals, superoxide radicals, ABTS radicals and total antioxidant capacity of CAMF and CAMR extracts

Plant extract	EC50 (lg/ml)			Total antioxidant activity
	Scavenging activity on DPPH radicals	Scavenging activity on ABTS radicals	Scavenging activity on Superoxide	
CAMF	59 ± 0.5	33.7 ± 0.3	52.3 ± 0.3	47.9 ± 0.5
CAMR	81.3 ± 0.6	44.1 ± 0.2	50.1 ± 0.3	25.7 ± 0.8
Silymarin	54.3 ± 3.3	25 ± 0.5	54.3 ± 0.4	76.2 ± 2.5
Ascorbic acid	43.3 ± 0.7	14.4 ± 0.02	23.73± 0.05	

CAMF- *Cassia auriculata* methanolic flowers extract
CAMR- *Cassia auriculata* methanolic roots extract

Anti-inflammatory activity

Table 3: The effect of various extracts of *Cassia auriculata* in carrageenan induced inflammatory in rats

Groups (mg/kg, p.o.)	**Carrageenan induced rat paw edema Volume in ml (% Inhibition)**				
	0	60	120	180	240
Control 5 ml saline	1.10 ± 0.013	1.53± 0.021	1.99± 0.016	2.38 ±0.015	2.57 ± 0.015
Diclofenac 150	1.06 ± 0.029	1.27± .024** (16.99)	1.18 ±0.019** (40.70)	1.07±0.028**(55.04)	1.17 ±0.033** (54.47)
CAMF 200	1.09 ± 0.030	1.28 ±0.039** (15.03)	1.26 ±0.055** (36.68)	1.15 ±0.060** (51.68)	1.28 ±0.021** (50.19)
CAMF 400	1.06± 0.036	1.30 ±0.040** (15.03)	1.20 ±0.021** (39.69)	1.09 ±0.034** (54.20)	1.17 ±0.037** (54.47)
CAMR 200	1.12 ± 0.015	1.33 ±0.029** (13.07)	1.30 ±0.026** (34.67)	1.18 ±0.018** (47.43)	1.36 ±0.014** (47.08)
CAMR400	1.08 ± 0.014	1.32 ±0.026** (13.72)	1.22 ±0.034** (35.67)	1.13 ±0.015** (52.52)	1.24±0.023** (51.95)

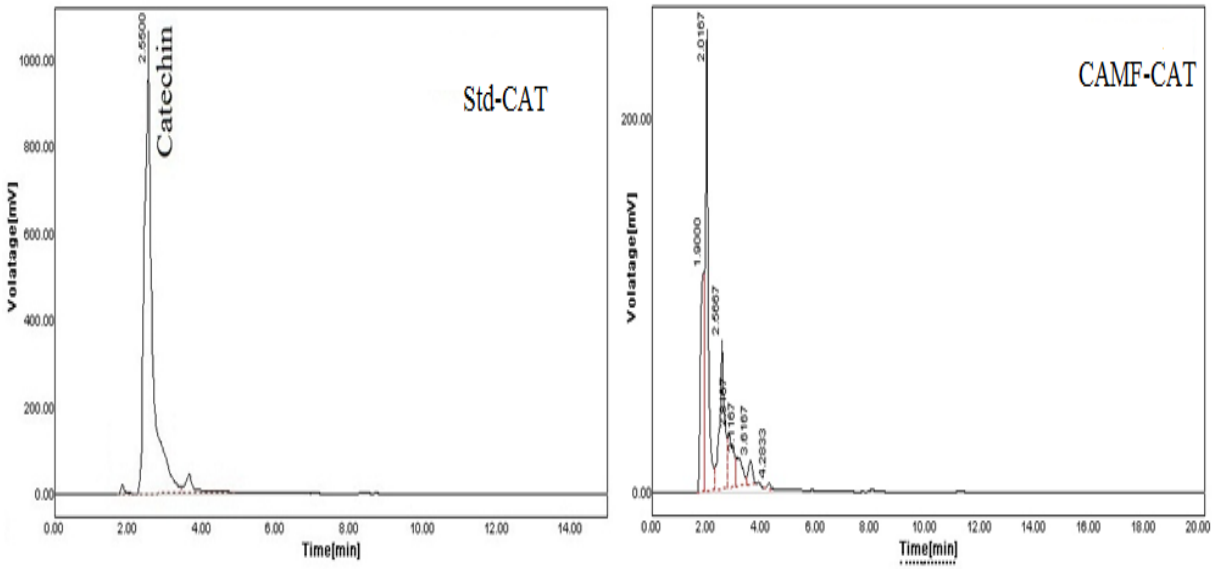
Values are expressed as mean ± SEM, n = 6
Data analysed by One way ANNOVA followed by Dunnette's test
** P< 0.01, * P< 0.05
CAMF- *Cassia auriculata* methanolic extract of flowers
CAMR- *Cassia auriculata* methanolic extract of roots

Table 4: The effect of various extracts of *Cassia auriculata* in chronic inflammatory in rats

Groups	Dose mg/kg p.o.	Weight of dry cotton pellet granuloma (mg)	% Inhibition
Control	Saline5 ml	80 ± 2.8	----------
Phenylbutazone	150	34 ± 1.6**	57.05
CAMF	200	38 ± 1.2**	51.96
CAMF	400	35 ± 2.2**	55.76
CAMR	200	43 ± 2**	45.74
CAMR	400	37 ± 1.4**	53.84

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

Fig. 1: HPLC chromatogram of an authentic standard catechin (CT), b catechin identified in CAMF extract



RESULTS

Standardized extracts of the *Casssia auriculata* showed significant amount of total phenolics and flavonoids. The total phenolic and flavonoid content of *Cassia auriculata* extracts was found in same pattern. The CAMF extract showed highest phenolic and flavonoid content as compared to CAMR extract (Table1).

The extracts exhibited significant antioxidant activity in the DPPH, ABTS and Superoxide radical scavenging assays. The CAMF extract showed maximum scavenging ability as compared to CAMR extract and comparable to the reference standard ascorbic acid and silymarin. Further HPLC analysis of the CAMF extract confirmed the presence of an important polyphenolic constituent, catchine (Rt = 2.5 min) (Fig. 1).

The extracts exhibited significant antioxidant activity in the DPPH and ABTS assays. The EC_{50} values obtained for DPPH and ABTS scavenging of the CAMF extract were 59 ± 0.5 and 33.7 ± 0.3 lg/ml, which was found to be the smallest among all the extracts and comparable to the silymarin and reference standard ascorbic acid (EC_{50} = 54.3 ± 3.3, 25 ± 0.5, 43.3 ± 0.7, and 14.4 ± 0.02 lg/ml, respectively). The total antioxidant capacity of the silymarin and CAMF (equivalent to ascorbic acid) was found to be the highest of all the extracts (76.2 ± 2.5and 47.9 ± 0.5lg/ml at 1000 lg/ml), and was concentration-dependent. In the superoxide radical scavenging assay, CAMF showed maximum scavenging ability as compared to CAMR extract.

Administration of carrageenan (0.1 ml, 1%, p.o.) in rats induced a marked increase in the paw volume as compared to normal controls indicating paw volume. Pre-treatment of the rats with CAMF and CAMR extracts at doses of 200 and 400 mg/kg prior to carrageenan administration caused a significant change in the volume of paw in a dose dependent manner. However, CAMF extract 400

mg/kg showed highest anti-inflamatory activity (Table 3) almost comparable to the diclofenac (5mg/kg) treated group.

While in cotton pellet-induced sub-chronic inflammation, Cotton pellets weighing 20±1 mg were autoclaved and implanted subcutaneously into both sides of the groin region of each rat. The CAMF and CAMR extracts at doses of 200 and 400 mg/kg administerd orally for seven days. However, CAMF extract 400 mg/kg showed highest anti-inflamatory activity (Table 4) almost comparable to the phenylbutazone (150mg/kg) treated group.

DISCUSSION AND CONCLUSION

The antioxidant and free radical scavenging potential of CAMF extract is more likely to be attributed to high total polyphenolic content and different chemical compounds present in them as confirmed by phytochemical analysis. The scavenging action against various radicals and inhibition of inflammation by CAMF extract may be due to the aforementioned phenolics. The free radical scavenging activity of the crude drug extracts was evaluated by DPPH assay. The DPPH radical scavenging activity of the ethyl acetate extract revealed high antioxidant activity considering the fact that quenching properties were obtained only from the crude extract. Scavenging of DPPH radical is related to the inhibition of lipid peroxidation[26, 27].

The phosphomolybdenum assay is a quantitative method to evaluate water-soluble and fat-soluble antioxidant capacity (total antioxidant capacity). The CAMF extract demonstrated electron-donating capacity showing its ability to act as chain terminators, transforming relative free radical species into more stable non-reactive products[28].

Superoxides are produced from molecular oxygen due to oxidative enzymes of body as well via non-enzymatic reaction such as auto-oxidation by catecholamines. The scavenging activity towards the superoxide radicals ($O_2^{\cdot -}$) is measured in terms of inhibition of generation of $O_2^{\cdot -}$. In present study, superoxide radical reduces NBT to blue colored formosan that is measured at 590 nm. The result shows that CAMF extract has potent scavenging activity with increasing percentage inhibition. The probable mechanism of scavenging the superoxide anions may be due to the inhibitory effect of extracts towards generation of superoxide in the *in vitro* reaction mixture.

$ABTS^{\cdot +}$ is a blue chromophore produced by the reaction between ABTS and potassium persulfate. Addition of the plant extract to this pre-formed radical cation reduced it to ABTS in a concentration-dependent manner. The results were compared with those obtained using ascorbic acid and the EC_{50} value demonstrates that the CAMF extract is a potent antioxidant.

The lipid peroxidation is accelerated when free radicals are formed as the results of losing a hydrogen atom from the double bond in the structure of unsaturated fatty acids. Scavenging of free radicals is one of the major antioxidation mechanisms to inhibit the chain reaction of lipid peroxidation. Keeping in view the high polyphenolic content and distinct radical scavenging properties of CAMF and CAMR extracts, the studies were further extended to in vivo conditions using carrageeenan and cotton pellets induced inflammation in rats.

The inflammatory response involves many effector mechanisms which produce a multiplicity of vascular and cellular reaction and thus requiring various models to characterize the exact inhibitory role of drug in question. It was also reported that the anti-inflammatory effect of several agents result in the partial inhibition of inflammation mediator release [29]. Carageenan-induced paw edema is widely used for determining the acute phase of inflammation, which again gives characteristic biphasic response. The early phase lasts 60 min and is associated with release of histamine, serotonin and bradykinin. Late phase occurs within 60 min after carageenan injection and lasts for 3 h. This phase is associated with neutrophils originated free radicals, such as hydrogen peroxide, superoxide, and hydroxyl radicals, as well as prostaglandin release [30]. It is evident from results (Table 3) that CAMF is effective in both early as well as late phase of inflammation and gives dose-dependent inhibition of rat paw edema. The maximum inhibition of 54.69% and 52.52% was given by CAMF and CAMR, 3 and 4 h respectively after carrageenan administration.

The chronic inflammation is a reaction when the acute response is insufficient to eliminate pro-inflammation agent. The chronic inflammation induces proliferation of fibroblast and the infiltration of neutrophils and exudation. The cotton pellet granuloma method is frequently used to investigate the proliferative phase of inflammation. It is evident from cotton pellet granuloma test (Table 4) that CAMF and CAMR at the dose of 400 mg/kg have 55.76% and 53.84% inhibition respectively in comparison to the 57.05% inhibition demonstrated by phenylbutazone at 150 mg/kg. Both the extracts shown dose-dependent inhibition and significant effects were observed at all level of dose range tested. Similar to acute inflammation, CAMF was found more potent than CAMR in chronic inflammatory condition. Therefore, data presented here indicate that CAMF might affect interferon-γ pathway leading to inflammatory events. To elucidate correct mechanism of inhibition, however, further study is anticipated.

Thus, it can be concluded that possible mechanism of anti-inflammatory activity of *Cassia auriculata* may be due to its free radical-scavenging

and antioxidant activity, which may be due to the presence of flavonoids and phenolic compounds in the extracts. It is further concluded that CAMF extract with maximum inhibition of free radicals is the most potent extract as compared to CAMR extract. Our findings support the reported therapeutic use of this plant as an anti-inflammatory agent in Indian system of medicine. Further studies are required for better understanding of the mechanism of action.

ACKNOWLEDGEMENT

Authors would like to thank Dr.R.B.Jadhav for his precious guidance. Authors also would like to thank Dr.D.A.Patil, who authenticated plant material. The authors are thankful to Dr.S.J.Surana, the principal, R.C.Patel Institute of pharmaceutical Education and Research, Shirpur, Dist. Dhule and Dr. A.S.Jain, the principal, Shri. D. D. Vispute College of Pharmacy & Research Center, Devad-Vichumbe, Near New Panvel for providing facilities.

CONFLICT OF INTEREST

There are no competing interests amongst authors. The authors declare that there are no conflicts of interest.

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