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IN *VITRO* ANTIOXIDANT AND *IN VIVO*-ANTI-INFLAMMATORY ACTIVITY OF *CASSIA SOPHERA* LINN

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ABSTRACT

Cassia sophera (CS), from Caesalpiniaceae, is a shrub found in the topics. In India it has been popularly used for the treatment of inflammatory diseases, asthma and diabetes. Dried herbal parts of CS were first extracted with 95% ethanol (CSEXT) and subjected for sequential fractionation with different solvents such as chloroform (CSF1), ethyl acetate (CSF2), and eventually with ethanol (CSF3). Different fractions demonstrated the presence of anthraquinones and flavonoids. In this study, we have evaluated the effects of different fractions of CS in antioxidant activity (DPPH, Reducing power, hydroxyl radical scavenging activity) and anti inflammatory (carrageenan induced edema), analgesic (acetic acid induced writhes) activities. CS fractions demonstrated in vitro scavenging of hydroxyl (24- 95%) and DPPH radicals (IC_{50} 0.9- 0.2 mg/ml**)**. The reducing power of CSF3 found to be concentration dependent. All fractions showed significant ($p<0.05$) and dose dependent anti inflammatory activity, as indicated by a reduction in carrageenan induced paw edema highest activity was exhibited by CSF3, similarly CSF3 showed greater protection (69.7%) than other fractions of CS against acetic acid induced writhing. The results revealed that CS contains substances like flavonoids which modulate the oxidative stress, might have shown antioxidant, anti inflammatory and analgesic activity.

Keywords: *Cassia sophera;* 1, 1-Diphenyl-2-picrylhydrazyl (DPPH); Carrageenan; Anti-inflammatory activity**;** Antioxidant activity.

INTRODUCTION

It is commonly accepted that in a situation of oxidative stress, reactive oxygen species (ROS) such as super oxide (O_2^-), hydroxyl (OH^-) and peroxyl radicals are generated. The ROS play an important role in the pathogenesis of various serious diseases, such as neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis, cataracts, and inflammation[1].

The use of traditional medicine is widespread and plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs. Several examples have revealed the fact that the plants which contain antioxidant potential demonstrate the beneficial effects in inflammatory diseases e.g. *Ledum groenlandicum* extracts possess antioxidant and anti-inflammatory activities, which supports its ethnopharmacological use[2]. Administration of *Pteleopsis suberosa* Engl. which possess antioxidant activity significantly reduced carrageenan-induced paw edema in dose-dependent manner [3].

Cassia sophera is medicinally important plant belonging to family caesalpiniaceae. This plant was selected for this study, because of their use in local traditional medicine for the treatment of inflammatory diseases, psoriasis, cough, arthritis, diabetes and convulsions of children[4]. The chemical analysis of the seed of *C. sophera* revealed the presence of ascorbic acid, dihydroascorbic acid and β-sistosterol[5]. *C. sophera* has shown inhibitory activity against ringworm[6]. The seed extracts of *C. sophera* were reported to having hepatoprotective activity in rats (Bilal et al., 2005). Aqueous and methanol extracts of seeds of *C. sophera* was shown to exhibit significant hypoglycemic activity against alloxan diabetic rabbits. Relatively, little work has been done on the phytochemistry of *C. sophera*. Antioxidant principles like Flavone-8-C-glycoside and anthraquinone

have been identified in leaves of *C. sophera*[7, 8, 9]

Taking into consideration the traditional claim of CS being used in inflammatory diseases and the chemical constituents which may prove beneficial in reducing oxidative stress; the present study was planned to investigate the antioxidant and anti inflammatory activity of different fractions of CS.

MATERIALS AND METHOD

Plant material

The leaves of CS were collected from Junner (Pune) district of Maharashtra state, India and identified at the Botanical serve of India, Pune. The sample of plant has been deposited in the department of Pharmacognocy at Dr. D.Y. Patil I.P.S.R., Pune.The voucher specimen no of plant is SSDH-1. Leaves were shade dried and coarsely ground to make powder. The powdered material was extracted with 95% ethanol, dried and then dried extract was adsorbed on silica gel (60-120) and fractionated successively with chloroform, ethyl acetate and finally with ethanol by using soxhlet apparatus.

Animals

Swiss albino mice of either sex (20-25 g) were used for present study. Animal were kept under a 12 h light/ 12 dark cycle, with free access to food and water.

Phytochemical studies

All the fractions (CSEXT, CSF2, CSF1, and CSF3) were subjected for qualitative chemical estimation to asses major phytochemical entity and flavonoids, glycosides were fond to be presents.

Acute toxicity studies

The acute toxicity studies for extracts and fractions of leaves were performed as per OECD guideline 423 by using albino mice. Overnight fasted mice of either sex (18-23 g) were administered with different fractions of CS at 5000 mg/ kg, p.o. After 24 hrs no mortality was found. 250, 500, and 750 mg/ kg, p. o. doses were selected for the further study.

Evaluation of anti-oxidant activity

DPPH radical scavenging activity

The ability of the extracts to scavenge DPPH radicals was determined by using following method[10]. 50 µl aliquot of each extract, in 50 mm Tris-HCl buffer (pH 7.4), was mixed with 450 µl of Tris–HCl buffer and 1.0ml of 0.1mM DPPH in methanol. After 30 min incubation in darkness and at ambient temperature, the resultant absorbance was recorded at 517 nm. The percentage inhibition was calculated.

Hydroxyl radical scavenging activity

The degradation of Deoxyribose generated by Fenton reaction was measured spectrophotometrically in the presences and absence of test compound[11]. The final reaction mixture in each test tube consisted of 0.3 ml each of Deoxyribose (30 mM), ferric chloride (1mM), EDTA (1 mM), H_2O_2 (20mM), in the phosphate buffer having pH 7.4 and 0.3 ml of test compound at different concentration. the test tube were incubated for 30 min at 37^{o}C after incubation, trichloro acetic acid (0.5 ml, 5%) and the thiobarbeturic acid (0.5 ml, 1%) were added and the reaction mixture was kept in boiling water bath at 30 min. it was then cooled and the absorbance was measured at 532 nm. The result was expresses as a % of scavenging of hydroxyl radical.

Reducing power activity

The reducing power of CS fractions was determined[12]. Extracts at different concentrations in 1 ml of distilled water were mixed with 2.5 ml of phosphate

buffer (0.2M, pH 6.6) and 2.5 ml potassium ferricyanide [K_3Fe (CN) $_6$] (1%), and then the mixture was incubated at 50^0C for 30 min. Afterwards, 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of upper layer solution was mixed with 2.5 ml distilled water and 0.5 ml $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Evaluation of anti-inflammatory activity

Carrageenan induced paw edema in mice

The previously described method of carrageenan-induced paw edema assay in mice was used in this work[13]. Paw edema was induced in the hind paw of mice by the subplantar injection of 50 µL of carrageenan, (1 % w/v). The contralateral paw was injected with the same volume of the vehicle and used as control. The course of the edema was monitored by measuring the thickness of footpad swelling at 1, 2, 3 and 4 h after carrageenan injection by using a vernier caliper. Animals received dose of extract and all fraction (250 mg/kg, 500 mg/kg 750 mg/kg p.o.), or distilled water (control animals), 1hrs before the carrageenan administration. Indomethacin (10 mg/kg) was used for animals of standard group.

Evaluation of analgesic activity

Acetic acid induced writhing in mice

The previously described method of acetic acid induced writhing in mice was used in this work[14]. In the writhing test 0.6 % of acetic acid (10 ml/kg i.p.) was injected and number of writhes and stretching with a jerk of the hind limb were counted for a period of 15 min. Animals received dose of CS extract and all fractions (250 mg/kg, 500 mg/kg 750 mg/kg p.o.) or distilled water (control animals). Acetylsalicylic acid (25 mg/kg p.o) were administered orally, 30 min before acetic acid injection, for animals of standard group.

Formalin induced pain

Pain was induced by injecting 0.05 ml of 2.5% formalin in distilled water in the sub-plantar of the right hind paw of rats[15]. Animals received dose of CS extract and all fractions (250 mg/kg, 500 mg/kg 750 mg/kg p.o.), indomethacin (10mg/kg), and 1% CMC (p.o.) 30 min prior to injecting formalin. The amount of time spent licking the injected paw was indicative of pain. The number of lickings from 0 to 5 min (first phase) and 15–30 min (second phase) were counted after injection of formalin. These phases represented neurogenic and inflammatory pain responses, respectively (Hunskaar and Hole, 1987).

Statistical Analysis

The results are presented as Mean and ± S.E.M. The statistical significance of differences between the groups was obtained using analysis of variance (ANOVA) complemented by Dennett's test. P-values less than 0.05 and 0.01 considered to be significant. IC_{50} value was determined to be the effective concentration at which free radicals were scavenged by 50%. The IC_{50} value was obtained by interpolation from linear regression analysis.

RESULTS

For present study leaves of CS were used to extract with (95%) ethanol. 30 gm of ethanol extract was used for further fractionation. The extract was fractionated first with chloroform and then ethyl acetate remaining was washed with ethanol and considers being ethanol fraction. After fractionation, the percentage yield of chloroform, ethyl acetate, ethanol

fraction was found to be (16.14%), (3.1%), (8.35%) respectively.

DPPH radical scavenging activity

All the fractions were capable of scavenging DPPH radicals at pH 7.4 in a dose-dependent fashion. From the estimated IC_{50} values, that CSF3 was the most potent scavenger followed by CSEXT, CSF2 and CSF1 (Table-1). The results of the DPPH radical scavenging assay reveal that these fractions, especially CSF3 was most capable of scavenging free radicals(IC_{50}= 0.2 ± 0.1 mg/ml) in solution at pH 7.4 and may prevent initiation of free radical-mediated chain reactions by preventing the abstraction of hydrogen from susceptible substrate in oxidative reactions.

Table 1: DPPH radical scavenging activity

GROUPS	IC_{50} (mg/ml)
CSEXT	0.9 ± 0.2
CSF1	0.7 ± 0.1
CSF2	0.4 ± 0.1
CSF3	0.2 ± 0.1

Where, CSEXT- Ethanol extract of CS, CSF1- Chloroform fraction, CSF2- Ethyl acetate fraction, CSF3- Ethanol fraction.

Hydroxyl radical scavenging activity

Hydroxyl radicals are very reactive, can be generated in biological cells to the Fenton reaction. CS fractions exhibited concentration dependant scavenging activity against hydroxyl radical generated in Fenton reaction system (Table-2). CS treatment demonstrated scavenging of hydroxyl radicals ranging from 24.46 % to 95.46%.

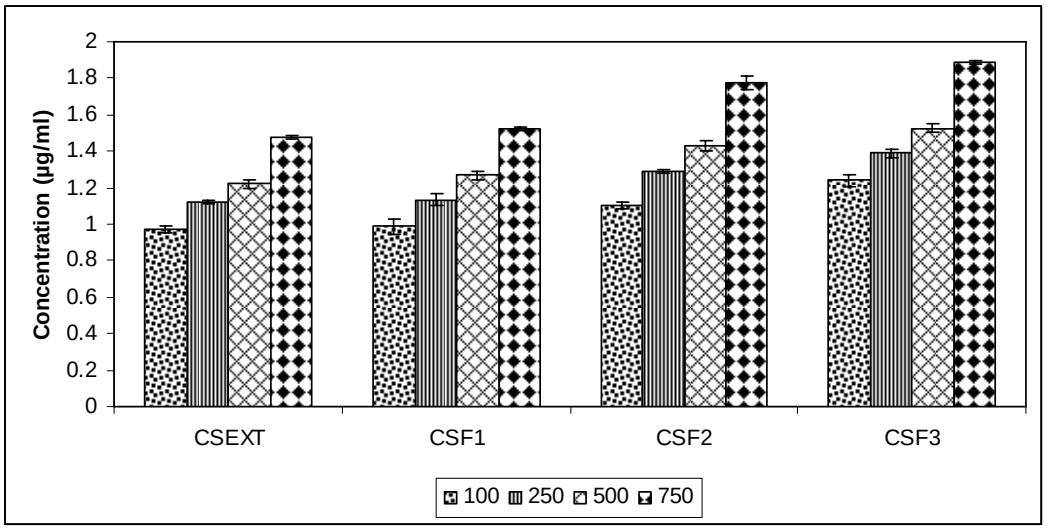
Table 2: Hydroxyl radical scavenging assay.

Concentration (µg/ml)	% Scavenging of Hydroxyl Radical			
	CSEXT	CSF2	CSF1	CSF3
10	34.61±0.61	26.19 ±1.82	24.46±2.25	35.15±1.41
50	55.09±0.27	49.65±1.32	47.55±1.58	57.51±1.19
100	76.51±0.22	66.38±1.93	65.79±1.35	78.50±1.72
250	80.96±0.18	75.59±0.98	74.69±0.87	83.23±1.18
500	85.11±0.70	81.50±1.35	80.59±1.94	86.22±1.54
750	92.70±0.29	89.45±2.184	88.21±1.44	93.92±1.27
1000	94.79±0.21	92.07±1.99	90.84±1.91	95.46±1.22

Where, CSEXT- Ethanol extract of CS, CSF1- Chloroform fraction, CSF2- Ethyl acetate fraction, CSF3- Ethanol fraction.

Reducing power assay

For the measurements of the reductive ability of CS, the Fe^{3+} – Fe^{2+} transformation was investigated in the presence of CS fractions. In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of each antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form[16]. The reducing power of CS fractions enhanced with increasing concentration of samples. The absorbance of the samples increased together with the reducing power (Fig.1). The reducing power of CSF3 was found to be concentration dependent and showed significantly higher than that of the control ($p<0.05$). The reducing power of fractions was found to be in the order like CSF3 > CSEXT > CSF2 > CSF1.

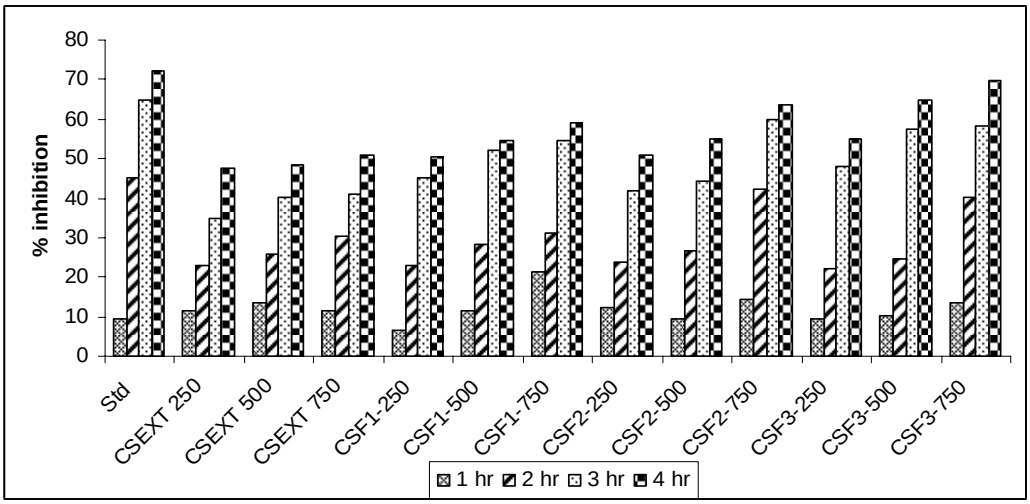


Results are presented as mean ± SEM. Where, CSEXT- Ethanol extract of CS, CSF1- Chloroform fraction, CSF2- Ethyl acetate fraction, CSF3- Ethanol fraction.

Fig. 1: Reducing power of different fractions of *CS*

Carrageenan induced paw edema in mice

When compared with the control, treatment with CS, significantly ($P<0.05$) reduced the paw edema from 2nd hrs after Carrageenan injection. Pretreatment with CS fractions (250, 500 and 750 mg/kg) showed a dose-dependent effect. There was significant activity showed by CSF3 fraction than other fractions such as CSEXT, CSF2, and CSF1. However, 10 mg/kg indomethacin significantly suppressed paw edema from 2nd hrs and remains significant up to the 4th hr. The determination of inhibition percentage showed (Fig 2) that administration of CSF3 at the dose of 750 mg/kg produced a comparable effect with indomethacin (69.7% and 72.1% respectively) 4 hr after carrageenan injection.



Where, CSEXT- Ethanol extract of CS, CSF1- Chloroform fraction, CSF2- Ethyl acetate fraction, CSF3- Ethanol fraction.

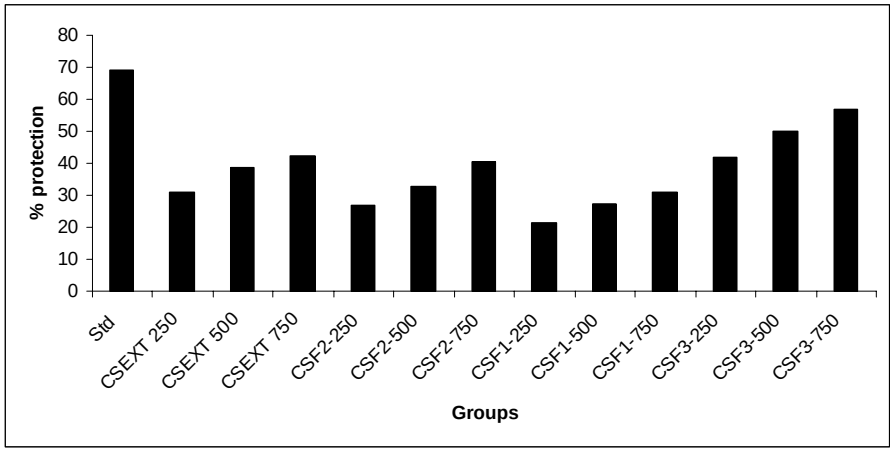
Fig. 2: Carrageenan induced paw edema in mice

Acetic acid induced writhing

CS fractions showed inhibition of the writhing response induced by acetic acid dose dependant (250, 500 and 750 mg/kg) (Fig. 3), which resulted in greater inhibition of stretching episode, when compare with the control. CSF3 showed greater protection (56.7%) than other fractions of CS. However, acetylsalicylic acid demonstrated 69.2 % protection against writhing in mice.

Formalin induced pain

CS extract and its fractions had analgesic effects on both first (0–5 min) and second phases (15–30 min) of formalin induced pain. These phases corresponded to neurogenic and inflammatory pains, respectively. CSF3 inhibited both, neurogenic and inflammatory pain at $P < 0.05$ at every dose level whereas higher doses of CSEXT, CSF1, CSF2 significantly $P < 0.05$ blocked the inflammatory pain. Indomethacin showed highest activity in blocking inflammatory pain and did not show significant activity in neurogenic pain. CSF3 was found to inhibit the pain resulting from inflammation better than the neurogenic induced pain.



Where, CSEXT- Ethanol extract of CS, CSF1- Chloroform fraction, CSF2- Ethyl acetate fraction, CSF3- Ethanol fraction.

Fig. 2: Acetic acid induced writhing

Table 3: Formalin induced pain

Groups	Pain Score	
	0-5 min	15-30 min
	65.12 ± 2.43	101.98 ± 2.19
Indomethacin	58.76 ± 3.21	55.22 ± 2.17**
CSEXT 250	62.34 ± 1.98	100.29± 3.10
CSEXT 500	55.67± 2.09	92.49 ± 3.16
CSEXT 750	54.22 ± 2.11*	87.16 ± 3.16**
CSF1 250	64.56 ± 1.92	97.12 ± 4.19
CSF1 500	61.23 ± 2.66	94.23± 2.98
CSF1 750	59.01 ± 1.02	89.10± 1.09*
CSF2 250	62.45 ± 3.19	96.30± 1.94
CSF2 500	58.82 ± 2.87	90.13± 2.63*
CSF2 750	56.24± 3.22	88.71 ± 2.87*
CSF3 250	55.19 ± 1.09*	86.19± 2.16**
CSF3 500	52.56 ± 2.11 **	73.27± 2.77**
CSF3 750	49.22 ± 2.19**	66.19 ± 3.71**

Results are presented as mean ± SEM. (n=6), ANOVA followed by Dunnett test. *p<0.05, **p<0.01 when compared with Control.

DISCUSSION AND CONCLUSION

All the fractions under evaluation of anti-inflammatory action were subjected to phytochemical study and showed to contain flavonoids. Flavonoids are one of the most numerous and widespread groups of phenolic in plants, exhibiting a range of biological and pharmacological effects such as antioxidant and anti-inflammatory[17]. In this study, extracts and fraction were subjected for phytochemical screening and biological activity. CSF3 and CSEXT were found to be more active in both *in vitro* antioxidant and *in vivo* anti-inflammatory and analgesic tests.

Inflammatory diseases are accompanied by the chronic release of cytokines and reactive oxygen and nitrogen species, which may be involved in increased tissue injury[18]. In acute and chronic inflammation, high concentrations of reactive oxygen species (ROS) are produced which generate an oxidative imbalance and decrease the capacity of the endogenous antioxidant enzymes such as super oxide dismutase (SOD) which may contribute to tissue necrosis. Some important pro-inflammatory roles for O_2^{--} include endothelial cell damage and increased micro vascular permeability, formation of chemo tactic factors such as leukotriene B4, oxidation and DNA single strand damage. In living organisms various ROS can form in different ways, including normal aerobic respiration, stimulated polymorph nuclear leukocytes and macrophages, and peroxisomes. These appear to be the main endogenous sources of most of the oxidants produced by cells. Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents, and pesticides[19]. Free radicals involved in the process of lipid peroxidation are considered to play a cardinal role in numerous chronic pathologies.

Considering the role of oxidative stress and ROS generation in the pathophysiology of inflammation, the fractions were assessed against DPPH radical serving as the oxidizing substrate, which can be reduced by an antioxidant compound to its hydrazine derivative *via* hydrogen donation, and as the reaction indicator molecule[20]. The potential hydroxyl radical scavenging ability of CS fraction might be due to active hydrogen donor ability of hydroxyl substitution and presence of the constituents with high molecular weight and proximity of many aromatic rings and hydroxyl groups in the structure of flavonoids and glycoside like molecules which prove more important for free radical scavenging. CSF3 scavenged the DPPH and hydroxyl free radical at physiological pH more significantly as compare to other fractions of CS. CS exhibited dose dependent increase in reducing power which in turn suggests the antioxidant potential of the plant.

The therapeutic applications of flavonoids on inflammation have previously been reported. Inflammation is important in many serious diseases, including cancer, Alzheimer's and AIDS. Therefore, intake of flavonoids is very important in the management of these diseases. In addition, flavonoids are known to prevent the synthesis of prostaglandins. Biochemical investigations on the mechanism of action of flavonoids have shown that these compounds can inhibit a wide variety of enzymes. On the other hand Arachidonic acid is indigenous compounds of the cell membrane with a task to protect the cell. The release of arachidonic acid is closely related to the cyclooxygenase (CO) and 5-lipoxygenase (LO) enzyme systems. The

ability of flavonoids to inhibit both CO and LO pathways of the arachidonic acid metabolism have been suggested to contribute to anti inflammatory action[21].

Carrageenan is the sulphated polysaccharide obtained from the seaweed, which is widely used phlogistic agent which shows signs and symptoms of inflammation, which can be assessed as increase in paw thickness in mouse as a result of increased inflammation, edema and increased vascular permeation. Inflammation produced by carrageenan is a tri phasic response. In the first phase of inflammation, histamine and serotonin like inflammatory mediators are involved which cause the edema and redness. In the second phase, different cytokines and kinins get released in response to the inflammation produced and the mediators already secreted at the localized site. In the third phase, the COX enzyme plays pivotal role and there is production of prostaglandins which induces pain[22]**.** In the present study, CSF3 showed inhibition of paw thickness at 3rd and 4th hour after carrageenan injection which probably suggested that CSF3 inhibit the prostaglandin formation in the third phase of inflammation.

The study indicated that CS has both peripheral and central analgesic properties. Its peripheral analgesic activity was deduced from its inhibitory effects on chemical induced nociceptive stimuli[23]. The acetic acid induced abdominal contraction elucidated peripheral activity[24], while the formalin test investigated both[24]. Acetic acid causes an increase in prostaglandins such as PGE_2 and PGF_2, serotonin, and histamine in the peritoneal fluid which brings about characteristic writhing in mice. Drugs that act primarily on the central nervous system inhibit both phases equally while peripherally acting drugs inhibit the late phase[25]. The formalin test is a very useful method for not only assessing antinociceptive drugs but also helping in the elucidation of the action mechanism. The neurogenic phase is probably a direct result of stimulation in the paw and reflects centrallymediated pain with release of substance P while the late phase is due to the release of histamine, serotonin, bradikynin and prostaglandins[26]. CSF3 was able to block both phases of the formalin response but the effect was more prominent in the second phase[27].

In the present study, CSF3 showed maximum protection against acetic acid induced writhing followed by other fractions probably explained the peripheral analgesic potential of CSF3 prostaglandin inhibitory activity.

The CSF3 was found to be more active in both antioxidant and anti-inflammatory activities. The study confirms the antioxidant and anti-inflammatory activities of *CS.*

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