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www.ijapgr.com/archives/**Research article****Evaluation of *in-vitro* anti-inflammatory activity of *Coreopsis lanceolata* L. flower petals****Anoop M V,* Anees P, Naseeha Ismayil, Ramjish Kaprat, Raheela V, Shibla Shirin Nadakkavail**

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(Received: 24th February 2015;Accepted: 29th March 2015)**Abstract**

Coreopsis lanceolata L. is perennial evergreen herb belongs to the family Compositae having yellow colored daisy like flowers. The present study was to evaluate *in-vitro* anti-inflammatory activity of *C. lanceolata* flower petals by inhibition of Cyclooxygenase, 5-lipoxygenase level and by estimation of cellular nitrite levels and nitric oxide synthase. The total ethanolic and aqueous extracts of flower petals were prepared and subjected to preliminary phytochemical analysis for the presence of various phytochemicals. Total ethanolic extract found to contain maximum of flavonoids by aluminium chloride colorimetric assay. Anti-oxidant activity study by using nitric oxide scavenging activity showed the total ethanolic extract has maximum activity ($IC_{50} = 125$ mcg/ ml). The evaluation of anti-inflammatory activity by inhibition of Cyclooxygenase (86.84% at 100 mcg/ ml), 5-lipoxygenase (64.15 % at 100 mcg/ ml) and by determination of cellular nitrite level (220.275 mcg) and inducible nitric oxide synthase (0.074 ± 0.002 nm) showed the total ethanolic extract is a good candidate for anti-inflammatory activity.

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Kerala, Indiaamv198610@gmail.com**KeyWords***Coreopsis lanceolata* L,
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5- Lipoxygenase, nitrite

INTRODUCTION

The mechanisms of inflammation involve a series of events in which the metabolism of arachidonic acid plays an important role. It can be metabolized by the Cyclooxygenase (COX) pathway to prostaglandins and thromboxane A₂, or by the 5-lipoxygenase (5-LOX) pathway to hydroperoxy-eicosatetraenoic acids (HPETE's) and leukotrienes (LT's), which are important biologically active mediators in a variety of inflammatory events. Upon appropriate stimulation of neutrophils, arachidonic acid is cleaved from membrane phospholipids and can be converted to leukotrienes and prostaglandins through 5-LOX or COX pathways respectively. Inhibition of 5-LOX and COX leads to decreased production of LTs and PGs, such a drug would have the potential to provide anti-inflammatory and analgesic effects with a reduction in the GI side-effects. Furthermore, inflammatory processes also involve reactive oxygen species started by leukocyte activation. Therefore, screening of antioxidant properties may provide important information about the potential activity of a drug on inflammatory processes¹.

Coreopsis lanceolata is perennial evergreen herb grows in small clumps but forms extensive colonies. The yellow daisy like flowers occurs singly at top elongated peduncles. Traditionally very less scientific investigation has been carried out on this plant, literature review shows the presence of antioxidant compounds on ethyl acetate extract², and the antioxidant activities were proved by Diphenylpicrylhydrazyl radical scavenging assay and superoxide

dismutase-like activity assay³. Nematicidal activities of ethyl acetate extract of the plant were proved⁴. The literature review shows that the plant is a good candidate for antioxidant related activity such as anti-inflammatory. This is leading as to the requirement of anti-inflammatory activity studies.

MATERIALS AND METHODS

Collection and authentication of plant material

Coreopsis lanceolata flower were collected from Botanical garden, Calicut university campus, Calicut, Kerala and was authenticated by botanist Mr. A.K Pradeep, Department of Botany, Calicut university, Calicut, Kerala and the voucher specimen number 88442 was kept in Department of Botany, Calicut university for future references.

Extraction

Yellow coloured petals were separated from fully expanded flowers of *Coreopsis lanceolata* and dried under shade and are powdered. 500 g of powder is extracted with 500ml of 90% ethanol using soxhlet apparatus at 60 - 70° C for 48 h. The extract thus obtained is concentrated with the help of vacuum evaporator. The marc obtained after ethanolic extraction were subjected to extraction with water by reflux method, the extract obtained after this extraction is concentrated. The total ethanolic extract and aqueous extract were stored at refrigerator and was subjected to preliminary

phytochemical screening and biological activity studies.

Preliminary phytochemical screening

The total ethanolic extract and aqueous extract of flower petals was subjected to various chemical tests for the investigation of presence of various chemical constituents⁵.

Estimation of total flavonoid content

Total flavonoids content of the extracts were determined by Aluminium chloride colorimetric method using Rutin as standard⁶. 10 mg Rutin was weighed and made up to 10 ml with methanol in a 10 ml standard flask. From the above solution (1 mg/ ml), 1 ml was pipetted out and made up to 10 ml with methanol to get 100 mcg/ ml rutin standard solution (stock solution). From the stock solution, solutions of concentration 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mcg/ ml were prepared. To each of these, 4 ml water was added followed by 0.3 ml 5% sodium nitrate. After 5 minutes, 0.3 ml of 10% aluminium chloride solution and at the 6th minute 2 ml of 1M sodium hydroxide was added. Then total volume was made up to 10 ml with distilled water. A blank was prepared without the addition of aluminium chloride solution. The solutions were mixed well and the absorbance was measured against the blank at 510 nm using UV-VISIBLE spectrophotometer. A standard graph was plotted using various concentrations of rutin and their corresponding absorbance.

Preparation of sample solution

10 mg of the sample extracts were weighed dissolved in methanol and made up to 10 ml with methanol. 1 ml was pipetted out from each dissolved samples and 4 ml of water followed by 0.3 ml of sodium nitrate was added. After 5 minutes, 0.3 ml of 10% aluminium chloride solution and at the 6th minute 2 ml of 1M sodium hydroxide was added, mixed well and the absorbance was measured at 510 nm and the values were interpreted in the standard graph of rutin to get the milligram equivalents of rutin.

In-vitro antioxidant activity

A. Nitric oxide scavenging assay

Preparation of standard

The standard used here was Ascorbic acid. 50, 100, 150 and 200 mcg/ ml solution of standard in methanol was used for assay.

Preparation of sample

Sample solution was prepared from total ethanolic and aqueous extracts of *C. lanceolata* petals dissolved in methanol. 10 mg of each extract were dissolved in methanol and solutions of 50, 100, 150 and 200 mcg/ ml concentration were prepared.

Estimation procedure

In this assay 0.5 ml of Sodium nitro prusside (5 mmolL⁻¹) in phosphate buffered saline pH 7.4, was mixed with different concentration of the extract (50, 100, 150 and 200 mcg/ ml from a stock concentration of 100 mg/ ml methanol) and incubated at 25°C for 180 minutes. A control without the test compound, but an equivalent amount of methanol was

taken. After 3 h, 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthyl ethylene diaminedihydrochloride) was added and incubated for 30 minutes for color development. Absorbance of the chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with N-1-naphthyl ethylene diaminedihydrochloride was measured at 546 nm and the percentage scavenging activity was measured with reference to the standard. The scavenging activity on the nitric oxide was expressed as inhibition percentage using the following equation:

Percentage Nitric oxide Scavenging =

$$\frac{\text{Absorbance of Control} - \text{Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

The tests were carried out in triplicate. The extract concentration providing 50% inhibition (IC₅₀) was calculated from the graph of inhibition percentage plotted against extract concentration^{7, 8}.

***In-vitro* anti-inflammatory activity**

Preparation of cell lines

RAW 264.7 macrophage cell lines was cultured in DMEM [HIMEDIA] media, supplemented with 10% heat inactivated FBS, antibiotics (Penicillin and Streptomycin) and 1.5% sodium bicarbonate. The media was filtered using 0.2 μm pore sized cellulose acetate filter (Sartorius) in completely aseptic conditions. The cells were then grown till 60% confluency followed by activation with 1 μl LPS (1 μg/ml). LPS stimulated cells were exposed with different concentrations

of samples such as 10 mcg/ml, 50 mcg/ml and 100 mcg/ml, from a stock of 1 mg/ml and incubated for 24 h. The anti-inflammatory effects of samples were determined by assessing the inhibition of COX, LOX, and iNOS and nitrate levels spectrophotometrically. The isolation was done by spinning at 6000 rpm for 10 minutes. Supernatant was discarded and 200 μl of cell lysis buffer (1 M Tris HCl, 0.25 M EDTA, 2 M NaCl, 0.5% Triton) was added. The incubation was done for 30 minutes at 4°C and enzymes assay was done in pellet suspended in a small amount of supernatant.

A. Assay of Cyclooxygenase

The assay mixture contained Tris- HCl buffer, glutathione, hemoglobin & enzyme. The assay started by the addition of arachidonic acid and terminated after 20 min incubation at 37°C by addition of 0.2 ml of 10% TCA in 1 N HCl, mixed and 0.2 ml of TBA was added and contents heated in a boiling water bath for 20 min, cooled and centrifuged at 1000 rpm for 3 min. The supernatant was measured at 632 nm for COX activity⁹.

B. Assay of 5- Lipoxygenase

70 mg of linoleic acid and equal weight of tween 20 was dissolved in 4 ml of oxygen free water and mixed back and forth with a pipette avoiding air bubbles. Sufficient amount of 0.5 N sodium hydroxide was added to yield a clear solution and then made up to 25 ml using oxygen free water. This was divided into 0.5 ml portions and flushed with nitrogen gas before closing and kept frozen until needed. The reaction was carried out in a quartz cuvette at 25°C with 1cm light

path. The assay mixture contain 2.75 ml tris buffer of pH 7.4, 0.2 ml of sodium linoleate and 50 mcl of the enzyme. OD was measured in 234 nm⁹.

C. Estimation of Cellular Nitrite Levels

To 0.5 ml of cell lysate, 0.1 ml of sulphosalicylic acid was added and vortexed well for 30 minutes. The samples were then centrifuged at 5,000 rpm for 15 minutes. The protein-free supernatant was used for the estimation of nitrite levels. To 200 mcl of the supernatant, 30 mcl of 10% NaOH was added, followed by 300 mcl of Tris-HCl buffer and mixed well. To this, 530 mcl of Griess reagent was added and incubated in the dark for 10–15 minutes, and the absorbance was read at 540 nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained¹⁰.

D. Inducible Nitric Oxide Synthase determination

Serum was homogenized in 2 ml of HEPES buffer. The assay system contained substrate 0.1 ml L-Arginine, 0.1 ml manganese chloride, 0.1 ml 30 mcg dithiothreitol (DTT), 0.1 ml NADPH, 0.1 ml tetrahydropterin, and 0.1 ml oxygenated hemoglobin and 0.1 ml enzyme (sample). Increase in absorbance was recorded at 401 nm¹¹.

RESULTS AND DISCUSSION

Preliminary phytochemical screening

The extractive values of two extracts were found out and were tabulated in table 1. The qualitative and quantitative analysis of extracts of *C. lanceolata* were carried out and extracts showed the presence of various chemical constituents such as alkaloids, glycosides, phenolics, flavonoids, tannins, saponins, carbohydrates and steroids. This shows high level of its possible medicinal value. Phytochemical screening showed the presence of antioxidant components phenolics and flavonoids, presence of these compounds might be responsible for the use of plant in ameliorating inflammatory ailments¹².

Estimation of total flavonoids

Estimation of total flavonoids in the extracts was carried out by Aluminium chloride colorimetric method. The absorbance values obtained for different concentration were plotted and the standard graph is shown in figure 1. The absorbance obtained for the *C. lanceolata* petal extracts were recorded and the total flavonoids content in rutin equivalents are given in table 2. From the table it is clear that the total ethanolic fraction has the highest total flavonoid content.

Flavonoids are ubiquitous secondary metabolites in plants and possess a wide range of therapeutic uses such as antioxidant, anti-inflammatory, ant mutagenic, ant carcinogenic, free radical scavenging activities and also decrease cardiovascular complications. So the total ethanolic extract was found to a source of wide range of potent photochemical.

Tab 1 .Extractive value of different extracts

Sl. no	Extracts	Color and consistency	Extractive value (%w/w) on dry weight basis
1.	Total Alcoholic (TEE)	Dark green sticky mass	18.00
2.	Aqueous (AQE)	Brown powder	10.6

Tab 2.Total Flavonoid content of different extracts

Sl.no	Extracts	Absorbances (510nm)	Flavonoids content (mcg/ml) (Rutin equivalent)
1.	Total Alcoholic (TEE)	1.244±0.003	57.12
2.	Aqueous (AQE)	0.454±0.001	20.27

n=3, absorbance expressed as mean± S.D

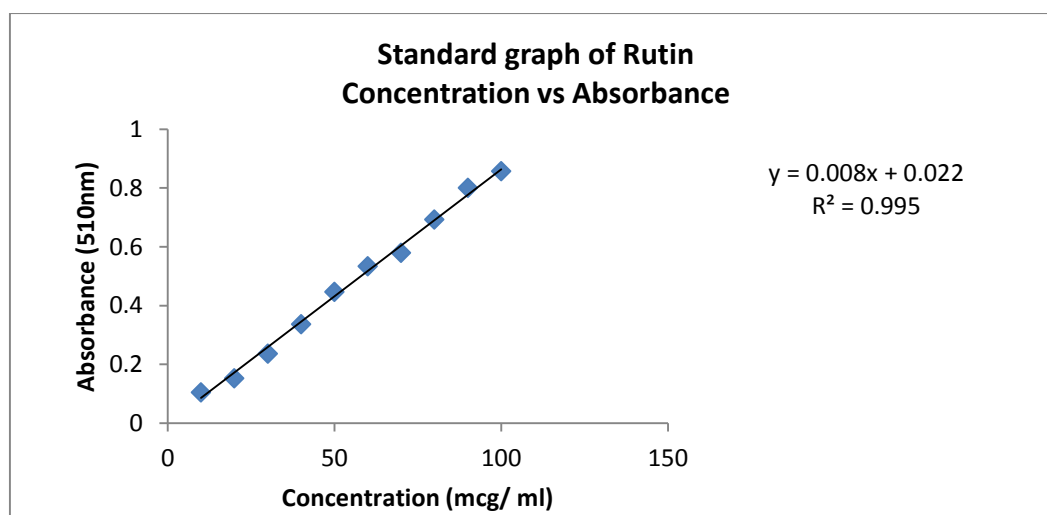


Fig 1. Standard graph of rutin

Anti-oxidant activity

A. Nitric oxide scavenging assay

The percentage inhibition obtained in the different concentration of extracts

was compared to that of standard ascorbic acid were calculated and is shown in figure 2. The calculation of

IC₅₀ values of standard ascorbic acid and extracts were tabulated in table 3. This assay shows that the total ethanolic extract has more Nitric oxide scavenging activity than aqueous extract. It has been determined that the antioxidant effect of plant products is mainly due to phenol compounds, such as flavonoids, phenol acids, tannins and phenol diterpenes. The potent antioxidant activity exhibited by the total alcoholic extract shows that they contain phytochemical compound with antioxidant properties i.e. due to the high concentration of phenolics and flavonoids.

In-vitro anti-inflammatory activity

A. Cyclooxygenase inhibitory assay

The effects of *C. lanceolata* petal extracts on production of prostaglandins were determined by the inhibition of Cyclooxygenase activity. The results are tabulated in table 4. The comparisons of the anti-inflammatory activity of extracts are given in the figure 3. From the table, we can suggest that total ethanolic extract has higher Cyclooxygenase inhibitory activity when compared to the aqueous extract.

B. 5-lipoxygenase inhibitory assay

The effects of *C. lanceolata* petal extracts on production of leukotrienes were determined by the inhibition of 5-lipoxygenase activity and the results

are shown in table 5 and figure 4. From the table, we can suggest that Total ethanolic extract and aqueous extracts has got good 5-lipoxygenase inhibitory activity comparing to standard Ibuprofen.

C. Estimation of Cellular Nitrite Levels

The effects of *C. lanceolata* petal extracts on cellular nitrite level were determined and the results are tabulated in table 6. The result showed the total ethanolic extract has good activity

D. Determination of Inducible nitric oxide synthase

The effects of *C. lanceolata* petal extracts on cellular nitric oxide synthase level were determined and the results are tabulated in table 7. The result showed the total ethanolic extract possess good activity.

In the present study, results indicate that the extracts of *Coreopsis lanceolata* possess good anti-inflammatory properties; however the total ethanolic extract showed the best anti-inflammatory activity by inhibiting COX inhibition and 5-LOX inhibition, the extract also showed an inhibition in cellular nitrite level and reduction in inducible nitric oxide synthase level. These activities may be due to the strong occurrence of polyphenolic compounds. The total ethanolic fraction contains maximum of polyphenolics, flavonoids and terpenoids, serve as free radical inhibitors or scavenger or acting possibly as primary oxidant thereby inhibiting inflammation¹⁴.

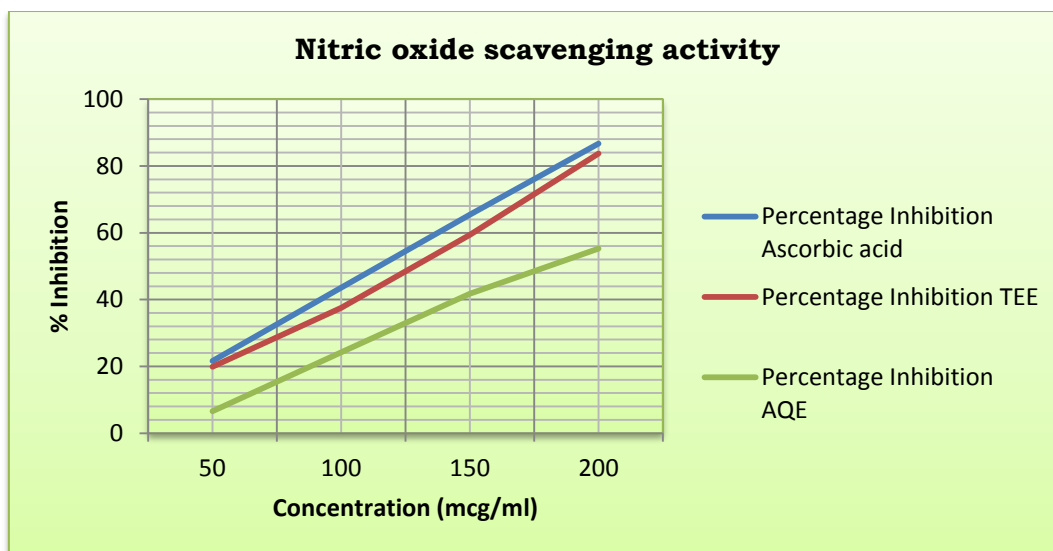


Fig 2. Calculation of IC₅₀ value of ascorbic acid, total alcoholic and aqueous extracts

Tab 3. Calculation of IC₅₀ values using Nitric oxide scavenging assay

Sl. no	Sample	IC ₅₀ value (mcg/ml)
1	Standard (Ascorbic acid)	115±0.005
2	Total Alcoholic extract (ALE)	125±0.003
3	Aqueous extract (AQE)	185±0.005

n=3 Values expressed as Mean ± SD

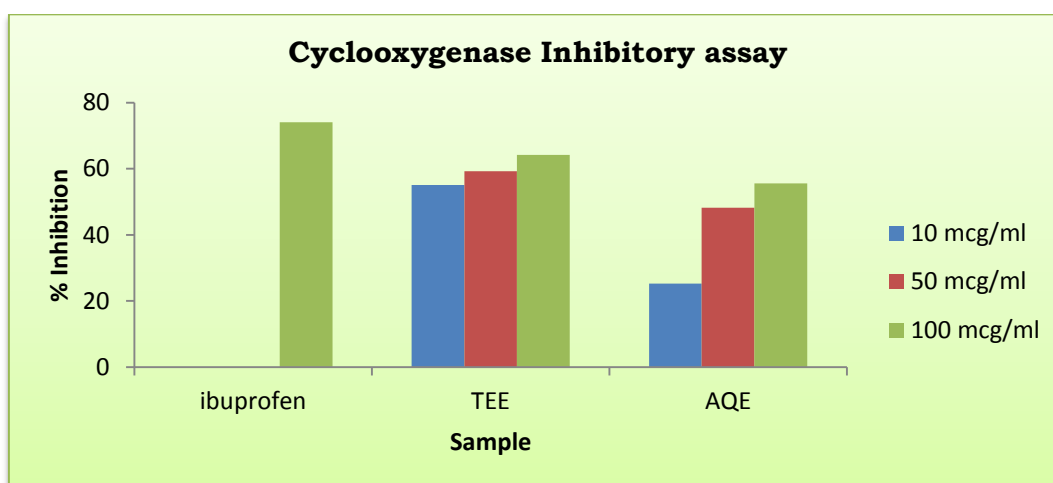


Fig 3. Cyclooxygenase inhibitory assay

Tab 4. Cyclooxygenase inhibitory assay

Sl.no	Sample	Concentration (mcg/ml)	Absorbance (632nm)	% inhibition
1	Control	-	0.076±0.60	-
2	Standard (Ibuprofen)	100	0.007±0.90	90.79
3	Total Ethanolic Extract (TEE)	10	0.03±1.32	60.53
		50	0.021±0.61	72.37
		100	0.01±1.08	86.84
4	Aqueous (AQE) extract	10	0.071±0.55	06.57
		50	0.054±0.05	28.95
		100	0.038±0.68	50.00

n=3 absorbance expressed as Mean ± SD

Tab 5. 5-lipoxygenase inhibitory assay

Sl.no:	Sample	Concentration (mcg/ml)	Absorbance (234 nm)	% inhibition
1	Control	-	0.463±0.85	-
2	Standard (Ibuprofen)	100	0.12±1.13	74.08
3	Total Alcoholic Extract (EAE)	10	0.208±1.48	55.06
		50	0.189±0.61	59.18
		100	0.166±0.74	64.15
4	Aqueous (AQE) extract	10	0.346±0.56	25.27
		50	0.24±0.78	48.16
		100	0.206±1.35	55.51

n=3 absorbance expressed as Mean ± SD

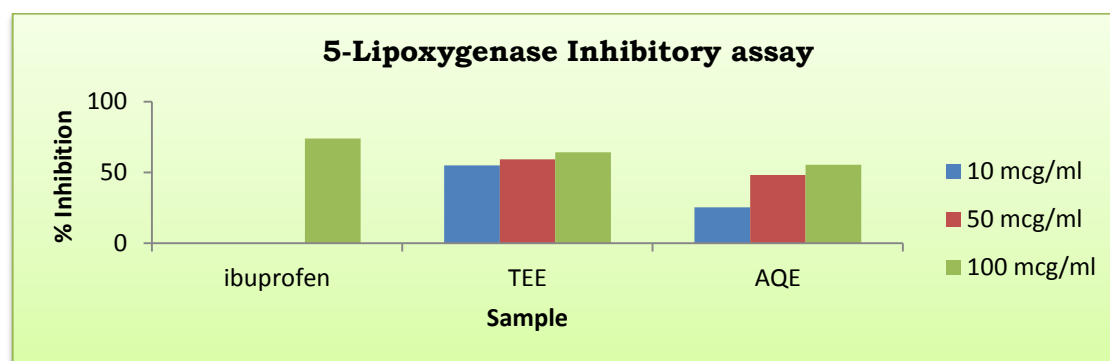


Fig 4. 5-lipoxygenase inhibitory assay

Tab 6. Cellular nitrite level estimation

Sl. No	Sample	Concentration (mcg/ml)	Absorbance	Concentration (mcg)
1	Control		0.3995	1985
2	Total Alcoholic extract (TEE)	10	0.0996	493.02
		50	0.0668	330.66
		100	0.0445	220.275
3	Aqueous extract (AQE)	10	0.0949	469.755
		50	0.0917	453.915
		100	0.0851	421.245

Inflammation is a complex physiopathological response to different stimuli. The inflammatory process involves the activity of inflammatory mediators such as neutrophil derived free radical, reactive oxygen species (ROS), nitric oxide (NO), prostaglandins and cytokines¹⁵. This over production leads to tissue injury by damaging macromolecules, lipid per oxidation of membrane and tissue damage play important role in pathogenesis of many inflammatory diseases. Thus the free radicals are important mediators that provoke or sustain inflammatory processes and consequently, their neutralization by antioxidants and radical scavengers can attenuate inflammation¹⁶.

During the course of inflammatory response, large amount of NO formed by nitric oxide synthase (iNOS) in activated macrophages surpass the physiological amount of NO, which are usually made by neuronal form of NOS (nNOS) or constitutive form of NOS (eNOS), these NO nitrosylates macromolecules. It also causes increased vascular permeability, vaso

dilation, tissue and endothelial damage leads to inflammation^{11, 12}.

Arachidonic acid (AA) is metabolized in the body through two main metabolic pathways with the enzymes: Cyclooxygenases (COX) and 5-lipoxygenases (5-LOX) into prostaglandin I₂, prostaglandin E₂, thromboxane A₂ and leukotrienes. It has been suggested that the inhibition of both prostaglandins and leukotriene production might have synergistic effects and achieve optimal anti-inflammatory activity so the dual inhibition of the COX and 5-LOX pathway could produce a wider spectrum of anti-inflammatory effects. In the past few decades, several compounds have been developed to block both COX and 5-LOX, but their use was abandoned owing to liver toxicity. Prototype experimental dual inhibitors have proved effective in preventing the production of both PGs and LTs and the consequent inhibition of migration and activation of inflammatory cells (mainly PMN, monocytes and macrophages) into inflamed sites. Importantly, the inhibition of migration of

inflammatory cells towards the affected sites has translated into a reduction of tissue damaging or necrosis in a model of tissue damage. 5-LOX/COX blockers have an excellent preclinical GI pharmacological safety profile¹⁶.

The total ethanolic extract showed pronounced Nitric oxide scavenging activity, showed good inhibitory activity against COX and 5-LOX enzyme mediated metabolism of arachidonic acid and pronounced activity in decreasing cellular nitrite level and inducible nitric oxide synthase. Therefore ability of the extract to scavenge free radical during inflammation and inhibition of COX and 5-LOX might be responsible for their inhibitory effect on progression of inflammatory reaction¹⁵.

CONCLUSION

Coreopsis lanceolata flower petals were selected for the study. Phytochemical studies showed the presence of alkaloids, glycosides, phenolics, flavonoids, steroids, terpenoids and saponins. The antioxidant compounds, flavonoids were quantitatively estimated and the total ethanolic extract showed the maximum of these compounds. Antioxidant activity studies using Nitric oxide showed that the total ethanolic and aqueous extracts could be promising sources of natural antioxidants. Anti-inflammatory studies using Cyclooxygenase, 5-lipoxygenase inhibitory activity, and Cellular nitrate level estimation and Inducible nitric oxide synthase determination showed that total ethanolic extract has good anti-inflammatory activity.

A detailed research on different pharmacological activities can be carried out such as Hepatoprotective activity, Analgesic activity, and Antipyretic activity. Isolation of more active constituents possessing anti-inflammatory can be assessed.

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