

Anti-inflammatory and Anti-arthritis Activity of Active Constituents Present in *Calophyllum inophyllum* Leaves Extract

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ABSTRACT: *Calophyllum inophyllum* is used for the treatment of various diseases, namely inflammation, arthritis, diabetes, bacterial infection, etc. We planned to isolate the active constituents of ethanol extract of *Calophyllum inophyllum* leaves and evaluated their anti-inflammatory and anti-arthritis activity. The petroleum ether, ethanol and aqueous extract were prepared from leaves of *Calophyllum inophyllum*, and screened for photochemical analysis. The different flavonoid compound isolated from ethanol extract, and further evaluated their anti-inflammatory and anti-arthritis activity. The maximum phytoconstituents along with flavonoids were observed in ethanol extracts of leaves of *Calophyllum inophyllum*. The eight different fractions (F1 to F8) were isolated from ethanol extracts of *Calophyllum inophyllum* leaves. The F6 and F7 indicate the presence of flavonoids, tannins & phenolic compounds. The findings indicate that the F6 and F7 isolated from *Calophyllum inophyllum* leaves extract exhibited moderate to highly anti-inflammatory and anti-arthritis activity. It suggests that the anti-inflammatory and anti-arthritis activity of F6 and F7 due to the presence of polyphenol and flavonoids.

Keywords: *Calophyllum inophyllum*, anti-inflammatory, anti-arthritis, isolated compound

Introduction

The drugs commonly in use for the treatment of inflammation and arthritis include glucocorticoids like cortisone and prednisone etc., NSAIDs like Ibuprofen and naproxen etc., disease-modifying anti-inflammatory and anti-rheumatic drugs like Methotrexate (MTX) and leflunomide etc., and newer therapies such as biological response modifiers like tumor necrosis factor, alpha blocking agents, anti-CD 20 therapy (rituximab) and abatacept which are often required to inhibit or halt the underlying immune processes. NSAIDs are used to control the rheumatoid arthritis; it leads to inhibit COX and LOX used for the metabolic modulation of arachidonic acid¹. However, besides high costs, all of these drugs are associated with numerous side effects, severe adverse reactions (like ulceration, perforation, gastric irritation, haematochezia, angioedema, hepatic failure, headache, hemolytic anemia, hyperglycemia, osteoporosis, immunodeficiency-related problems) and toxicity, including some risk of infections in

subsets of patients who are being treated with biological response modifiers. In recent days, researchers are directed towards traditional system of medicine for the discovery of drugs that are long acting anti-inflammatory agents displaying minimum side effects^{2, 3}. In India, many Ayurvedic practitioners are using various indigenous plants for the treatment of different types of arthritic conditions. Although the application of these medicaments has a sound tradition and a rational background according to the Indian system of medicine, perhaps it is essential to investigate the rationality of their use in modern scientific terms⁴. A compound obtained from plant imparts a remarkable role to cure and control various diseases from ancient times. The numerous studied has been conducted and validated the secondary metabolites presents in the plants imparts pharmacological activity to combat numerous disease. A plant containing flavonoids or polyphone is good choice to use as anti-arthritis drugs. The flavonoids smoothly inhibit the production of free radical, and lead to impede COX

(cyclooxygenase) and LOX stimulation. Consequently, flavonoids control the inflammation and arthritis¹. *Calophyllum inophyllum* is the most versatile medicinal plants having a wide spectrum of biological activity. *Calophyllum* showed potent antidiabetic, anticancer, diuretic, anti-inflammatory, antimicrobial, laxative, astringent, emollient, anthelmintic and antiepileptic activities. The plant was also reported for its anti-fungal activity, anti-microbial activity, cardiac activity, anti-inflammatory, anti-arthritis and anti-fertility activity⁵⁻⁹. We planned to isolate the flavonoids from *Calophyllum inophyllum* leaf extracts. Further the anti-inflammatory and anti-arthritis potential of flavonoids fraction isolated from *Calophyllum inophyllum* extracts were evaluated.

MATERIAL AND METHODS PREPARATION OF EXTRACTS

500 grams of coarsely powdered of *Calophyllum inophyllum* leaves were packed separately in Soxhlet extractor and extracted using petroleum ether, ethanol and distilled water successively. The extracts were then concentrated to dryness under reduced pressure and controlled temperature, respectively, and they were preserved in a refrigerator. The ethanol and aqueous extract were selected for further study.

PHYTOCHEMICAL ANALYSIS

Qualitative chemical tests were performed to determine the presence of alkaloids, carbohydrates, cardiac glycosides, polyphenols, saponins, tannins and terpenoids^{10, 11}.

ISOLATION OF COMPOUNDS FROM

Calophyllum inophyllum extract

The *Calophyllum inophyllum* extract was subjected to column chromatography using silica gel (60-120 mesh size), and eluted with the following solvent ratios of Hexane: dichloromethane (DCM), 100:0, 75:25, 50:50, 25:75, 0:100, then with 75:25, 50:50, 25:75, 0:100, DCM: Ethanol (Eth). Finally eluted with 75:25, 50:50, 25:75, 0:100, Eth: Methanol (MeOH). The fractions (25 ml) were collected from the column. The elute collected were monitored by thin layer chromatography (eluent: DCM-MeOH, 9:1 and 3:2) for homogeneity and the similar fraction were pooled together. The eight different fractions were collected and dried. The fraction F1, F2 and F3 were containing waxy material; the fractions F4 and F8 were powder but quantity was very little.

The yield of fraction F5, F6 and F7 were 315 mg, 440 mg and 365 mg, respectively. The three fractions were further analyzed for phytochemical screening to determine the nature of isolated compound¹².

ANTI-INFLAMMATORY ACTIVITY

The Carrageenan-induced oedema and cotton pellet-induced granuloma model were performed to assess the anti-inflammatory activity of isolated compounds of *Calophyllum inophyllum* leaves extract.

CARRAGEENAN-INDUCED OEDEMA

Albino Wistar rats of either sex weighing between (150-200 gm) were divided into various groups and six animals in each group. The groups were as follows:

- Group I (control group) – Treated with distilled water
- Group II – Treated with standard drug Indomethacin at 10 mg/kg body weight
- Group III – Treated with F6 at 50 mg/kg body weight
- Group IV – Treated with F7 at 50 mg/kg body weight

Acute inflammation was produced by injecting 0.1ml of 1% carrageenan suspension in normal saline into the subplantar region of right hind paw after 60 minutes of drug administration. The control group was administered only distilled water. The isolated compound and standard drugs administered intraperitoneally 1 h before carrageenan and suspension administration. A mark was made on the leg at the malleolus to facilitate uniform dipping at subsequent readings. The volume of paw oedema was measured with the help of plethysmograph by mercury displacement method immediately before and five hours after the drug administration¹³⁻¹⁵. The inhibition of oedema in various treated groups was then calculated by using statistical analysis.

COTTON PELLET-INDUCED GRANULOMA MODEL

Albino Wistar rats of either sex weighing between (150-200 gm) were divided into various groups and six animals in each group. The groups were as follows:

- Group I (control group) – Treated with distilled water
- Group II – Treated with standard drug Indomethacin at 10 mg/kg body weight
- Group III – Treated with F6 at 50 mg/kg body weight
- Group IV – Treated with F7 at 50 mg/kg body weight

The animals were grouped as described above to study the anti-inflammatory activity. The groups were fasted and treated with drugs/doses similar to that of carrageen an- induced hind paw edema. Sterile cotton pellets each weighing 30 ± 5 mg were prepared and sterilized in a hot air oven at 123°C for 3 h. Each animal was placed under light ether anesthesia and subcutaneously implanted with four cotton pellets, one each into both the axillae and the groin region under aseptic conditions. The drugs were administered orally for seven days starting from the day of implantation of the pellets. All the animals had free access to drinking water and food. On the 8th day, all the animals were sacrificed and the implanted cotton pellets were recovered, cleaned of surrounding tissues, and blotted with filter paper. These cleaned pellets were weighed and dried in a hot air oven overnight at 70°C and the dry weights were noted^{16, 17}.

ANTI-ARTHRITIS ACTIVITY

One week before the commencement of the experiment, the rats were randomly divided into five groups of six rats per group. On day 0 rats were injected with 0.1 ml of Freund's complete adjuvant (FCA) into the sub plantar (s.p) region of the left hind paw of all the animals. This consists of *Mycobacterium butyricum* suspended in heavy paraffin oil by thoroughly grinding with a pestle and motor to give a final concentrate of 0.6 mg/ml. Administration of test compounds and standard drug was started on the next day and continued for 28 days. The paw was marked with ink at the level of the malleolus laterals and paw volumes were recorded by the plethysmometer immediately after injection and on 7th, 14th, 21st and 28th day^{18,19}. The experimental rats were randomly divided into five groups of six rats per group and treated as follows:

- Group I (control group) – Arthritic rats treated with distilled water
- Group II – Arthritic rats treated with standard drug Indomethacin at 10 mg/kg body weight
- Group III – Treated with F6 at 50 mg/kg body weight
- Group IV – Treated with F7 at 50 mg/kg body weight

HEMATOLOGICAL SCREENING

On the 28th day blood samples for hematological assays were obtained through ocular puncture of the rats and collected in EDTA-treated sample bottles. The White blood cell (WBC),

Hemoglobin (Hb) and erythrocyte sedimentation rate (ESR) were determined^{18, 19}.

STATISTICAL ANALYSIS

The results are expressed as mean \pm SEM of six independent experiments. Statistical significance between the groups was evaluated by one-way analysis of variance (ANOVA) followed by Dunet's test. A $P < 0.05$ value was considered as statistically significant.

RESULTS AND DISCUSSIONS

In the present study, *Calophyllum inophyllum* was selected for isolation of active constituents from extract and check anti-inflammatory and anti-arthritis activity of active constituents.

PHYTOCHEMICAL SCREENING OF

Calophyllum inophyllum

Preliminary phytochemical investigations of the extracts of leaves of *Calophyllum inophyllum* revealed the presence of flavonoids, tannins, phenolic compounds, alkaloids, glycosides, fats and carbohydrates. The details are presented in table 1.

From the result of phytochemical screening, the petroleum ether extract of leaves of *Calophyllum inophyllum* exhibited the presence of fats and oils. Alkaloids, glycosides, carbohydrates, flavonoids, tannins, proteins and polyphenol were found in ethanol extracts of leaves of *Calophyllum inophyllum*. Similarly glycosides, carbohydrates, flavonoids, tannins and polyphenol were existing in aqueous extracts of leaves of *Calophyllum inophyllum*. The maximum phytoconstituents were observed in ethanol extracts of leaves of *Calophyllum inophyllum* (Table 1). Hence ethanol extracts of *Calophyllum inophyllum* was selected for further separation of compound as this extract revealed the presence of flavonoids and phenolic compounds.

Isolation of compound from ethanol extract of *Calophyllum inophyllum*

The eight different fractions were collected and dried. The fraction F1, F2 and F3 were containing waxy material; the fractions F4 and F8 were powder but quantity was very little. The yield of fraction F5, F6 and F7 were 315 mg, 440 mg and 365 mg, respectively. The three fractions were further analyzed for phytochemical screening to determine the nature of the isolated compound.

PRELIMINARY PHYTOCHEMICAL ANALYSIS OF ISOLATED FRACTION

The phytochemical investigation of F5 of *Calophyllum inophyllum* leaves revealed the presence of alkaloids, glycosides and carbohydrates. The F6 and F7 indicate the presence of glycoside, flavonoids, tannins & phenolic compound (Table 2). Many investigations have proven that varieties of flavonoid molecules possess anti-inflammatory activity in various animal models of inflammation. Especially, some flavonoids were found to inhibit chronic inflammation of several experimental animal models. Thus, it may be valuable to continuously evaluate the anti-inflammatory activity of flavonoids, not only for establishing anti-inflammatory mechanisms, but also for developing a new class of anti-inflammatory agents. The F6 and F7 containing polyphenol and flavonoids compound and these organic substances impart chief role in anti-inflammatory and anti-arthritis activity. Hence this result supports us to evaluate the anti-inflammatory and anti-arthritis activity of the F6 and F7. Anti-inflammatory studies Carrageen an-induced oedema the effect of the F6 and F7 isolated from *Calophyllum inophyllum* on paw oedema is presented in (Table 3). The animals administered only distilled water, the sub plantar injection of carrageen an produced a local oedema that increased progressively from 0.31 ml after the first hour to reach a maximum within 4 h. The administration of fraction F6 and F7 (50 mg/kg) revealed significant ($P < 0.05$) reduction in oedema in the rats compared with the same time of the distilled water treated group. Indomethacin (10 mg/kg) produced a significant ($P < 0.05$) decrease in oedema at the 2 hour compared with the same time of the distilled water treated group. The effect of the isolated compound in this model may be attributed to the inhibition of the release of pro-inflammatory mediators of acute inflammation, especially prostaglandins. Phenols are the most widespread secondary metabolite in the plant kingdom. There is abundant literature regarding medicinal plants establishing relations between anti-inflammatory and phenol/flavonoid content. It has been documented that the flavonoids and polyphenol inhibit lipid per oxidation and the inflammatory mediators Cyclo-oxygenase (COX) -1 and -2 to produce anti-inflammatory effect. Several researches suggest that the

combination of secondary metabolites with flavonoids, compounds produces synergistic pharmacological activity²⁰. Although no record of chemical constituents isolated and characterized from *Calophyllum inophyllum* were found, and the methods used for the identification of phytochemical constituents are preliminary in nature. The anti-inflammatory effects recorded for isolated compound *Calophyllum inophyllum* in this study, caused by the total polyphenolic and flavonoids constituents present in the plants. The phytochemical study of the isolated compound justifies the above statement. The results obtained in this study established the anti-inflammatory actions for the isolated compound. However, the mechanism of these actions is uncertain, and the flavonoids and polyphenol imparts chief role for the anti-inflammatory activity.

COTTON PELLETT-INDUCED GRANULOMA MODEL

The results revealed that the isolated fraction of *Calophyllum inophyllum* shows dose dependent inhibition of weight of both wet and dry cotton pellets. The mean number of this action²¹. However, mechanistic studies measuring specific cytokine levels may help elucidate this reasoning. No steroidal anti-inflammatory drugs (NSAID) for instance indomethacin employed in this study are proved to inhibit cyclooxygenase enzymes I and II, which is implicated in the production of inflammation mediating agent prostaglandin E2 (PGE2) from arachidonic acid. Therefore, the pattern of anti-inflammatory activity exhibited by the isolated compound was similar to that of indomethacin. The result of the study supports the anti-inflammatory activity of isolated compounds is due to the presence of flavonoids.

ANTI-ARTHRITIS ACTIVITY

Adjuvant Induced Chronic Arthritis

Experimental models have suggested that mycobacterial infections can trigger autoimmune arthritis, mainly through T-cell mediated responses. Arthritis was induced in rats by injecting dead mycobacteria in liquid paraffin. There was a significant increase in rat paw volume in FCA injected control rats when compared to the standard and fraction treated rats. Isolated compound treatment at the dose the progression of arthritis was confirmed in our study by scoring total arthritis lesions. The inflammation associated with AIA is mainly

dependent on prostaglandin E2 (PGE2) generated by cyclooxygenases (COXs). Besides, the role of cytokines like TNF- α and IL-1 has also been implicated in this model. Now from the results observed it was found that the F6 and F7 treated arthritic animals showed decreased inflammation of joints^{22, 23}. Therefore, the anti-arthritis action of isolated compounds may be mediated by prostaglandin and/or cytokine inhibition. These results are also in line with reports that anti-inflammatory action of *Calophyllum inophyllum* leaves has been attributed to polyphenolic component. The inhibition of lipid per oxidation, capillary permeability and fragility, and enzymes such as phospholipase A2, cyclooxygenase, and lipoxygenase may be due to tannins and polyphenols components. In the present study, arthritic control rats showed a reduced RBC count, reduced Hb levels, and an increased erythrocyte sedimentation rate (ESR). All these symptoms indicate an anemic condition. The F6 and F7 treated groups showed a significant recovery from the induced anemia. The significant increase in leukocyte count in adjuvant induced arthritic rats may be due to the stimulation of the immune system against the invading antigens and significant decrease in FBP8, FBP9, FBP10, FCM6, FCM7 and FCM8 treated groups showed its immunomodulation effect. This clearly indicates the anti-arthritic activity of *Calophyllum inophyllum* isolated compound. This study confirmed that the flavonoid fraction obtained from *Calophyllum inophyllum* leaves extract are responsible for its anti-arthritis activity and the effects observed are attributable due to the presence of flavonoids in the plant. Possible mechanism and the identification of the bioactive component responsible for anti-arthritis activity.

CONCLUSION

In the present study an attempt was made to isolate the various active constituents and evaluate their anti-inflammatory and anti- arthritis activity. It was concluded that the F6 and F7 isolated from *Calophyllum inophyllum* of 50 mg/kg exhibited significant reduction in rat paw edema volume compared to control group. Table 5 demonstrated the effect of F6 and F7 isolated from *Calophyllum Freund's* adjuvant model induced arthritis. After 28 days it was found that F6 and F7 significantly shows dose dependent inhibition in paw thickness, i.e. the

chronic inflammation induced by adjuvant shows decrease in paw thickness. The administration of F6 and F7 isolated from *Calophyllum inophyllum* on Freund's adjuvant induced arthritis animals enhanced the levels of RBC and Hb compared to control animals (Table 6). The WBC count and ESR were significantly reduced after administration F6 and F7 compared to control animals. In the present study, experimental arthritis was reliably established with repeated and daily subcutaneous plantar injection of 0.1 ml of FCA over a period of 28 days, which was characterized by the plantar edema formation which maximized on day 7 of the treatment and subsequent increase for the remaining part of the study. The inflammation induced by FCA is primarily due to edema formation and cellular influx. decreases in weight of both wet and dry cotton pellets in rats, which received 50 mg/ kg body weight of the isolated compound was significant ($p < 0.05$) lower than those in the control rats (Table 4). The F6 and F7 demonstrated 49.55% and 57.56% inhibition, respectively in weight of wet cotton pellets. The F6 and F7 demonstrated 40.98% and 48.62% inhibition, respectively in weight of dry cotton pellets. Non-steroidal anti-inflammatory drugs decrease the size of granuloma which results from cellular reaction by inhibiting granulocyte infiltration, preventing generation of collagen fibers and suppressing mucopolysaccharides. The isolated mpounds of *Calophyllum inophyllum* showed significant anti-inflammatory activity in cotton pellet induced granuloma and thus found to be effective in chronic inflammatory conditions, which reflected its efficacy in inhibiting the increase in the number of fibroblasts and synthesis of collagen and mucopolysaccharides during granuloma tissue formation. The suppression of the T helper 1 (Th-1) T-lymphocyte pathway, which releases inflammatory cytokines such as interleukin-12 and interferon-g, may also be responsible for leaves extract exhibited moderate to highly anti-inflammatory and anti-arthritis activity. It suggests that the anti-inflammatory and anti- arthritis activity of F6 and F7 due to the presence of polyphenol and flavonoids. This scientific study revealed the efficacy of the isolated compound and it would definitely have a wide scope in future.

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