Original Article

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In vivo and *in silico* anti-arthritic studies of chlorogenic acid from the rhizome of *Lasia spinosa* (L.) Thwaites (Araceae)

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ABSTRACT

Aim: The aim of the study was to examine the anti-arthritic function of methanol extract of *Lasia spinosa* (MELS) and chlorogenic acid (CA) in the rats of Freund's complete adjuvant (FCA) mediated arthritis by *in vivo* and *In silico* studies. **Materials and Methods:** The MELS was subjected to acute oral toxicity in rats and tested against FCA induced arthritis in rats. Large-scale isolation and chromatographical analysis with spectral review verified that CA is responsible for the pharmacological effects reported. The efficacy of MELS and CA against CFA-induced arthritis was subsequently tested for hematological, biochemical, and *in vivo* anti-oxidant parameters in rats on the last day of the research. The interleukin-6 (IL-6) and tumor necrosis factor-α expressions in the paw tissue were determined by Western blotting technique. Ankle joint histopathological and radiological studies were also conducted. **Results:** MELS and CA dosage based on anti-arthritis, which was obvious in comparison with an arthritis control group with decreased paw volume, joint diameter, and body weight. MELS and CA showed significant anti-arthritic activity by rising RBC and Hb levels and decreasing WBC and platelet levels. The anti-arthritis function has also been verified by the altered biochemical and anti-oxidant parameters MELS (400 and 200 mg/kg) and CA (5, 10 mg/kg). The activity was further confirmed by histopathological radiological tests with *in silico* analysis. **Conclusion:** This study confirms the ethnomedicinal use of *L. spinosa* rhizomes in the treatment of arthritis. It also indicates the strong anti-arthritic effects of CA. Additional clinical trials are, however, required to show the effectiveness of CA in different immuno-inflammatory conditions.

Keywords: *Lasia spinosa* (L.) Thwaites, Anti-arthritic, *In silico*, Chlorogenic acid, α-TNF

INTRODUCTION

Rheumatoid arthritis (RA) is a persistent joint disease occurring 2.5% of the world's total population.[1] This results in a significant loss of quality of life and the resulting degradation that has a significant socio-economic impact.[2,3] The facilitating incident of RA is accompanied by an auto-immune response inflammatory reactions of the synovial membrane; a joint structure usually made up of macrophage and fibroblast-like cells known as synoviocytes.[4] RA pathogenesis often includes reactive oxygen species (ROS) which trigger cartilage destruction either by direct degradation of the matrix or by triggering the matrix metalloproteinase (MMP).[5]

The most common forms of RA are under the category of inflammatory immune arthritis (IIA).[6] The cytokines, proteinases, oxygen derivatives, and interleukins (IL) are inflammatory mediators found in the blood plasma and synovial fluid during IIA that have been linked to the inflammation

and cartilage destruction. These mediators are synthesized by immune cells and released into an inflamed joint.[7] In the pathogenesis of RA, there was a significant role of NF-κB signaling.[8] In the nuclear DNA, the responsive element to NF-κB is transcribed and produced cytokines such as tumor necrosis factor-α (TNF-α), IL-6, and IL-1β.[9] The primary inflammatory reaction during RA is due to the synthesis and release of histamine, leukotrienes and prostaglandins (PG).[10] The PG's (PGG2 and PGH2) are synthesized from a fatty acid-derived substance arachidonic acid by COX-2 enzyme in response to immunological and chemical stimuli. The anti-inflammatory effect of many phytomedicines is because of inhibition of COX-2 and IL.

Rat Freud-induced arthritis (FA) is a typical human RA condition, causing weight loss, oxidative tissue degradation, and swelling/destruction joint-related inflammatory infiltration.[11,12] Arthritis develops through a cell-mediated auto-immune among mycobacterium capsule and proteoglycan cartilage within 12–14 days of adjuvant infusion.[4] Nonsteroidal anti-inflammatory medications, anti-rheumatic medicines, immunosuppressants, and anti-cytokines are commonly used in adults with RA to manage inflammatory symptoms/pain. The main concerns are persistent impoverishment, toxic effects, GIT disturbances, CVS abnormalities, autonomic dysfunction, and significant cost circumstances.[12] Researchers also paid particular attention to anti-inflammatory and anti-oxidant herbal drugs for the effective treatment of arthritis.[13]

Herbal medications were widely used for treating arthritis,[14] and it is well documented. The development of novel medical approaches was primarily focused on an ethnopharmacology understanding of medicinal plants and experimental research.[15,16] One such traditional plant is *Lasia spinosa* (Araceae) which is an annual plant, and it has long been traditionally used as an anti-inflammatory, anthelmintic, emmenagogue, anti-oxidant, ant diabetic, and antimalarial activities.[17,18] The plant *M. alliacea* has been reported to contain several phytoconstituents including p-hydroxybenzoic, 3-hydroxy-4-methoxy benzoic, 2-(4'- methoxyphenyl)-ethanol, adenine,[19] β-sitosterol acetate, and stigmasterol.[20] Therefore, the purpose of the current study was to examine the efficacy of methanol extract of *L. spinosa* (MELS) and isolated compound CA on FA-induced arthritis in rats and the possible mechanism of the action. The active site of the TNF-α and IL-1β was assessed and perform the molecular docking to study binding mechanisms of these compound.

MATERIALS AND METHODS

Collection of Plant Material

The whole plant of *L. spinosa* (L) Thwaites was collected from kommulamamidi, near kottapalli village, paderu in Visakhapatnam (Andhra Pradesh) in the month of November- December 2018. The plant materials were authenticated by Prof S.B. Padal, Department of Botany (Voucher specimen number 23302), Andhra University, and Visakhapatnam. The collected plants were processed. The processed rhizomes were sliced dried under shade for 10 days, coarsely powdered and passed through sieve 60# and stored in an airtight container for further study.

Extraction of Plant Material

The powdered rhizomes of the plant were employed for extraction. The powdered rhizomes (5 kg) were extracted 3 times by Soxhlet apparatus with hexane, ethyl acetate and methanol solvents. The resulting filtrate was pooled and dried under reduced pressure by rotary evaporator at 40°C (Buchi R-210, Switzerland), The extracts were collected and stored in a desiccator for further phytochemical and pharmacological studies.[5,21]

Isolation of Chlorogenic Acid (CA)

To isolate an anti-arthritic compound from MELS was performed on column chromatography using stationary phase as silica gel (1:2), 100–200 mesh, and packed in column (150 × 6 cm) using n-hexane solvent and 80 g of methanol extract was loaded on the top of silica gel. The column was eluted using non-polar to polar solvents in various proportions. A total of about 59 fractions measuring 200 mL each were collected and concentrated by the rotary evaporator. Weight of each fraction was measured. The above fraction was eluted with hexane: chloroform (80:20–0:100 v/v). Further, in 54th fraction was found to be pure as they showed a blue color spot on TLC eluted using chloroform: methanol:formic acid (15:3:2) solvent system and on spraying with methanolic sulfuric acid reagent (10%). The Rf value was 0.58 and yields were reported. This pure compound has been exposed to multiple spectroscopic techniques to describe the structure.

In Silico Studies

Preparation of crystallographic protein structures

The protein's atomic coordinates, TNF-α (PDB ID: 2AZ5) and IL-6 (PDB ID: 1ALU) were retrieved from the RCSB PDB site [Figure 1]. The charge assignment, solvation parameters, and fragmental volumes to the protein were performed using PyRx before study or docking. The protein molecule was further designed for molecular docking.[22-26]

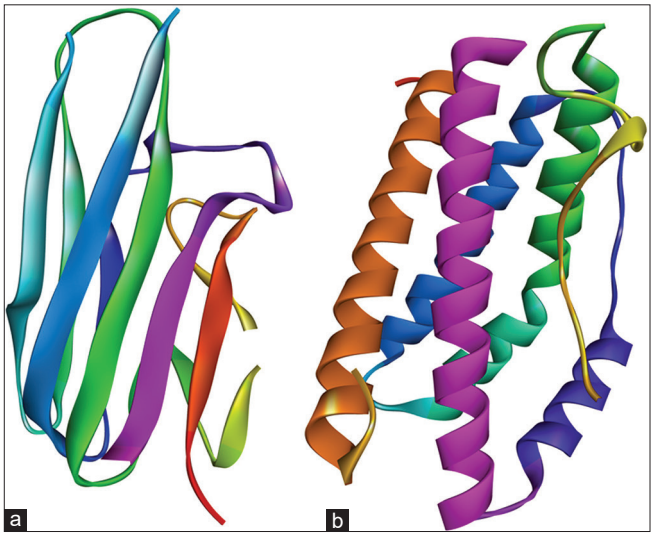

Figure 1: 3D structures of (A) TNF-α (PDB ID: 2AZ5) (B) IL-6 (PDB ID: 1ALU)

Ligands Preparation and Prediction of Drug Likeliness

From the PubChem database, CA 3D structure was obtained.[27] The drug likeliness properties of CA was analyzed using DruLito tool.

Compound Screening using PyRx Program

The auto-dock wizard was used as docking engine to molecular check all compound libraries with PyRx software.[28] The ligands were found versatile during the docking process, and the protein should be rigid. The grid parameter configuration file was created with PyRx with dimensions of (X = –19.409600, Y = 74.650750, Z = 33.849550for 2AZ5; X = –9.806735 Y = –16.273673, Z = 4.449286, for 1ALU). In this test, we predicted the active amino acids of proteins involved in interactions with the ligands. The tests for the root-mean-square deviation of less than 1.0 Å were deemed optimal and grouped to determine the desirable relation. The lowest (most negative) binding energy was recognized as the most binding ligand.

Analysis and Visualization

Biovia Drug Discovery Studio 2020 was used to examine the docking site visually, and the results were confirmed with AutoDock Vina.[29]

ADMET Analysis

The ligands ADMET with their pharmacokinetic properties must be investigated to establish their role within the body. The ADMET history of the ligands was studied, and admetSAR was used.[30,31]

Animals

In the study, Albino Wistar rats (170–200 g) were purchased from the Mahaver Company, Hyderabad and housed in a regular hygiene animal house at 25 ± 2°C and humidity (60 ± 10%) at 12 h of day and night with food and water *ad libitum*. Following the consent of the College, the research was conducted according to CPCSEA specifications with IAEC no. IAEC-11/AU-Pharm/2018-19.[5,32]

Acute Toxicity Studies

The acute oral toxicity study in compliance with the OECD Guidelines-423 was performed for MELS.[33] In brief, the extract was given orally to rats fasted overnight. For the first 24 h, animals were subjected to general clinical observations. For the early 4 h, they were strictly supervised. They have subsequently been kept for 14 days under daily surveillance. The doses of 1/5th and 1/10th of MELS were selected based on the acute toxicity test.

Freund's Complete Adjuvant (FCA)-Induced Arthritis

Arthritis was exacerbated by 0.1 ml of carrageenan adjuvant injected into the right paw. The animals were classified into various categories with six animals each:

- Group I: Non-arthritic, Distilled water-treated animals
- Group II: Arthritic animals treated with 0.5% FA in distilled water
- Group III: Animals treated with Ibuprofen (15 mg kg^{-1}, p.o.)
- Group IV: Animals treated with MELS (200 mg kg^{-1}, p.o)
- Group V: Animals treated with MELS (400 mg kg^{-1}, p.o.)
- Group VI: Animals treated with CA (5 mg kg^{-1}, p.o.)
- Group VII: Animals treated with CA (10 mg kg^{-1}, p.o.).

The dosage of all the groups began on the 12th day. Experimental parameters such as body weight, paw diameter and joint diameter were tested regularly (Days 0, 7, 14, and 28). The retro-orbital puncture was extracted on day 28 and used to measure cytokine levels and biochemical tests.

Arthritis Evaluation

Bodyweight changes

During therapy, all rat's body weight was documented for every 7th day.[34]

Estimation of Paw Diameter and Volume Changes

Paw diameter and edema were measured with plethysmometer on days 0, 7, 14, 21, and 28.[35,36]

The inhibition proportion was calculated using the formula.

Percentage inhibition of edema = (1–Vt)/Vc×100,

Where Vt = joint diameters of treated rats and Vc = joint diameters of control rats.

Biochemical Assays

On the 28th day, the rats were subjected to anesthesia using diethyl ether and separated their blood from retro-orbital plexuses and held them in proper blood tube. For the determination of hematologic parameters such as Hb, RBC, WBC, and ESR, standardized laboratory methods have been applied.

Anti-oxidants

Animals were anaesthetized by ether on the 28th day and slaughtered by cervical dislocation. Animal's liver was easily extracted and washed in a Tris buffer. The liver of each animal was sliced into small pieces and homogenized. For calculating lipid peroxidation (LPO), glutathione (GSH), and catalase, homogenates were used. All these were estimated according to the method described.[37-41]

Estimation of TNF-α, IL-6 Expression by Western Blotting

The paw tissue was centrifuged, and tissue homogenate was subjected to biochemical analysis using the procedure described in Panda *et al*. 2020.[42] The tissue proteins such as IL-6 and TNF-α expression levels were analyzed using Western blotting analysis.

The Reduced GSH, Nitric Oxide, LPO, and Catalase Activity

The paw tissue was centrifuged, and tissue homogenate was subjected to enzyme activity analysis using the procedure described in Ellman.[37,43]

Radiography and Histopathology

Legs of slaughtered rodents at the knee joints were amputated, and X-rays were taken for test and control animals to assess the level of arthritis incidence and then for microscopical histologic study.[35,44,45]

Statistical Analysis

The result is regarded as mean ± SEM. The results were evaluated using ANOVA one-way with the use of the Graph-Pad Prism 5 software to *t*-test Dunnet. $P < 0.05$ was found to be statistically significant.

RESULTS

Characterization Isolated Compound

^{1}H NMR (CDCl3, 500 MHz) δ 7.57 (1H, dd, J = 9.918 Hz, 15.569 and 25.787 Hz), 7.06 (H, t, J = 2.316 Hz and 4.425 Hz), 6.94 (H, td, J = 1.831 Hz and 3.662 Hz), 6.81 (H, dd, J = 1.608 Hz and 8.087 Hz), 6.26 (H, t, J = 16.479 Hz and 32.501 Hz), 5.37 (H, m) 4.20 (H, m), 3.73 (H, m), 2.32 (H, m), 2.19 (H, s), 2.15 (H, t, J = 3.357 Hz and 5.798 Hz), 2.05 (H, m). 13C (CDCl3, 500 MHz) δ 175.260, 167.294, 147.492, 145.497, 144.721, 125.941, 121.377, 114.768, 113.507, 113.507, 74.698, 72.563, 70.135, 69.975, 37.836, 36.459. (m/z) = 354, calculated for $C_{16}H1_80_9$ [Figure 2].

In Silico Studies

Drug likeliness properties

Drulito software was used to study the physicochemical properties of the isolated CA [Table 1]. The basic physicochemical properties of TPSA and AMR mainly include drug intake, distribution and penetration functions.

Molecular Docking Studies

Molecular docking was conducted on CA isolated from *L. spinosa* to discover a prospective anti-arthritic candidate against TNF-α and IL-6 (PDB ID: 2AZ5 and 1ALU). The CA was bound to the target proteins TNF-α and IL-6 and rated based on their dock results. For a detailed review, refer to Table 2 [Figure 3].

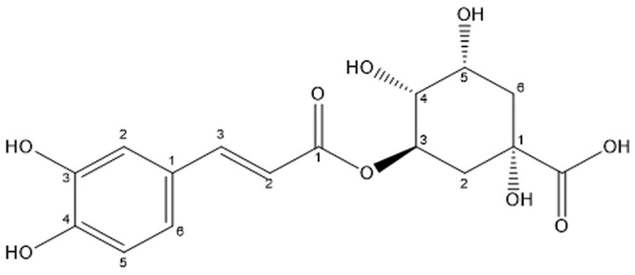

Figure 2: Structure of Chlorogenic acid isolated from Rhizomes of Lasia spinosa

ADME/T Evaluation Using admetSAR

The ligand ADMET properties were evaluated using the admetSAR method, and toxicity was estimated by toxicity estimation toxicity tool [Figure 4].[46,47] Exemplary human intestinal absorption (HIA) and blood-brain barrier penetration were found to be CA. Greater HIA indicates that the compound may be best absorbed through oral administration from the intestinal tract. The ligand showed a negative toxicity test for AMES, which indicates that the ligands are not mutagens. Comparing the LD50 concentration, the lower dose compound is lethal than, the higher LD50 compound Table 3 displays the different ADMET parameters obtained from the method admetSAR.

Acute Toxicity Studies

In Wistar albino rats, MELS were assessed for acute toxicity and animals were tracked for 24 h. MELS does not produce any fatality up to 2000 mg kg^{-1}, so 1/10th of dose, that is, 200 and 1/10th of the dose, that is, 400 mg kg^{-1} have been chosen for this research.

Effect of MELS and CA on the Weight of Adjuvant-induced Arthritic Rats

As shown in Table 4 and Figure 5, the weight reductions in treated arthritic rats in relation to negative control is prevented by the MELS (200 and 400 mg kg-1) and the CA (5 and 10 mg kg-1. Likewise, Ibuprofen, a therapy of the reference medication, substantially raised the rats' body weight in the same direction as MELS and CA.

Effect of MELS and CA on Paw Volume of Adjuvant-induced Arthritic Rats

The findings demonstrated a substantial increase ($P < 0.05$) in paw size after arthritis induction [Figures 6 and 7 and Table 5]. However, in contrast to harmful measures, MELS (200 and 400 mg/kg) and CA (5 and 10 mg/kg) greatly decrease paw swelling in treated arthritic groups. At higher doses, MELS and CA were more beneficial. The peak impact was observed at 400 mg kg^{-1} on the 28th day.

Hematological Parameters

Table 6 and Figure 8 demonstrates changes in hematology in adjuvant mediated arthritic rats. RBC count and hemoglobin declined significantly; WBC count and the ESR of arthritis rats improved considerably in contrast with the rats in the test. Higher doses of MELS and CA treatment showed major improvements to hematology of both adjuvant-induced arthritis progression and development.

Anti-oxidants

LPO, NO, and TPC amounts have risen and decrease in GSH and CAT level in the arthritic control group. The treated category of Ibuprofen demonstrated substantial reductions in levels of the LPO and NO compared with arthritis control and

Table 1: Physicochemical properties of active compounds and accordance with the rule of drug-likeliness

Ligand	MW	Logp	Alogp	HBA	HBD	TPSA	AMR	nRB
Chlorogenic acid	354.1	–0.7	–1.194	9	6	164.75	85.8	5

Table 2: Interactions of COVID-19 Main Protease (PDB ID: 6LU7) amino acid residues with ligands at receptor sites

Ligand	Target	Binding affinity, ΔG (Kcal/mol)\	Amino acids involved and distance (Å)	
			Hydrogen binding interactions	Hydrophobic interactions
Chlorogenic acid	2AZ5	–6.6	ALA A: 22 (3.72), GLY A: 24 (3.33), ASP A: 140 (3.30), LYS A: 65 (5.70)	-
	1ALU	–6.6	THR A: 43 (3.86), ASP A: 160 (4.44), GLN A: 156 (5.47), ARG A: 104 (4.25), GLN A: 159 (5.04, 5.49), ASN A: 103 (3.46)	LYS A: 46 (4.47, 6.43), ARG A: 104 (3.71)

Table 3: ADME/T properties of isolated chlorogenic acid

Compound	HIA	BBB	AMES toxicity	Carcinogenicity	LD_{50} in rat (mg/kg)
Chlorogenic acid	0.7433	0.5663	Non AMES Toxic	Non-carcinogen	3526.44

Table 4: Effect of MELS and CA on body weight changes in CFA-induced arthritis. Each value is mean±S.E.M (n=6)

Groups	Dose (mg/kg)	Initial body weight (g) Day 0 (mean±SEM)	Final body weight (g) Day 28 (mean±SEM)
Normal control	-	138.26±6.51	145.86±3.22
Arthritic control	-	133.71±7.07	128.62±4.58$^{@}$
Ibuprofen	15	136.32±8.15	148.22±3.85$^{@}$
MELS	200	136.17±8.37	137.11±6.22*
MELS	400	134.71±6.93	138.45±5.25$^{\#}$
CA	5	136.97±8.27	142.54±3.91$^{\#}$
CA	10	135.97±8.07	146.22±4.92$^{\#}$

$^{@}P<0.001$, $^{\#}P<0.01$, $^{*}P<0.05$ when compared to normal control

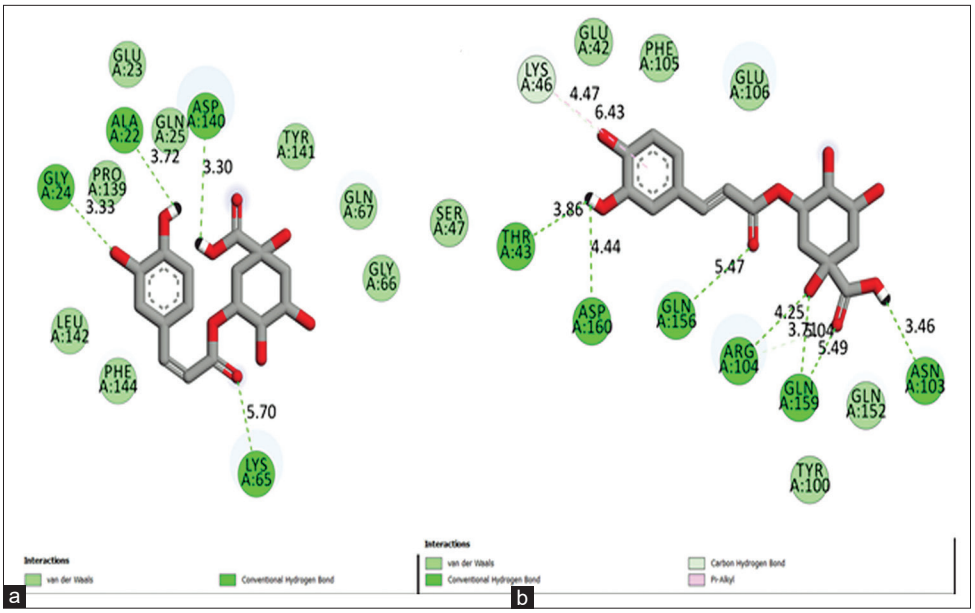

Figure 3: 2D Interactions of Chlorogenic acid (CA) with (A) α- TNF (PDB ID: 2AZ5) (B) IL-6 (PDB ID: 1ALU)

a marked rise in GSH, CAT compared with arthritis control. The MELS (50 and 100 mg/kg) and CA (5 and 10 mg/kg) therapies demonstrated a substantial improvement from arthritis in GSH, CAT, and TPC [Table 7].

Western Blotting

In the MELS, CA, and Ibuprofen treated groups, the inflammatory marker proteins such as α-TNF and IL-6 expressions were significantly reduced as compared to arthritic control groups [Figure 9].

Effect of MELS and CA on X-ray Analysis of Hind Limbs in FA-induced Arthritis

Radiographical changes in RA parameters are useful measures for diagnosing the severity of the disease. The swelling of soft tissue is early radiographic, although remarkable X-ray changes

such as bone degradation and the spread of joint gaps can be seen mainly during the late stages (final stages) of arthritis. Figure 10 illustrates rat joints' radiographic characteristics in the arthritic adjuvant model. In adjuvant-induced arthritic rats, swelling of soft tissue with joint spacing was observed suggesting arthritic osseous death. The standard medicines treated with Ibuprofen prevented this ossic destruction, and there was no joint swelling. Similar to histopathological study, 28-day treatment by MELS (200 and 400 mg/kg) and CA (5 and 10 mg/kg) showed a substantial decrease in osteoarthritis by demonstrating less inflammation of soft tissue and expansion of the joint area in the cohorts treated with 14-days.

Effect of MELS and CA on FA Histological Evaluation of Induced Arthritis

The histology of normal rats shows the intact bone structure without noticeable cell invasions and synovial tissue vasculature. Arthritic rats showed mononuclear cell infiltration of typical redness and granuloma and vascular tissue in synovial tissues in the negative control group, accumulation of fibrous tissue and invasion of synoviocytes in the subchondral bone. In the Ibuprofen-treated group, the microsections shows tissue composed of normal bony trabeculae lined by periosteum and also contains haemopoietic elements in normal number and maturation. No necrosis and new bone formation seen. In MELS (200 mg/kg) and CA (5 mg/kg), administered rat joints, moderate redness, fibrin deposits, and bone degradation were observed whereas in the rats with MELS (400 mg/kg) and CA (10 mg/kg) therapy, mainly, composed of normal trabeculae with hemopoietic elements in normal number and a few scattered inflammatory cells were observed. No necrosis or new bone formation occurs [Figure 11].

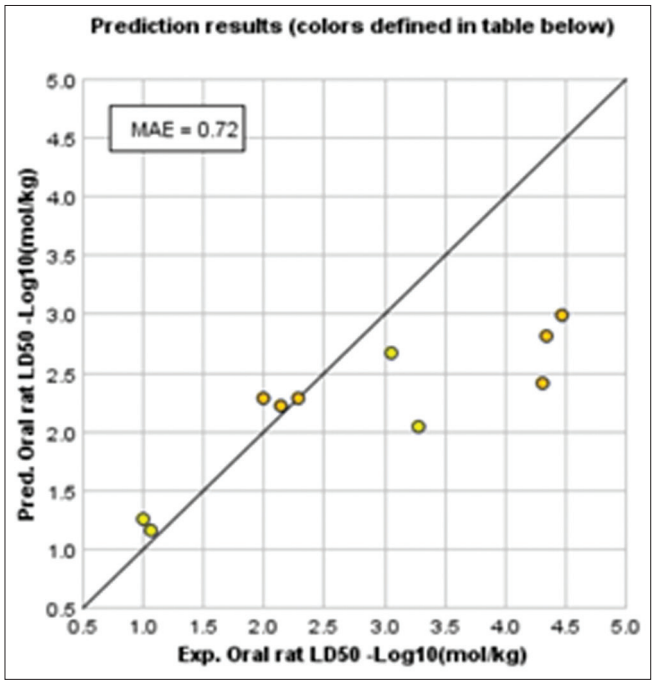

Figure 4: Predicted oral acute toxicity in rats by Toxicity Estimation Toxicity Tool (TEST)

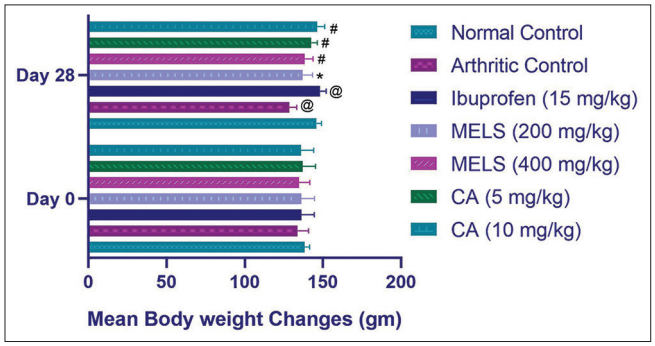

Figure 5: Effect of MELS and CA on body weight of CFA induced arthritis at day 0 and day 28

DISCUSSION

Alternative medicines are becoming more effective in the treatment of rheumatism. Many medicinal plants ease RA symptoms, the results of which compared to conventional medicinal agents available.[16,48-50] Acute toxicity studies have shown that the extract is non-toxic at a dose of 2000 mg/kg. RA

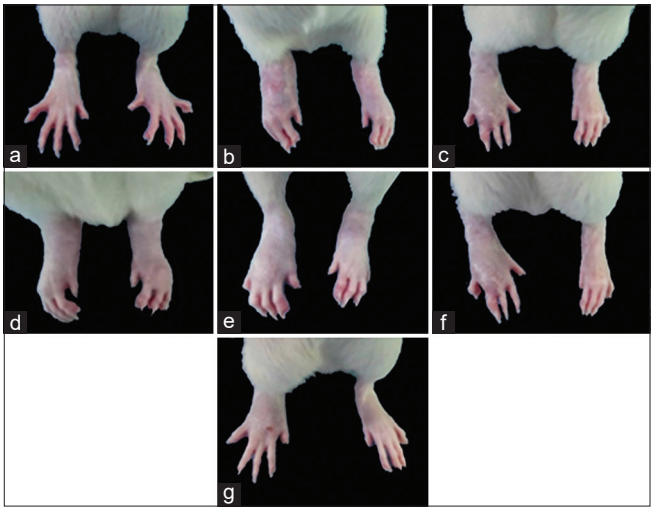

Figure 6: Representative photographs of the right hind paw of healthy control and arthritic rats on day 28 after adjuvant injection. (A) Normal control. (B) Arthritic control. (C) Ibuprofen treated (15 mg/kg) (D) MELS (200 mg/kg) treated. (E) MELS (400 mg/kg) treated (F) CA (5 mg/kg) (G) CA (10 mg/kg)

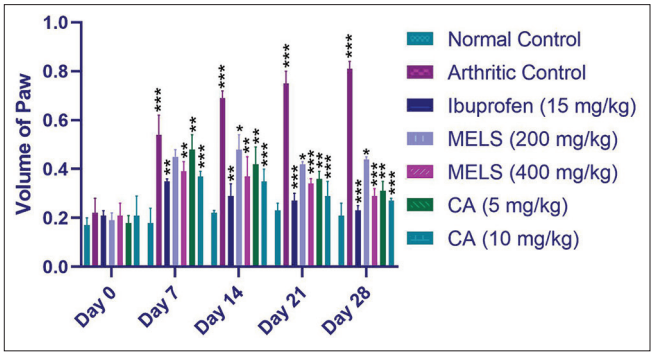

Figure 7: Effect of MELS, CA and ibuprofen on paw volume of CFA induced inflammation Each value is Mean ± S.E.M (n = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared to normal control

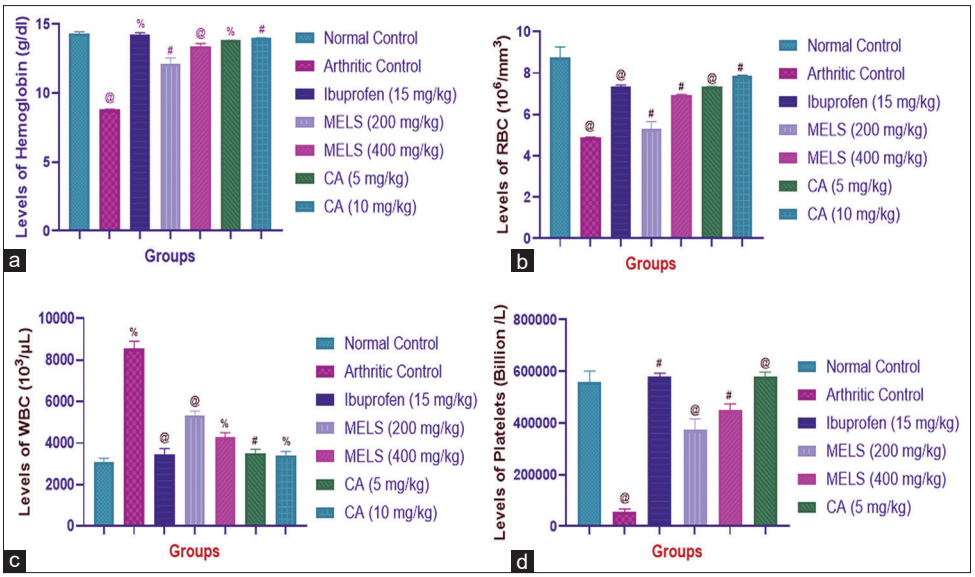

Figure 8: Effect of MELS, CA and ibuprofen on haematological parameters

Table 5: The influence of the MELS and CA on the entire volume of paw of Freund's mediated adjuvant (ml)

Group	Day 0	Day 7	Day14	Day 21	Day 28
Normal Control	0.17 ± 0.03	0.18 ± 0.06	0.22 ± 0.01	0.23 ± 0.03	0.21 ± 0.05
Arthritic Control	0.22 ± 0.06	0.54 ± 0.08***	0.69 ± 0.03***	0.75 ± 0.05***	0.81 ± 0.03***
Ibuprofen	0.21 ± 0.02	0.35 ± 0.01**	0.29 ± 0.05**	0.27 ± 0.03***	0.23 ± 0.02***
MELS (200 mg/kg)	0.19 ± 0.03	0.45 ± 0.03	0.48 ± 0.06*	0.42 ± 0.01*	0.44 ± 0.01*
MELS (400 mg/kg)	0.21 ± 0.05	0.39 ± 0.04**	0.37 ± 0.08**	0.34 ± 0.02***	0.29 ± 0.03***
CA (5 mg/kg)	0.18 ± 0.03	0.48 ± 0.06**	0.42 ± 0.07**	0.36 ± 0.03**	0.31 ± 0.04**
CA (10 mg/kg)	0.21 ± 0.08	0.37 ± 0.02***	0.35 ± 0.05***	0.29 ± 0.06***	0.27 ± 0.01***

*P< 0.05, **P< 0.01, ***P< 0.001 against the control groups as contrasted to normal group

Table 6: Effect of oral administration of MELS and CA on Haematological parameters in arthritic rats on day 28

Group	Hemoglobin (g/dl)	RBC (10^6/mm^3)	WBC ($10^3/\mu$L)	Platelets (Billion/L)
Normal Control	14.32±0.13	8.75±0.52	3100±168.1	557881±42869.1
Arthritic control	8.82±0.01$^{@}$	4.89±0.02$^{@}$	8548±362.1$^{\%}$	57283±10647.8$^{@}$
Ibuprofen (15 mg/kg)	14.22±0.16$^{\%}$	7.37±0.05$^{@}$	3428±312.9$^{@}$	580613±12622.3$^{\#}$
MELS (200 mg/kg)	12.12±0.42$^{\#}$	5.32±0.03$^{\#}$	5326±216.2$^{@}$	372922±43678.3$^{\#}$
MELS (400 mg/kg)	13.39±0.22$^{@}$	6.92±0.05$^{\#}$	4281±224.1$^{\%}$	448792±26371.9$^{@}$
CA (5 mg/kg)	13.82±0.02$^{\%}$	7.35±0.01$^{@}$	3528±178.2$^{\#}$	561482±10387.2$^{\#}$
CA (10 mg/kg)	14.01±0.01$^{\#}$	7.89±0.01$^{\#}$	3489±213.9$^{\%}$	579289±18674.1$^{@}$

The findings for each study groups are represented as mean±SEM. The statistical significance was conducted using the ANOVA approach in one direction, accompanied by the evaluation by Bonferroni. $^{@}P$<0.001, $^{\%}P$<0.01, $^{\#}P$<0.05 in comparison with normal control

is a persistent inflammatory disorder that affects about 1% of the population of the developing world. Due to this overlap in physiology, adjuvant-induced arthritis is commonly used in the study of the efficacy of anti-inflammatory drugs as a RA paradigm.

The present research demonstrated an anti-arthritic impact on both inflammation parameters of MELS (400 and 200 mg/kg) and CA (5 and 10 mg/kg) therapy. The inflammation was substantially reduced relative to the arthritic control group, as the diameter and thickness of the paw reduced. The present research found that the paw volume in rats threatened by FCA rises with knee stiffness. The fall in body weight is attributed to a decreased absorption of nutrients by the bowels during inflammation.[51] Consequently, MELS and CA restoring the bodyweight of rats will enhance nutrient absorption via the intestines of rats.

Ibuprofen (NSAID) was being used as a pain-relieving drug for RA - disorders. In this study, ibuprofen administration demonstrated effective anti-arthritic efficacy against FA-induced arthritis by inhibiting TNF-α and IL-6. Nevertheless, side effects, such as nausea, diarrhea, pain, and increased

Table 7: MELS and CA impact in Freund's adjuvant mediated arthritic rats on antioxidant levels

Group	NO (μmol/L)	Lipid peroxide (μmol/L)	Catalase (μmol/L)	MDA (μmol/L)	GSH (μmol/L)	Total protein carbonyl content (nmol/mg)
Normal control	6.483±0.414*	4.136±0.198**	5.899±0.397**	5.212±0.721*	2.518±0.012*	0.527±0.081*
Arthritic control	10.421±1.119	9.782±1.313	2.901±0.152	6.725±0.263	0.739±0.140	1.670±0.056
Ibuprofen	6.956±1.021***	5.962±2.891**	5.221±0.928***	5.282±0.186***	2.385±1.650***	0.451±0.049**
MELS (200 mg/kg)	7.893±1.279**	7.319±1.561***	6.002±0.462**	5.891±0.387**	4.212±0.324**	0.986±0.012*
MELS (400 mg/kg	7.4826±1.09***	6.814±1.680*	5.532±0.532**	5.755±0.512***	3.681±3.761*	0.623±0.032***
CA (5 mg/kg)	7.136±1.546**	6.218±2.121*	5.300±1.825***	5.512±0.360*	3.142±2.542**	0.590±0.145**
CA (10 mg/kg)	6.796±1.412*	5.762±1.183**	5.183±1.462*	5.482±0.126*	2.228±3.547**	0.432±1.101**

The findings are displayed as medium±SEM. The results have been evaluated using single-way variance analysis and the Dunnett test. *P<0.05, **P<0.01, ***P<0.001 when compared with arthritic control group

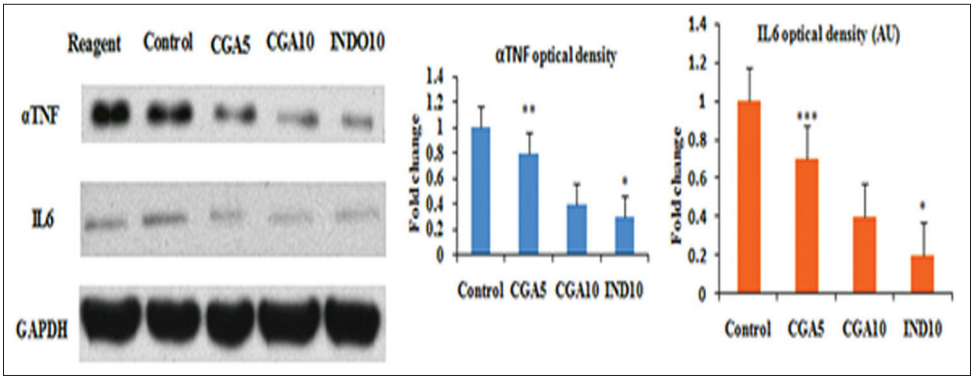

Figure 9: Immunoblot images of α-TNF and IL-6 signalling with their quantification

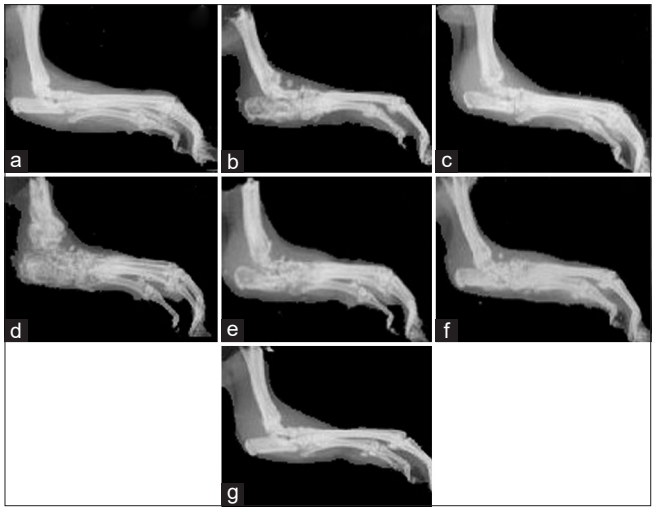

Figure 10: Radiology of hind legs in adjuvant induced arthritic rats. (A) Normal control (B) Arthritic control. (C) Ibuprofen 15 mg/kg treated. (D) MELS 200 mg/kg treated (E) MELS 400 mg/kg treated (F) CA 5 mg/kg (G) CA 10 mg/kg

blood pressure, have been reported. Such related side-effects limit its therapeutic use to combat arthritis. However, MELS's side effects are not established at this point.

Throughout arthritis, the bone deformation and joint impairment caused by inflammatory reaction associated with hyperalgesia induced by PG and other endogenous mediators.[52] FA-induced arthritis rates are shown to improve nociception when inflammatory mediators such as TNF-α and IL1-β are released.[53] MELS and CA groups showed substantial paw volume decreases in contrast with the control group. The day after the adjuvant injection, there was a substantial loss of weight, but afterwards, the usual weight recovery was maintained for rats. The results of this study also shown that the extent of inflammation and loss in body weight is closely related to each other. The findings indicated that MELS therapy has anti-inflammatory consequences, as shown by a significant decrease of paw edema of arthritic animals.

The result showed decreased rates of Hb, premature degradation of RBC and reduced rates of erythropoietin from bone marrow in arthritic rats were documented.[54] The most important causes are abnormal iron storage and the failure of the bone marrow to respond to anemia[55] in the reticuloendothelial system and the synovial tissue.[56] The MELS and CA therapy increase the RBC count and hemoglobin rates, suggesting that the anemia is fully restored. The significant increase in leukocyte count in adjuvant-induced arthritic rats can be attributed to the stimulation and decrease of the immune system against an invasive antigen in the treated MELS and CA groups. In arthritic control groups, the number of ESRs which has significantly increased has been significantly reversed by MELS, CA and standard drug,

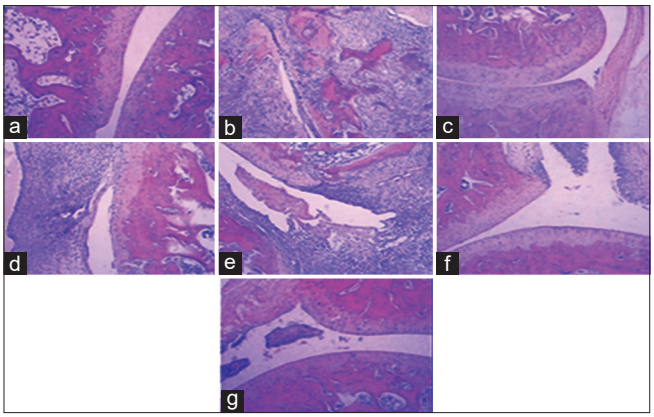

Figure 11: Histopathological photos of ankle joints stained with H&E. (×100) (A) Healthy control. (B) Arthritic control. (C) Ibuprofen 15 mg/kg treated. (D) MELS 400 mg/kg administered. (E) MELS 200 mg/kg treated (F) CA 5 mg/kg treated (G) CA 10 mg/kg treated

Ibuprofen, which restore its importance in arthritic conditions back to almost normal.

To avoid and neutralize free radicals, the body has an important anti-oxidant function. A variety of endogenous anti-oxidant enzymes, such as CAT included when the equilibrium is lost between the development of ROS and anti-oxidant defense, "oxidative stress" results that deregulates the cellular activity of a sequence of events leading to different pathological conditions.[57]

The reduced CAT in RA is due to their inactivation with H_2O_2 and indicates that rheumatics and oxidative stress can be caused by these enzymes.[58] GSH is usable in oxidized and reduced interconvertible forms. The reduced GSH, in turn, retains the cellular level of the active Vit-C type. GSH plays a major function in cell and tissue structure protection.[59] The reduced GSH levels are correlated with several pathological disorders. This could be for a variety of reasons. Oxidative stress, for example, may cause GSH loss by oxidation.

LPO may thus play a part in the pathogenesis of the RA.[58] In this analysis, the LPO level of CFA-induced arthritis rats for MELS and CA decreased significantly and possibly suggested prevention of cell destruction by minimizing oxidative stress. In the current MELS and CA research, levels of CAT and GSH will increase dramatically by preventing H_2O_2 inactivation of these enzymes or reducing oxidative stress.

Inflammatory mediators such as IL-6, IL-1β, and TNF-α play a key role in pathogenesis by promoting immune cell infiltration and triggering MMP release. Inflammatory cells with specific anti-inflammatory mediators produced for inflammation alleviation are used to excrete large volumes of pro-inflammatory cytokines. They lead to several characteristics of arthritis, including synovial tissue inflammation, synovial growth, and cartilage and bone damage. IL-6, IL-1β, and TNF-α are shown to be a crucial role in RA pathogeny of several pro-inflammatory factors. It was also hypothesized that IL-6 and IL-1β contribute to arthritis so that downregulation of the production of these cytokines can be an effective way in which RA therapy can be conducted. Overproduction of TNF-α, IL-1β or IL-6 in Freud adjuvant arthritis serum rats have also been discovered, which is the pathological mechanism, and clinical representation has significant similarity to human RA In this analysis, MELS and CA reduced the serum levels of TNF-α, IL-1β, and NF-kβ significantly in comparison to the arthritis model group. Moreover, the suppressive effects of the high-dose MELS and CA group were higher than the low dose. Such results showed that the anti-inflammatory effects of MELS had been related to its TNF-α and IL-6 level inhibition.

We investigated MELS' chemical profile with LC-MS to detect the chemical responsible for *L. spinosa*'s major anti-arthritis effect. The key components in MELS were 12 phenolic acid derivatives, particularly CA. As previously mentioned, the biological compounds found in the *L. spinosa* could be responsible for by CA.[60,61]

In medicinal chemistry, molecular docking has become an extremely robust tool for deciding the prevalent binding mode(s) of a ligand with the three-dimensional protein. This *in silico* technique can be used to predict a relationship between a small and a protein at the atomic level, to determine the actions of the small molecules in the active position of the target protein. In the anti-arthritic test by chosen ligands, the results encouraged us to recognize the interaction of this compound with the active site of TNF-α. It is a cell-signaling protein that is implicated in inflammation and acute process reactions that ultimately contributes to certain inflammatory health conditions such as RA. TNF-α was present in higher levels in patients with RA, or Crohn's disease-6 is one of the synthesized cytokines produced from immunocytes and known as an important inflammatory mediator.[62]

Online studies using molecular docking programs have become an effective means of promoting experimental medications, partially due to the desired time and budgeting pricing of *in silico* drug testing, relative to traditional laboratory trials. We used a computerized protein-ligand system of docking using free software programs as well as virtualized interactions between the different ligands with the IL-6 and α-TNF in this study.

ΔG indicates insightful ligand docking on active protein site, method of molecular communication such as hydrogen bonding, hydrophobic communication, as well as the required amino acid electrostatic communication, which is a ligand docking phase in favorable shape. The binding affinity of CA is –6,6 kcal/mol with α-TNF and IL-6.

CONCLUSION

MELS (400 mg/kg) and CA (10 mg/kg) ultimately decrease the rat paw volume and restores the hematological and histological defects in adjuvant-induced arthritis rats. Still, radiological tests confirmed CA's anti-arthritic role in FA-induced arthritis. Their capacity to deregulate amounts of TNF-α and IL-6 may be attributed. Interestingly, CA displayed more substantial anti-arthritic impact on FA-induced arthritis in rats. Further study was required to predict the other phytoconstituents responsible for the anti-arthritic activity.

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