Original Article

Thai Journal of Pharmaceutical Sciences

Inhibitory effects of *Manosa alliacea* in Freund's adjuvant arthritis on inflammatory markers and its confirmation by *In-silico* strategy

D. S. N. B. K. Prasanth[1], Sk. Aminabee[2], A. Lakshmana Rao[3], Chakravarthi Guntupalli[1], Alavala Rajasekhar Reddy[4], Umasankar Kulandaivelu[4], S. N. Koteswara Rao[5], P. Rajeshwari[6]

[1]Pharmacognosy Research Division, K L College of Pharmacy, Koneru Lakshmaiah Education Foundation, Guntur, Andhra Pradesh, India, [2]Department of Pharmacology, V. V. Institute of Pharmaceutical Sciences, Krishna, Andhra Pradesh, India, [3]Department of Pharmaceutical Analysis, V. V. Institute of Pharmaceutical Sciences, Krishna, Andhra Pradesh, India, [4]Pharmaceutical Chemistry Research Division, K L College of Pharmacy, Koneru Lakshmaiah Education Foundation, Guntur, Andhra Pradesh, India, [5]Pharmaceutics Research Division, K L College of Pharmacy, Koneru Lakshmaiah Education Foundation, Guntur, Andhra Pradesh, India, [6]Pharmacology Research Division, K L College of Pharmacy, Koneru Lakshmaiah Education Foundation, Guntur, Andhra Pradesh, India

Corresponding Author:
Dr. D. S. N. B. K. Prasanth, Pharmacognosy Research Division, K L College of Pharmacy, Koneru Lakshmaiah Education Foundation, Vaddeswaram, Guntur, Andhra Pradesh, India.
Ph: +91-7382027437.
E-mail: dsnbkprasanth@kluniversity.in

Received: Mar 30, 2021
Accepted: May 02, 2021
Published: Dec 04, 2021

ABSTRACT

For the assessment of the curative effect of *Manosa alliacea* on Freud's adjuvant (FA) arthritis on Swiss albino rats. Methanol extract from *M. alliacea* (MEMA) was administered orally at 200 mg/kg and 400 mg/kg, 28 days after FA immunization. For control and treatment groups, paw volume, body weight, hematological parameters, X-ray, and histological tests were measured. In addition, reverse transcription-polymerase chain reaction (RT-PCR) was used to measure the levels of various inflammatory markers. *In vitro*, DPPH and H_2O_2 tests were used to evaluate the antioxidant capability. MEMA decreased paw volume and paw thickness, bodyweight considerably ($P < 0.05$, 0.01, 0.001), compared to hematological anomalies of arthritis control. X- rays tests and histological tests did not reveal significant structural changes in the rat ankle joints administered with MEMA. The levels of expression tumor necrosis factor - α, NF-Kβ, IL-1β, and COX-2 were significantly suppressed in the treatment groups. The *in-silico* study has shown that a number of chemical components in the plants under study can effectively bind to various inflammatory targets. That is why we say *M. alliacea* is a good source for treating rheumatoid arthritis.

Keywords: *Manosa alliacea, In silico, In vivo*, Freund's adjuvant arthritis

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, paralytic joint disease that affects 1% of the adult population worldwide.[1] This results in a significant loss of quality of life and the resulting degradation that has a significant socio-economic impact.[2,3] An inflammatory reaction to synovial membrane inflammation, joint lining, which is typically made up of macrophage, and fibroblast-like cells, was activated and called synoviocytes.[4] Free radicals, in particular reactive oxygen species (ROS), also involve RA pathogenesis and cause cartilage destruction either through a direct degradation of the matrix or through activation of 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's) 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] The RA is associated with the significant role of Nuclear Factor Kappa (NF-κB) signalling.[8] The inflammatory stimuli phosphorylate and degrade the IκBα with subsequent translocation of NF-κB into the nucleus. In the nuclear DNA, the responsive element to NF-κB is transcribed and produced cytokines such as tumor necrosis factor (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 IL, whereas the late phase is due to the release of prostaglandins (PGs).[10] The PGs (PGG2 and PGH2) are synthesized from a fatty acid-derived substance arachidonic acid (AA) by the 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 (Freud's adjuvant [FA]) is a typical human RA condition, causing weight loss, oxidative tissue degradation, and swelling/destruction joint-related inflammatory infiltration.[11,12] Arthritis develops within 12–14 days of adjuvant injection through a cell-mediated autoimmune between mycobacterium capsule and cartilage proteoglycan.[4] Non-steroidal anti-inflammatory medications, anti-rheumatic medicines, immunosuppressants, and anti-cytokines are commonly used in adults with RA to manage inflammatory symptoms/pain. The primary worries are impoverished chronic efficacy, potential toxicity, gastrointestinal tract disorders, cardiovascular disorders, immunodeficiency, and high cost.[12] Researchers also paid particular attention to anti-inflammatory/antioxidant herbal drugs and raising side effects to treat RA. Botanical remedies have recently been promoted in the U.S. for the treatment of arthritis, especially following the removal of FDA authorized anti-inflammatory drugs.[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] One such traditional plant is *Manosa alliacea* (Bignoniaceae) which is an annual plant, and it has long been traditionally used as an analgesic, anti-inflammatory, anti-arthritic, anti-pyretic, purgative, vermifuge, anti-rheumatic, anti-malarial, pneumonia, and other respiratory disorders.[16-18] The chemical constituents and efficiency of extracts from *M. alliacea* had been intensively explored in earlier research. The plant *M. alliacea* has been reported to contain several phytoconstituents including β-amyrin, β-sitosterol, ursolic acid, β-sitosteryl-d-glucoside, apigenin, luteolin, 7-O-methylscutellarein, apigenin-7-glucuronide,[19,20] alliin,[21] 9-methoxy-α-lapachone, 4-hydroxy-9-methoxy-α-lapachone, 19-hydroxy hexatriacontane-18-one,[22] glycyrrhetol, β-peltoboykinolic acid, 3β-hydroxyurs-18-en-27-oic acid[23] and possess anti-hyperlipidemic,[24] antifungal,[25] antimicrobial,[26] biocide.[27]

Therefore, the purpose of the current study was to examine the efficacy of adjuvant-induced arthritis in rats for methanol extract *M. alliacea* (MEMA) and the possible mechanism of the action. In addition, the active site of cyclooxygenase 2 (COX-2), the TNF - α, NF-κB -light-chain-enhancer of activated B cells (NF – kB) and IL-1β has been molecularly docked to investigate binding mechanisms of such compounds.

MATERIALS AND METHODS

Collection of Plant Material

M. alliacea's dried whole plant from Tirupati, Andhra Pradesh, India has been collected. These have been certified in the laboratory by Dr. K. Madhava Chetty's Department of Botany and University of Sri Venkateswara. A voucher specimen (1498) is kept in the laboratory.

Extraction of Plant Material

M. alliacea (2.5 kg) was grounded and extracted by maceration at room temperature using petroleum ether, methanol ethyl acetate (10 l × 3), petroleum ether. The extract was filtered and concentrated under a decreased pressure of 40°C on the rotary evaporator, which resulted in crude extracts, that was screened for phytochemical analysis subsequently.[28]

Antioxidant Activity

DPPH assay

The free radical scavenging activity of each extract was estimated by 1.1-diphenyl-2-picrylhydrazyl (DPPH) assay.[29] A fresh methanol DPPH solution (0.2 mM) was packaged and incubated in the dark 2 h before the test. The raw extracts and even standard ascorbic acid is dissolved separately in their respective extraction solvents. At a differing concentration, 0.05 ml of each sample solution was then transferred into the 96-well plates. A multi-channel pipette was used to add the methanol solution of DPPH (0.195 ml). The resulting mixture was evaluated by a 540 nm micro-plates test after 1 h of dark incubation. A radical activity for DPPH scavenging has been calculated by the gram dry weight equivalent ascorbic acid (mg T.E./g dry weight):

DPPH radical scavenging activity = Absorbance $_{test}$ – Absorbance blank/Absorbance $_{blank}$ × 100.

Hydrogen peroxide assay

Dehpour's updated approach calculated the extract behavior for scavenging in relation to hydrogen peroxide radicals.[30] The solution for hydrogen peroxide (40 Mm) was made using the pH 7.4 phosphate buffer, and its amount was measured using a ultraviolet (UV) spectrophotometer to determine the absorbance at 560 nm. Add 0.1 mg/ml of the extract and absorption, determined by using a UV spectrophotometer at 560 nm, to a blank solution with a hydrogen peroxide-free phosphate tampon. The percentage of hydrogen peroxide scavenging extract and the standard compound was determined with the formula:

Hydrogen peroxide scavenging activity = Absorbance $_{blank}$ – Absorbance $_{sample}$/Absorbance $_{blank}$ × 100

Gas chromatography–mass spectrometry (GC-MS) analysis

MEMA has been evaluated by GC-MS. The research was carried out with Agilent Technologies 6890 series gas chromatography and (Agilent) 5973 Agilent Chemstation powered mass select

detector. A capillary screen for eHP-5MS. At 1.0 mL/min flow rate and 37 cm/s linear speed, the carrier gas was extremely pure helium. The temperature of the injector is 250°C. The temperature of the initial oven was 60°C and was projected to increase at 10°C/min to 280°C, with 4 min per stage kept. A split ratio of 20: 1 is added to the injections of 2 µL. The mass spectrometer was operated with the following voltages: ion source temperature 230°C, quadruple temperature 150°C, solvent delay 4 min, and scan scale 50–700 amu, for the mADE ionization of 70 eV electrons and electron multiplier voltage of 1859. Contrasting processing periods and mass spectral data and separation rates with the NIST database defined the compounds.[31]

Experimental animals

Female Wistar rats (170–200 g) were used in work. The animals were imported from Mahaver Company, Hyderabad, and housed in an animal house with standard hygiene at 25 ± 2°C, humidity (60 ± 10%) with a time of 12 h of day and night, with food and water ad libitum. Following the consent of the College Institutional Animal Ethical Committee (1847/P.O/Re/S/16/CPCSEA), the research was conducted according to CPCSEA specifications.

Acute toxicity studies

We acute oral toxicity study in compliance with the OECD Guidelines-423 for MEMA.[32] 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 first 4 h, they were strictly supervised. They have subsequently been kept for 14 days under daily surveillance. The extract dosage was 1/8th of the accepted average effective dose from the acute toxicity test.

Freund's complete adjuvant-induced arthritis

Arthritis was caused by the injection of 0.1 ml of the adjuvant in the right paw. The animals have been divided into six categories, each containing six animals:

- Vehicle control: Non-arthritic animals treated with distilled water
- Adjuvant-Induced Arthritis: Arthritic animals treated with 0.5% CMC in distilled water
- Ibuprofen (15 mg/kg): Arthritic animals treated with Ibuprofen (15 mg/kg, p.o.)
- MEMA (200 mg/kg): Arthritic animals treated with MEMA (200 mg/kg, p.o.).
- MEMA (400 mg/kg): Arthritic animals treated with MEMA (400 mg/kg, p.o.).

The dosage of all the groups began once a day on day 12. The evaluation was performed routinely in experimental parameters such as body weight, paw length, and joint diameter (Day 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 rats' body weight was documented every 7th day.[33]

Measurement of changes in paw diameter and volume

Paw thickness and edema were measured with water plethysmometry on days 0, 7, 14, 21, and 28.[34,35]

The inhibition proportion was calculated using the formula.

$$\text{Percentage inhibition of edema} = (1-V_t)/V_c \times 100.$$

Where Vt and Vc are the joint diameters of treated and control rats.

Biochemical assays

For the determination of hematologic parameters such as hemoglobin (Hb), red blood cells (RBC), white blood cells (WBCs), and ESR, standardized laboratory methods have been applied. Blood samples for TNF - α and COX-2 mRNA expression were collected.

Radiography and histopathology

Legs of killed rats were amputated at the knee joints and X - rays were taken for test and control animals to assess the level of arthritis incidence and then for microscopical histologic study.[34,36]

Determination of mRNA expression levels of TNF-α, NF-kB, IL-1β, COX-2

The TRIzol process has been employed with the use of a Nanodrop Spectrophotometer for complete RNA removal from the whole blood.[37] The package manufacturer's procedure (Thermo scientific, USA) was used to perform cDNA Syntheses. This method used 500 ng total RNA, mixed with 0.5 µg 100 µM dt18 oligo and no-nucleases-free water, per reaction. The resulting mixture was chilled on the ice at 65°C for 5 min. Five solution buffers of the net reaction method were eventually applied to the mixture (250 mM KCl, 20 mM $MgCl_2$, 250 mM Tris-HCl (pH = 8.3), 50 mM DTT); 10 mM dNTP mix; 20 RiboLock RNase inhibitor units; and, of course, 200 M-MuLV reverse Transcriptase enzymes. The mixture was processed in a thermal cycler at 42°C for 1 h. The reaction was stopped for 5 min in due course by heating at 70°C. The Bio-Rad Device has been used to amplify and measure the PCR product for a RT-PCR. Briefly, the reaction blend was developed using a gene-specific primary CDNA mold, SYBR Green Master (2 teas) PCR combination, as well as nuclear-free water and was automated for 45 denaturation cycles (95°C), rinding (60°C), and termination (72°C). This thermal cycler was used for these purposes. Both tests have been done three-fold. For the creation of different primers manually, the genes of the markers used were picked from the Ensembl Genome Database using online feedback (v.0.4.0). Table 1 displays the sequences of the primers.[38]

Docking Studies

Preparation of protein

α-TNF (PDB ID: 2AZ5), NF-KB (PDB ID: 1SVC), IL-1β (PDB ID: 9ILB) and COX-2 (PDB ID: 4COX), X-ray crystal structures were downloaded from the RCSB PDB database [Figure 1].[39,40] Using the graphical UI. UI program "Auto-Dock Tools," docking simulations were prepared, run, and analyzed. The receptor was used to prepare protein in docking tests, Kollman atom

Table 1: Designed primers and amplified bands

Marker	Forward/Reverse	Sequence	Amplified band (Base Pair)
TNF-α	Forward Primer	TCGTAGCAAACCACCAAGCA	181
	Reverse Primer	GTGAGGAGCACATAGTCGGG	
NF-KB	Forward Primer	GCAACTCTGTCCTGCACCTA	203
	Reverse Primer	CTGCTCCTGAGCGTTGACTT	
IL-1β	Forward Primer	CAGGATGAGGACCCAAGCAC	84
	Reverse Primer	CAGGTCGTCATCATCCCACG	
COX-2	Forward Primer	GTGACTGTACCCGGACTGGA	171
	Reverse Primer	TGGATTGAATTCGAAGGAAGGG	

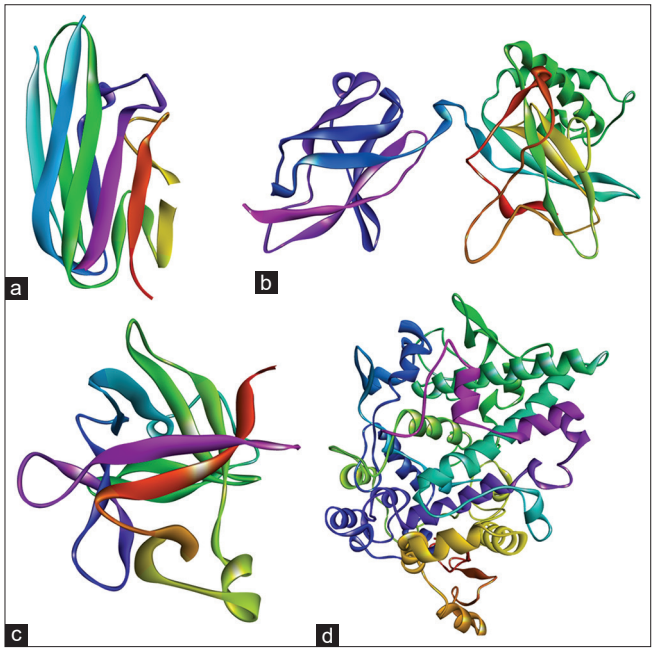

Figure 1: Three Dimensional Structure of the molecular targets. (a) α- tumor necrosis factor (PDB ID: 2AZ5) (b) NF-kB (PDB ID: 1SVC) (c) IL-1β (PDB ID: 9ILB) (d) COX-2 (PDB ID: 4COX)

charges, solvation parameters, and polar hydrogen. As ligands are not peptides, Gasteiger was activated, instead fused non-polar hydrogens. AutoDock requires pre-calculated grid maps, one for each atom shape, in the docked ligand as it stores potential energy. This grid must surround the macromolecule region of interest (active site).[41]

Preparation of ligands and analysis of drug likeliness

PubChem database has been used to retrieve the crystal-3D structure of the active compounds of *M. alliacea*.[42] Drug-likeliness properties of ligands have been analyzed using DruLiTO software for the active compounds selected.[43]

Validation of target protein-ligand complex structures

Autodock 4.0 methodology was validated with the respective co-crystallized ligands of target proteins to ensure the virtual screening process. Autodock 4.0 represents a valid root-mean-square deviation (RMSD) score and accurate binding with the target receptor. In this context, COX-2 (PDB ID: 4COX) was tested with its co-crystallized inhibitor Indomethacin and TNF-α (PDB ID: 2AZ5) was tested with its co-crystallized ligand, i.e., 6,7-Dimethyl-3-[(Methyl{2-[Methyl({1-[3-(Trifluoromethyl) Phenyl]-1h-Indol-3-Yl}Methyl)Amino]Ethyl}Amino)Methyl]-4h-Chromen-4-One.[44,45]

Compound screening using PyRx program

The autodock wizard used PyRx tools to perform molecular research of all compound libraries as the engine for docking.[46] The method has often been used to know/predict the amino acids that bind with ligands in the active site of the protein. In spatial RMSD, tests below 1.0Å were considered as optimal and were coupled to consider preferred binding. The lowest (most negative) binding energy is known as the optimum binding affinity ligand. The Biovia Drug Discovery Studio 2019 was used to scan the docking site visually, and Autodock Vina validates the findings.[47]

ADMET analysis

ADMET of the ligands is pharmacokinetic properties calculation that is required to be examined to establish their function inside the body. The ADMET inheritance of the ligands was studied, making use of admetSAR.[48,49]

Predictions for antiviral activity (PASS) computer program

Prophecy of *Cinnamon* for antiviral activity was created with the assistance of software, PASS. PASS is a computer system-based program utilized for the prognosis of various sorts of physiological actions for multiple compounds consisting of phytoconstituents. The estimated activity of a substance is predicted as probable activity (Pa) and probable inactivity (Pi). The substances that show Pa higher than Pi are indeed the only components deemed viable for a particular medical activity.[50-52]

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

Antioxidant Activity

DPPH is a stable free radical that exhibits full UV and visible absorption at 517 nm. It decreases in the concentration of antioxidants in the study, considered an indicator of

their antioxidant activity. Samples' capacity to scavenge radical DPPH was calculated on their concentration bases offering 50 percent inhibition (IC_{50}). The results of one way ANOVA test and post adhoc test indicate the significant difference of mean percentage scavenging between different concentrations of tested extracts. The scavenging activity of the methanolic extract pronounced at higher concentrations of 80 mg/ml and 100 mg/ml with a mean percentage of 55.66 ± 0.38 and 63.12 ± 1.26 for MEMA, respectively, which was lower compared with standard antioxidant, ascorbic acid but exhibited a similar pattern of concentration-dependent free radical scavenging effect. The IC_{50} values of MEMA (71.41) and ascorbic acid (38.46) were obtained using the linear regression equation. The radical scavenging ability, IC_{50} of extracts and ascorbic acid were presented in Table 2.

GC–MS of Methanol Extract of *M. alliacea*

GC–Analysis of MAME is shown in the Figure 2. Separation strategies, in combination with GC –MS, permitted effective component separation, as shown in Figure 3. Phytochemical compound identification was based on the peak area, molecular retention, and formula. Centered on the projected mass spectrophotometer, four compounds are Apigenin-7-O-methylglucuronide. MAME Scutellarin, Luteolin and Ursolic acid [Table 3].

Acute Toxicity Studies

In Wistar albino rats, MEMA was tested for acute toxicity and animals were monitored for 24 h. MEMA caused no deaths of up to 2000 mg/kg, so for the present study, 400 and 200 mg/kg were chosen.

Effect of *M. alliacea* on FA-induced Arthritis

At the end of the 28-day adjuvant study period, the volume of the paw in the arthritic control group increased to 2.473 ± 0.081 in arthritis induced. In all treatment groups, inflammatory edema and thickness of the paw have dramatically dropped ($P < 0.001$), which was represented in Tables 2 and 3, Figures 1 and 3. The sequence of anti-arthritic effects was the following: Ibuprofen >MEMA (400 mg/kg) >MEMA (200 mg/kg), in curing paw edema. During the study era, such as RA influences body weight, body weight changes were reported. Arthritic control rats reduced their weight from 204.2 ± 1.54 g to 148.52 ± 1.89 g on day 28. Prophylactic

Table 2: Dose-dependent DPPH free radical scavenging and H_2O_2 radical scavenging activity of different extracts of *M. alliacea*

Method	Treatment	Absorbance wavelength (nm)					IC_{50}
		20	40	60	80	100	
DPPH free radical scavenging (% Inhibition)	PELS	12.22±0.13	21.52±0.68	31.62±1.58	35.24±1.82	41.36±0.21	258.91
	EALS	16.22±0.66	28.54±1.52	33.36±0.38	39.46±0.51	46.84±0.67	107.43
	MELS	28.33±0.21	38.36±1.33	47.23±1.78	54.78±0.63	61.31±1.57	69.70
	Ascorbic acid	38.45±0.58	51.22±0.12	68.67±0.32	79.54±0.36	89.22±0.55	36.25
H2O2 scavenging activity (% Inhibition)	PELS	6.22±0.64	10.85±0.63	16.87±0.26	20.32±0.59	26.55±0.44	195.20
	EALS	18.33±1.41	24.81±0.49	31.28±0.83	38.32±0.33	43.87±0.36	117.81
	MELS	31.23±0.69	42.31±1.15	46.84±0.65	55.69±2.43	63.89±0.79	65.32
	Ascorbic acid	46.22±0.79	51.67±0.38	65.21±0.26	74.89±01.52	86.98±1.23	31.36

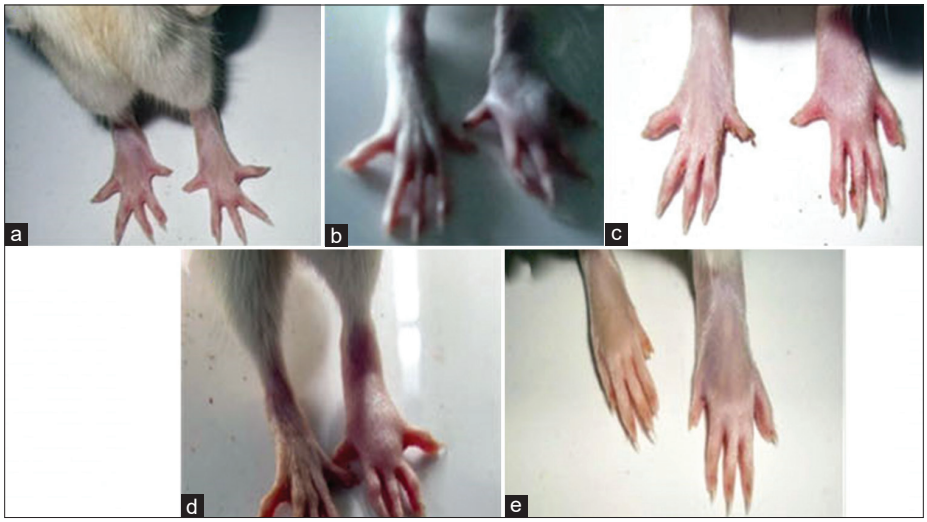

Figure 2: Representative photographs of the right hind paw of rats on day 28 after adjuvant injection. (a) Normal control. (b) Arthritic control. (c) Ibuprofen treated (15 mg/kg) (d) Methanol extract from *Manosa alliacea* (MEMA) (200 mg/kg) treated. (e) MEMA (400 mg/kg) treated

administration of Ibuprofen (15 mg/kg), MEMA (200 and 400 mg/kg) stopped reducing body weight during the trial period, as shown in Table 4.

Efect of MEMA on paw volume in FA-induced arthritis

The volume of rat paw in the FA is rising considerably in arthritic control rats compared to standard and test rats. Treatment for MEMA (200 and 400 mg/kg) demonstrably reduced the amount of rat paw edema relative to the arthritis group [Table 5 and Figures 2 and 4].

Hematological parameters

Table 6 and Figure 5 demonstrates changes in hematology in adjuvant-mediated arthritic rats. RBC count and Hb declined

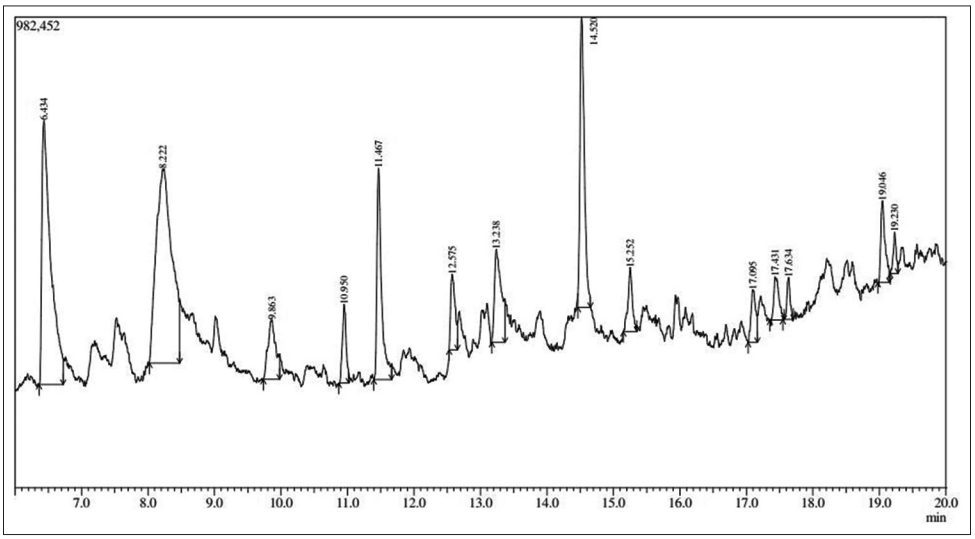

Figure 3: Gas chromatography–mass spectrometry spectral analysis of methanol extract of *Manosa alliacea*

Table 3: Biological active compounds derived from *M. alliacea*

Retention Time	Compound name	Area (%)	Molecular formula	Molecular weight
6.434	1,2-Dimethyl-3-nitro-4-nitroso benzene	1.83	$C_8H_8N_2O_3$	180.16
8.222	Scutellarin	2.39	$C_{21}H_{18}O_{12}$	462.4
9.863	1,2,3-Propanetriol	0.36	$C_3H_8O_3$	92.09
10.950	Trans. a-Chrysanthemal	0.23	$C_{10}H_{16}O$	152.23
11.467	Luteolin	0.76	$C_{15}H_{10}O_6$	286.24
12.575	Isosorbide	0.27	$C_6H_{10}O_4$	146.14
13.238	2,3-Dihydro-Benzofuran	0.50	C_8H_8O	120.15
14.520	Apigenin 7-O-methylglucuronide	1.01	$C_{22}H_{20}O_{11}$	460.4
15.252	Ursolic acid	0.22	$C_{30}H_{48}O_3$	456.7
17.095	Fucosterol	0.20	$C_{29}H_{48}O$	412.7
17.431	Neophytadiene	0.17	$C_{20}H_{38}$	278.5
17.634	Myristic acid vinyl ester	0.10	$C_{16}H_{30}O_2$	254.41
19.046	β-Amyrin	0.17	$C_{30}H_{50}O$	426.7

Table 4: Impact of MEMA in FA-induced arthritis on the weight of rats

Treatment groups	Day 0	Day 7	Day 14	Day 21	Day 28
Bodyweight (g)					
Arthritic control	204.2±11.54	172.68±18.12	168.22±13.54	156.2±18.12	148.52±11.89
Normal control	206.1±7.66^{ns}	213.2±3.82^{ns}	216.7±4.11^{ns}	223.4±3.89*	225.2±4.88*
Ibuprofen	208.2±2.59^{ns}	213.6±7.66*	218±6.21$	226.4±4.31#	232.66±5.12$
MEMA (200 mg/kg)	209.3±8.36^{ns}	203.6±11.15^{ns}	204.1±9.11*	211.5±14.12#	211.4±11.54#
MEMA (400 mg/kg)	211.8±10.83^{ns}	212.4±6.89*	221.8±12.08$	215.7±13.29*	218.7±14.73$

Mean±SEM (n=5) values were measured by ANOVA two way, followed by a snap test; P<0.05 was considered to be significant compared with arthritic ns control, non - relevant, $$P$<0.0001, #, P<0.001, *, P<0.05

Table 5: The influence of the MEMA on the entire volume of paw of Freund's mediated adjuvant (FA) (ml)

Group	Day 0	Day 7	Day14	Day 21	Day 28
Normal Control	0.19±0.03	0.18±0.06	0.21±0.02	0.21±0.08	0.20±0.06
Arthritic Control	0.25±0.04	0.58±0.05###	0.73±0.02###	0.84±0.02###	0.90±0.04###
Ibuprofen	0.23±0.05	0.38±0.02	0.32±0.04**	0.30±0.06***	0.28±0.06***
MEMA (200 mg/kg)	0.21±0.04	0.66±0.04	0.58±0.02*	0.52±0.06*	0.48±0.02*
MEMA (400 mg/kg)	0.23±0.02	0.42±0.02**	0.39±0.04**	0.34±0.04***	0.32±0.06***

###, indicates, $P<0.001$ against the normal group,* $P<0.05$, ** $P<0.01$, * ** $P<0.001$ against the control groups as contrasted

Table 6: Effect of oral administration of MEMA on Hematological parameters in arthritic rats on day 28

Group	RBC (millions/cmm)	WBC (thousands/cmm)	Hb (gm/dL)	ESR (mm/hr)
Normal Control	7.25 ± 0.55	9.80 ± 0.14	13 ± 0.25	1.25 ± 0.25
Arthritic control	3.46 ± 0.22###	21 ± 1.37###	9.07 ± 0.22###	47 ± 7.50###
Ibuprofen (15 mg/kg)	7.05 ± 0.42***	13.40 ± 0.33***	12.15 ± 0.09***	2.75 ± 0.47***
MEMA (200 mg/kg)	5.23 ± 0.35*	18.25 ± 0.39**	10.27 ± 0.63**	9.75 ± 1.31**
MEMA (400 mg/kg)	6.77 ± 0.56**	15.91 ± 0.88***	11.45 ± 0.26***	6.82 ± 0.42***

###indicates $P<0.001$ versus the normal group, *, $P<0.05$, ** $P<0.01$, *** $P<0.001$ versus the control group

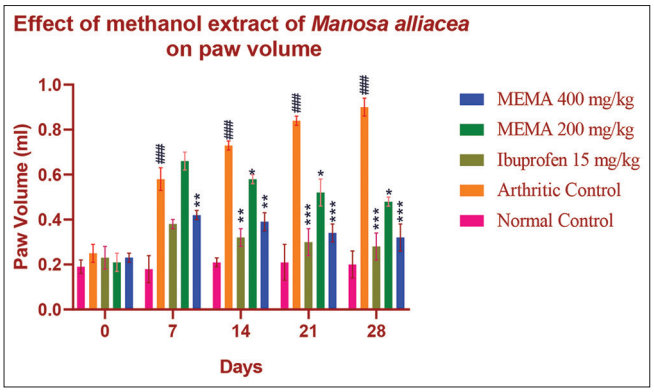

Figure 4: Impact of Methanol extract from *Manosa alliacea* on paw volume. ###shows $P<0.001$ versus normal group,*$P<0.05$, **$P<0.01$, * **$P<0.001$ versus control groups

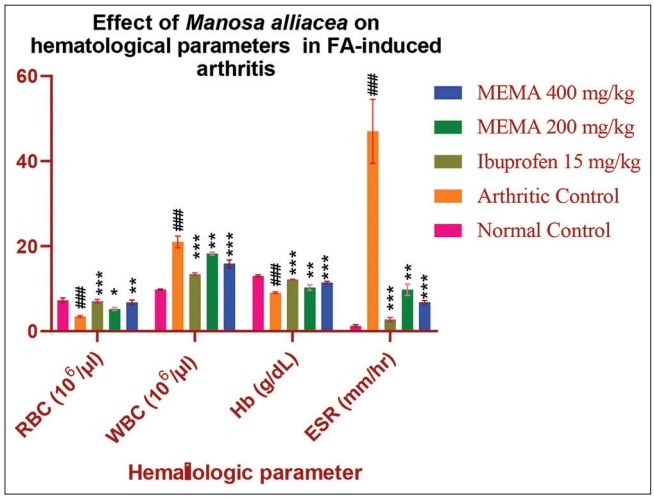

Figure 5: Effect of *Manosa alliacea* methanol extract on haematological parameters in FA-induced arthritis

significantly; WBC count and the ESR of arthritis rats improved considerably in contrast with the rats in the test. MEMA treatment showed significant improvements to the hematology of both adjuvant-induced arthritis progression and development.

Effect of M. alliacea 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 6 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 MEMA (200 and 400 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 MEMA (200 and 400 mg/kg).

M. alliacea's effect 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 show tissue composed of normal bony trabeculae lined by periosteum and also contains hemopoietic elements in normal number and maturation. No necrosis and new bone formation were seen. In MEMA (200 mg/kg) administered rat joints, moderate redness, fibrin deposits, and bone degradation were seen whereas in the rats with MEMA (400 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 occurred in the standard treatment group [Figure 7].

Effect of M. alliacea on the mRNA expression level of inflammatory mediators in CFA-induced arthritis

TNF - α, NF - kB, IL-1β, and COX-II expression levels in mRNA have been significantly increased in arthritic rats compared with normal rats. Fold changes, TNF-α (3.38 ± 0.525), NF-kB (4.82 ± 0.381), IL-1β (5.95 ± 0.128), and Cox-II (6.54 ± 0.318) were observed in arthritic rats, while ibuprofen, MEMA (200 and 400 mg/kg) substantially inhibited the production of these markers relative to arthritic control, as seen in Figure 8.

Computational Studies

Validation analysis of the target protein

For the COX-II and α-TNF, target structure (PDB- 4COX, 2AZ5) with the reported inhibitor as a co-crystallized ligand was retrieved from PDB for docking purpose. To further validate the docking methodology, the RMSD value was calculated. RMSD score of the co-crystalized (internal) ligand and extracted internal ligand of the docked target protein-ligand complex structure served as the control docking model as represented in Figure 9. The docking outcome showed that Autodock 4.0 determined the optimal orientation of the co-crystallized ligand. RMSD value 0.454913 and 0.68545 indicates that the methodology is accurate to predict the binding affinity for unknown ligands.

For NF-KB (PDB ID: 1SVC), IL-1β (PDB ID: 9ILB), target structure with the reported inhibitor is not available in the PDB collection or literature. We have utilized the active site prediction option of Discovery Studio Visualizer (DSV) 2020. After loading the protein structure to DSV, it reads the protein and highlights the probable active site, i.e., residue information in yellow color.

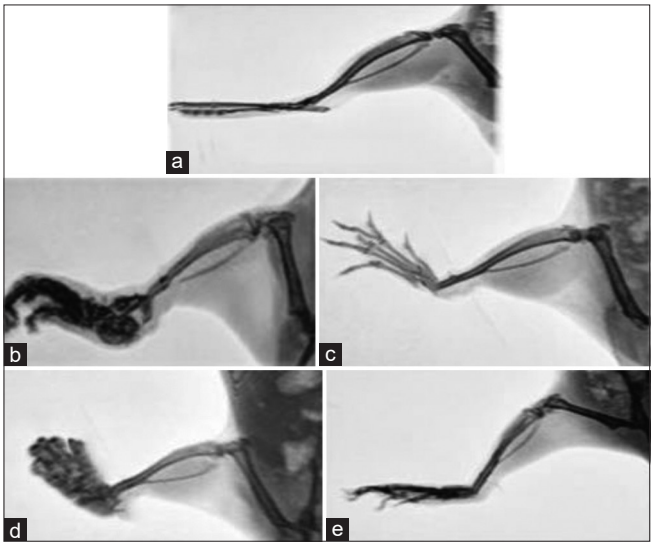

Figure 6: Radiology of hind legs in adjuvant-induced arthritic rats. (a) Normal control. (b) Arthritic control. (c) Ibuprofen 15 mg/kg treated. (d) Methanol extract from *Manosa alliacea* (MEMA) 200 mg/kg treated. (e) MEMA 400 mg/kg treated

Molecular Docking Studies

Docking researches revealed, Apigenin 7-O-methylglucuronide had the most excellent docking rating of −10.7 and −7.5 kcal/mol with 4-COX and 2AZ5, whereas β-Amyrin has the best correlation with 1SVC with a docking score of −8.4 kcal/mol and Ursolic acid had a good binding affinity of −7.4 kcal/mol with 9ILB.

Apigenin 7-O-methylglucuronide showed four hydrogen bond interactions; ASN A:34 (4.83), CYS A:37 (2.81), ASN A:39 (4.98), TYR A:130 (6.46) and also hydrophobic interactions ARG A:44 (5.26), PRO A:153 (4.70, 4.99), ARG A:469 (6.46) with respective to 4-COX and it forms five hydrogen bonds, i.e., GLN A:25 (3.24), ASN A:30 (3.84), ASP A:45 (2.86), ASN A:46 (3.83, 5.02), LEU A:26 (4.44) and hydrophobic interaction with LEU A:29 (3.82) with 2AZ5. The outcomes gotten by the autodock 4.0 are shown in Table 4, as well as the protein-ligand interactions revealing hydrogen, hydrophobic and electrostatic bonding are additionally published in Tables 7-10 and Figure 10.

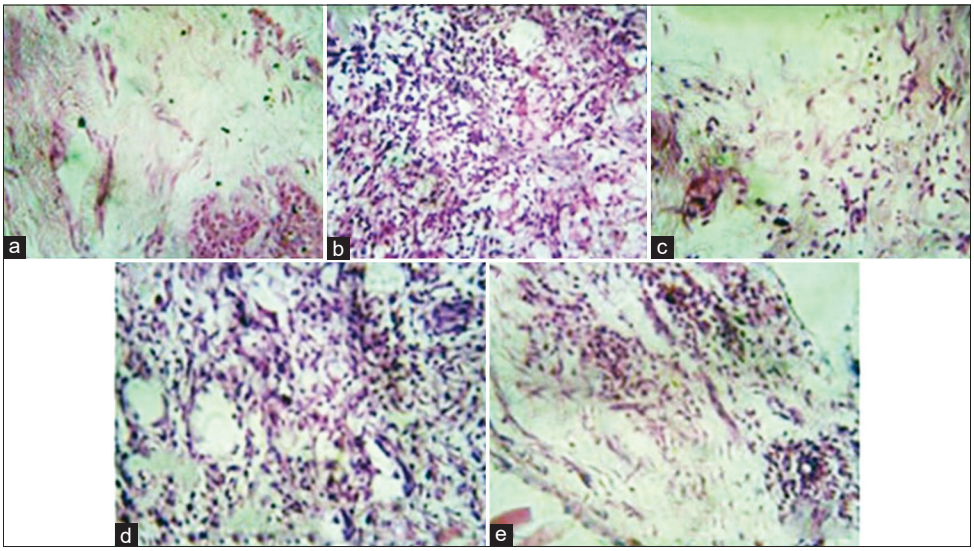

Figure 7: Histopathological photos of ankle joints stained with H&E. (×100) (a) Healthy control. (b) Arthritic control. (c) Ibuprofen 15 mg/kg treated. (d) Methanol extract from *Manosa alliacea* (MEMA) 400 mg/kg treated. (e) MEMA 200 mg/kg treated

β-Amyrin displayed binding affinity of -8.4 kcal/mol. It has no hydrogen bonds and forms good hydrophobic interaction with one main acid PRO P:71 (5.47) with NFkB (1SVC). In case of Ursolic acid, it forms hydrogen bonds with LEU A:62 (5.75), TYR A:68 (6.17), LYS A:65 (5.49), and no hydrophobic and electrostatic interactions with −7.9 kcal/mol.

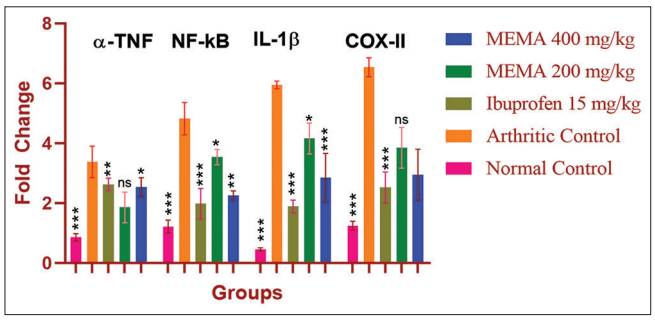

Figure 8: Effect of *Manosa alliacea* on mRNA expression levels of various inflammatory mediators (α-TNF, NF-kB, IL-1β, and COX-II) in adjuvant-induced arthritis. Values are represented by a single-way ANOVA and a Dunnet test as mean±SEM.*, $P < 0.05$, compared to Arthritic control * *, $P < 0.01$, compared to Arthritic control * * *, $P < 0.001$ versus Arthritic control and ns: Non - significant

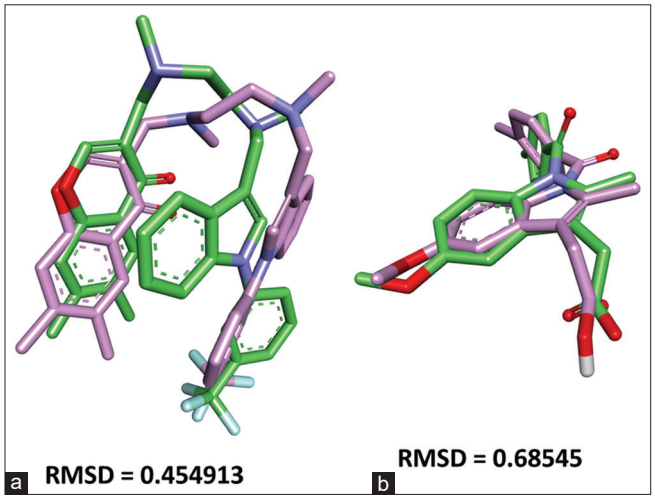

Figure 9: Data obtained in the validation of the molecular docking protocols for the receptors (a) Cyclooxygenase- II (PDB ID: 4COX) (b) α- TNF (PDB ID: 2AZ5). Pink – Native ligand; Green: Docked poses

Drug Likeliness

The physicochemical properties of the chosen four active compounds were studied on DruLiTo software. Except, lupeolin, reaming all the compounds had one deviation with Lipinski's rule [Table 11].

PASS

The biological activity spectra of previously identified phytoconstituents were obtained by online PASS version. These predictions were interpreted and used in a flexible manner and given in Table 12.

ADMET Analysis

Table 13 revealed the ADME properties of selected ligands. Here the expected properties of all substances were within the scope of Lipinski 's five drug law.

DISCUSSION

RA is an Inflammatory bone articulate disease associated with hyperalgesia and functional impairment caused by tissue damage.[53] Work during recent decades has shown the tissue damage caused by the penetration of neutrophil inflammatory cytokine (e.g. TNF - α and IL-1β) mediators. The production or release of these pro-inflammatory mediators has a wide range of arthritic models.[54]

Rather than human RA, adjuvant arthritis has many characteristics; for example, weight loss, oxidative tissue disorders and synovial membrane inflammatory infiltration with articulate swelling/destruction.[55] In this model, arthritis develops between mycobacterium capsule and cartilage proteoglycans within 10–14 days after FCA injection, through cells-mediated autoimmunity.[12] A bodyweight change is a helpful indicator for assessing the progression of the illness and how anti-inflammatory medications are treated.[56] Sub-plantar administration of FCA in this study caused high immune-inflammatory responses with reduced body weight. The lower body weight in FA-mediated animals may be attributed to lower absorption of glucose and leucine in the gut of animals. The insignificant increase in body weight during MEMA treatment reveals a slightly restored absorption capacity of the intestines of induced FA.[57]

Ibuprofen (NSAID) has been commonly used as a pain-relieving drug for inflammatory disorders consistent with

Table 7: Interactions of Cyclooxygenase (4COX) amino acid residues with ligands at receptor sites

Ligands	Binding Affinity, ΔG (Kcal/mol)	Amino acids involved and Distance (A°)	
		Hydrogen Binding Interactions	Hydrophobic Interactions
Apigenin-7-O-methylglucuronide	−10.7	ASN A: 34 (4.83), CYS A: 37 (2.81), ASN A: 39 (4.98), TYR A: 130 (6.46)	ARG A: 44 (5.26), PRO A: 153 (4.70, 4.99), ARG A: 469 (6.46)
Ursolic acid	−8.7	GLN A: 203 (5.20), HIS A: 388 (5.52)	VAL A: 295 (5.18), LEU A: 391 (5.05), PHE A: 395 (6.41), PHE A: 404 (5.51)
β-Amyrin	−8.8	ILE A: 124 (5.632)	-
Ibuprofen	−7.8	ARG A: 120 (5.96), TYR A: 355 (6.12)	TYR A: 385 (3.83), LEU A: 352 (5.72), PHE A: 381 (5.34), LEU A: 384 (4.88), TRP A: 387 (6.09), VAL A: 523 (4.50), ALA A: 527 (5.28)

Table 8: Interactions of α-TNF (2AZ5) amino acid residues with ligands at receptor sites

Ligands	Binding Affinity, ΔG (Kcal/mol)	Amino acids involved and Distance (A°)	
		Hydrogen Binding Interactions	Hydrophobic Interactions
Apigenin-7-O-methylglucuronide	−7.5	GLN A: 25 (3.24), ASN A: 30 (3.84), ASP A: 45 (2.86), ASN A: 46 (3.83, 5.02), LEU A: 26 (4.44)	LEU A: 29 (3.82)
β- Amyrin	−7.1	-	TYR A: 59 (5.59)
Ursolic acid	−7.1	GLU A: 23 (4.76), PHE A: 144 (5.33)	-
Ibuprofen	−5.4	-	PHE A: 144 (4.48, 5.15)

Table 9: Interactions of NF-KB (1SVC) amino acid residues with ligands at receptor sites

Ligands	Binding Affinity, ΔG (Kcal/mol)	Amino acids involved and Distance (A°)		
		Hydrogen Binding Interactions	Hydrophobic Interactions	Electrostatic Interactions
Apigenin-7-O-methylglucuronide	−7.4	CYS P: 119 (5.09), ASP P: 121 (3.66, 0.78), THR P: 146 (3.32), ARG A: 157 (5.08)	VAL P: 61 (6.10), LYS A: 149 (6.38), VAL P: 145 (6.38)	ARG P: 157 (5.87, 4.27)
Ursolic acid	−8.1	SER P: 74 (4.69)	-	-
β-Amyrin	−8.4	-	PRO P: 71 (5.47)	-
Ibuprofen	−5.5	SER P: 113 (4.43), ARG P: 157 (5.94)	TYR P: 60 (4.62), VAL P: 61 (4.29, 5.26), LEU P: 143 (6.23), LYS P: 149 (5.15), VAL P: 145 (4.30)	-

Table 10: Interactions of IL-1β (9ILB) amino acid residues with ligands at receptor sites

Ligands	Binding Affinity, ΔG (Kcal/mol)	Amino acids involved and Distance (A°)		
		Hydrogen Binding Interactions	Hydrophobic Interactions	Electrostatic Interactions
Apigenin-7-O-methylglucuronide	−7.7	GLU A: 25 (3.94), LEU A: 80 (3.32, 4.43), LEU A: 134 (4.60), THR A: 79 (3.55), SER A: 125 (4.46)	PHE A: 133 (4.44, 4.50), PRO A: 131 (4.24)	ASP A: 142 (7.06)
Ursolic acid	−7.9	LEU A: 62 (5.75), TYR A: 68 (6.17), LYS A: 65 (5.49)	-	-
β-Amyrin	−7.4	THR A: 79 (4.65)	-	-
Ibuprofen	−5.7	LEU A: 80 (4.37), LEU A: 134 (4.43, 3.39), PRO A: 78 (5.00)	MET A: 130 (4.05), PRO A: 131 (4.30), PHE A: 133 (4.84, 4.61)	-

Table 11: Physicochemical properties of the active compounds and accordance with the rules of drug-likeness

Ligands	MW	logp	Alogp	HBA	HBD	TPSA	AMR	nRB	No. of Violations
Apigenin 7-O-methylglucuronide	439.94	0.31	−1.962	11	0	71.06	117.49	5	1
Ursolic acid	407.98	8.954	1.484	3	0	17.07	132.26	1	1
Beta-Amyrin	375.99	11.546	3.012	1	0	0	132.32	0	1
Ibuprofen	187.99	3.4	1.961	2	0	17.07	64.11	4	0

Table 12: PASS Prediction of various phytoconstituents of *M. alliacea*

Main Predicted activity by PASS online	Apigenin-7-O-methylglucuronide		Beta-Amyrin		Ursolic_acid		Ibuprofen	
	Pa	Pi	Pa	Pi	Pa	Pi	Pa	Pi
Anti-inflammatory	0.744	0.011	0.843	0.005	0.864	0.005	0.901	0.004
Inflammatory Bowel disease treatment	0.237	0.108			-	-	0.398	0.027

RA. In this study, ibuprofen administration demonstrated effective anti-arthritic efficacy against FA-induced arthritis by inhibiting TNF-α and IL-1β. Nonetheless, it was reported to be correlated with side effects, including nausea, vomiting, diarrhoea, headache, itching, and elevated blood pressure. Such related side-effects limit its therapeutic use to combat

Table 13: ADME/T Properties of different compounds from *M. alliacea*

Compound	GPCR ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
Apigenin 7-O-methylglucuronide	0.02	−0.13	−0.11	0.34	−0.01	0.33
Ursolic acid	0.28	−0.03	−0.5	0.89	0.23	0.69
β-Amyrin	0.22	−0.05	−0.31	0.67	0.11	0.56
Ibuprofen	−0.17	−0.01	−0.72	0.05	−0.21	0.12

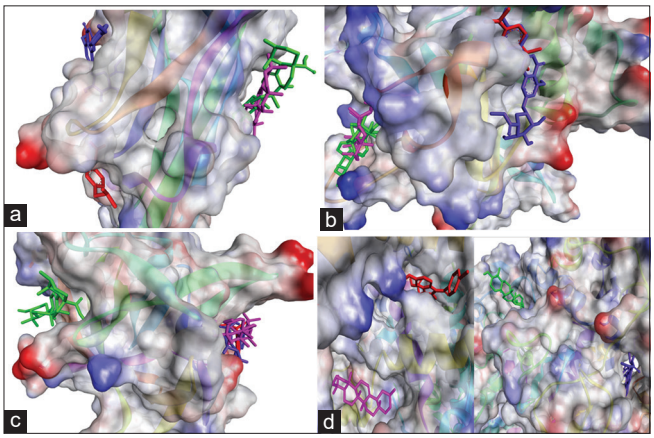

Figure 10: *In silico* Docked complexes of Ligand (Ball and Stick representation) with (a) α-TNF (b) NF-kB (c) IL-1β (d) COX-2 (Molecular representation). **Red**- Ibuprofen; **Blue:** Apigenin – O- methylglucuronide; **Pink:** β-Amyrin; **Green:** Ursolic acid

arthritis. However, MEMA's side effects are not established at this point.

The results of antioxidant tests showed that the methanolic extract had significant antioxidant potential and was able to exert tissue damage protection effects in RA.[58] The methanol extract showed an anti-arthritic effect, as shown in the data. It has been suggested to quench oxygen-derived free radicals by donating a hydrogen atom and an electric to the free radical by various naturally occurring antioxidants, phenol compounds and flavonoids. Accordingly, it is well known that *Manosa* species are abundant in diverse bioactive compounds, mainly flavonoids, tannins, phenols or essential oils that inhibit ROS, cyclooxygenase-2, 5-lipoxygenase and many immune-modulatory molecules including cytokines and chemokines for their several anti-inflammatories and anti-arthritic impact.[59]

GC – MS MAME Identification Confirmation Testing was by matching their mass spectrum with NIST Library edition 2005. Depending on the estimation of the mass spectrophotometer, four compounds are1,2-Dimethyl-3-nitro-4-nitroso benzene, Scutellarin, 1,2,3-Propanetriol, Trans-Chrysanthemal, Luteolin, Isosorbide 2,3-Dihydro-Benzofuran Apigenin 7-O-methylglucuronide, Ursolic acid, Fucosterol, β-Amyrin and Myristic acid vinyl ester and found in MAME.

In arthritis, inflammatory reaction leading to bone deformation and joint function disability is consistent with hyperalgesia, induced by PGs and other endogenous mediators.[60] Levels of FA-induced arthritis display increased nociception due to the release of inflammatory mediators such as TNF-α and IL1-β.[61] So this methodology is used for the calculation of MEMA dosage level between 200 and 400 mg/kg/p. o. MEMA controlled groups showed substantial paw volume decreases in contrast with the test group arthritic. 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 are closely related to each other. The findings indicated that MEMA therapy has anti-inflammatory consequences, as shown by a significant decrease of paw oedema of arthritic animals.

Decreased rates of Hb and RBCs combined with reduced rates of erythropoietin were documented due to reduced response to bone marrow erythropoietin and degradation of RBCs prematurely.[54] The results showed that the decline in RBC count and Hb level is anaemic in arthritic rats. The most important causes are abnormal iron storage and the failure of the bone marrow to respond to anaemia[62] in the reticuloendothelial system and the synovial tissue.[63] The MEMA therapy increases the RBC count and Hb rates, suggesting that the anaemia 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 MEMA groups. In arthritic control groups, the number of ESRs which has significantly increased has been significantly reversed by MEMA and standard drug, Ibuprofen, which restore its importance in arthritic conditions back to almost normal.

Inflammatory mediators such as IL-6, IL-1β and TNF - α play a key role in RA pathogenesis by promoting immune cell infiltration and triggering metalloproteinase matrix (MMP) release (Brennan *et al*., 2008). 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 (Zhang *et al*., 2002). In this analysis, MEMA extract 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 MEMA group were higher than the low dose. Such results showed that the anti-inflammatory effects of

MEMA had been related to its TNF - α, NF-kβ and IL-1β level inhibition.

Molecular docking has been turned in medicinal chemistry into a more and more robust method to determine the prevalent binding mode(s) of a ligand with a three-dimensional protein. This *in-silico* technique may be used to model the relationship between a small molecule and protein at the atomic stage, enabling the activity of small molecules to be defined both at the binding site of the target protein and to elucidate essential biochemical processes. The positive findings shown in the anti-inflammatory test by selected ligands have inspired us to consider how this compound is related to the active site of TNF-α. TNF-α is a cell-signalling protein (cytokine) which is involved in inflammation and acute process reaction that in effect contributes to many autoimmune disease clinical issues, including RA. TNF-α in people with RA or Crohn's disease was found in higher amounts. IL-1β is one of the cytokines that is synthesized, released from immunocytes and regarded as an essential inflammatory mediator.[64]

Online testing utilizing molecular docking programs has ended up being a significantly prominent method to the advancement of new medicines, partly due to the desired time and also budgeting prices of *in silico* medication testing compared to standard lab experiments. In this research, we used a computational protein-ligand docking method making use of open software programs as well as virtualized—interactions of the various ligands with the cyclooxygenase-2 and α-TNF.

ΔG indicates informative of ligand docking in the active site of a protein, kind of molecular communications, such as hydrogen bond, hydrophobic, as well as likewise electrostatic communications, with necessary amino acid, which is a step of ligand docking in favourable conformations. Out of all compounds, Apigenin 7-O-methylglucuronide has a good binding affinity of −10.7 and −7.5 kcal/mol with respect to 4COX and 2AZ5. β-Amyrin displayed binding affinity of −8.4 kcal/mol with 1SVC. In the case of Ursolic acid, it has good binding energy of −7.9 kcal/mol with 9ILB.

CONCLUSION

In the end, MEMA at a dose of 400 mg/kg, p.o, reduces rat paw oedema volume and normalized haematological abnormalities in adjuvant-induced arthritic rats in FA-induced arthritis. Still, radiological tests confirmed MEMA's anti-arthritic role in FA-induced arthritis. It can be attributed to their ability to de-regulate levels of TNF-α, NF-kβ, IL-1β and decrease levels of inflammatory enzyme COX-2. MEMA showed significant antioxidant potential against oxygen-free radicals. Interestingly, MEMA displayed more substantial anti-arthritic impact on FA-induced arthritis in rats with ample phenolics and flavonoids, as shown by both signs and pathology ratings. It is essential to isolate the other chemical compounds and investigate their anti-arthritis, possible mode of action and fundamental signalling pathways in the research of a new lead molecule in the treatment of arthritis.

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