Research Project

"Design of novel nanoparticulate herbal formulation for rheumatoid arthritis & studies on inflammatory pathways"

UGC approval No.

F. No. 42-701/2013 (SR) dated 25/03/2013

Submitted by:

Dr. Arulmozhi S.

Principal Investigator

Rationale and significance of the study:

Rheumatoid arthritis is a chronic systemic autoimmune disease, in which inflammation plays a fundamental role. Recently there is an increasing interest in the herbal drugs for the management of arthritis and many plants are screened for anti arthritic activity. Most of the reported studies have explored only the basic anti inflammatory/arthritic activity of crude extracts and failed to study the role of solubility of the active constituents which plays a major role in determining the therapeutic activity. 6-shagoal and wedelolactone from *Gingiber officinalis* and *Eclipta alba* respectively, are reported to be potent water insoluble anti-inflammatory agents. For relatively insoluble compounds the dissolution rate is often the rate-determining step in the overall absorption process. Moreover GIT is challenging environment for stability and transport of drugs due to the influence of pH and enzymes. Hence a systematic approach is needed to bring out an effective herbal formulation. Recently nano technology attracts global attention due to its wide application. However its application in herbal research is not much explored. Prompted by these findings we propose to develop a novel nano herbal formulation, standardized for above constituents with therapeutically proven efficacy at molecular level for the treatment of rheumatoid arthritis.

The rhizome of Ginger (*Zingiber officinale* Roscoe) one of the most heavily consumed dietary substances in the world. It has been widely used in Chinese, Ayurvedic and Tibb-Unani herbal medicines all over the world. Recently, ginger has received worldwide attention as a botanical dietary supplement because of its antioxidative, anti-inflammatory, and antitumor activities. These activities are mainly due to pungent bioactive principles present in ginger, gingerols and shogaols. Gingerols are not stable during storage or thermal processing; dehydrate to generate shogaols. Shogaols are mainly found in the dried and thermally treated roots, with 6-shogaol

being the most abundant followed by 8- and 10-shogaol. Although most of the animal studies with ginger extract showed antioxidative, anti-inflammatory, and antitumor activities, no report has considered the instability of gingerols during the thermal process and long-term storage will affect the chemical profile of the ginger extract used in their animal studies. They either did not quantify the levels of the active components in their raw material or simply considered gingerols as the active principles and have paid little attention to shogaols. Only Sang et al (2009) and Dugasani et al (2009) reported that 6-shogaol is having potent pharmacological actions than 6-gingerol.

Wedelolactone is one of the important phytoconstituent of *Eclipta alba* that has been identified as a anti-inflammatory agent. The plant is useful for the treatment of inflammation widely. However there is no standardized form of the plant is available for the rational treatment of arthritis. In fact the combination of above phytoconstituents have not been made available for the treatment of arthritis.

A survey of work one in the research area and the need for more research:

Eclipta alba (Linn.) Hassk [Synonym – *Eclipta prostrata* (Linn.)] (Family – Asteraceae) icommonly known as "Bhringarajah". The herb *Eclipta alba* is reported to be traditionally used to alleviate pain and inflammation (Gogte, 2000). He analgesic and antinflammatory potential of various extracts of Eclipta alba is also reported. Arunachalam *et al*., 2009, Sureshkumar *et al*., 2005, Tewtrakul *et al*., 2011, Leal *et al*., 2000. Sawant *et al*., 2004 Wedelolactone is the predominant components *Eclipta alba* (Liu *et al*., 2014) and is reported to be antiinflammatory (Fang., 2012), inhibits NF-kappaB (Yaun *et al*., 2013), 5-lipoxygenase (Wagner and Fessler, 1986) and inhibits trypsin; (Syed *et al*. 2003), making it a candidate drug for the prevention and

treatment of inflammation. Wedelolactone is reported to specifically suppress caspase-11 expression, a key regulator of proinflammatory cytokine IL-1β (Kobori *et al*.,2004) .

Zingiber officinale commonly known as ginger has been used to treat pain and inflammation in traditional systems of medicine. The main constituents of ginger are [6]- [8]- and[10] gingerol, and shogaol. All these constituents were shown to strongly inhibit COX-1 in disrupted rat basophilic leukemia-1 (RBL-1) cells (Tjendraputra *et al*., 2001).However shagaols are more potent inhibitor of inflammatory mediators and reactive oxygen species. (Ok *et al*. 2012).

Herbal medicines are known for their synergistic effects. There are reports for design of herbal compositions by mixing of various fractions enriched with active constituents from different medicinal plants. Considering the intense pharmacological and antiinflammatory potential of *Eclipta alba* and *Zingiber officinale*, it is worthwhile to enrich the extracts and to optimize the composition of fractions from these two plants for treatment of rheumatoid arthritis. Further, pharmacokinetic study of 6- shogaol from *Zingiber officinale* reported its poor bioavailability due to its high log p value 4.77 as well as it's rapid metabolism. Wedelolactone from *Eclipta alba*, is reported to be poorly absorbed limiting its clinical use. Hence there is need to enhance the solubility of 6-shogaol and wedelolactone from its extract. With proper approaches in design of formulation of this enriched extract, the bioavailability and therapeutic efficacy can be improved.

1. Dugasania S, Pichika M R, Nadarajah V D, Balijepalli M K, Tandra S, Korlakunta J N. Comparative antioxidant and anti-inflammatory effects of [6]-gingerol, [8]-gingerol, [10]-gingerol and [6]-shogaol Journal of Ethnopharmacology 127 (2010) 515–520.

2. Gogte V M.. Ayurvedic Pharmacology and Therapeutic uses of Medicinal Plants (Drvyagunvignyan), Published by Shri Ramakrishnan Bhartiya Vidya Bhavan Kulapati 2000, 1st edition pp 452
3. Arunachalam G, Subramanian N, Pazhani G P, RavichandranV. Anti-inflammatory activity of methanolic extract of *Eclipta prostrata* L. (Astearaceae) African Journal of Pharmacy and Pharmacology 2009,3(3):97-100.
4. Sureshkumar S, Sivakumar T, Chandrasekar M J N, Suresh B. Evaluation of Anti –Inflammatory Activity of *Eclipta alba* in Rats. Ancient Science of Life 2005, 24 (3), 1-6.
5. Tewtrakul S, Subhadhirasakul S, Tansakul P, Cheenpracha S Karalai C. Antiinflammatory Constituents from *Eclipta prostrata* using RAW264.7 macrophage cells. Phytotherapy Research, 2011, 25: 1313–1316.
6. Leal L K, Ferreira A A, Bezerr G A, Matos F J, Viana G S. Antinociceptive, anti-inflammatory and bronchodilator activities of Brazilian medicinal plants contaning coumarin: A comparative study. Journal of Ethnopharmacology 2000, 70:151–159.
7. Sawant M 1, Isaac J C, Narayanan S. Analgesic studies on total alkaloids and alcohol extracts of *Eclipta alba* (Linn.) Hassk. Phytotherapy Research. 2004,18(2):111-113.
8. Liu YQ, Zhan LB, Liu T, Cheng MC, Liu XY, Xiao HB Inhibitory effect of Ecliptae herba extract and its component wedelolactone on pre-osteoclastic proliferation and differentiation. Journal of Ethnopharmacology, 2014 18(157), 206-211.
9. Fang H D Y. Anti-Inflammatory Gallic Acid and Wedelolactone Are G Protein-Coupled Receptor-35 Agonists. Pharmacology, 2012;89:211–219.
10. Yuan F, Chen J, Sun P, Guan S, Xu J. Wedelolactone inhibits LPS-induced pro-inflammation via NF-kappaB Pathway in RAW 264.7 cells. Journal of Biomedical Science, 2013, 20-84.

11. Wagner H, Fessler B. In vitro 5-lipoxygenase inhibition by *Eclipta alba* extracts and the coumestan derivative wedelolactone. Planta Med 1986,52: 374-377.
12. Syed SD, Deepak M, Yogisha S, Chandrashekar AP, Muddarachappa KA, D'Souza P, Agarwal A, Venkataraman BV. Trypsin inhibitory effect of wedelolactone and demethylwedelolactone. Phytother apy Research. 2003,17(4):420-421.
13. Kobori M, Yang Z, Gong D, Heissmeyer V, Zhu H, Jung YK, Gakidis MAM, Rao A, Sekine T, Ikegami F, Yuan C, Yuan J. Wedelolactone suppresses LPS-induced caspase-11 expression by directly inhibiting the IKK Complex. Cell Death and Differentiation 2004, 11:123–130.
14. Tjendraputra E, Tran VH, Liu-Brennan D , Roufogalis BD, Duke CC. Effect of Ginger Constituents and Synthetic Analogues on Cyclooxygenase-2 Enzyme in Intact Cells Bioorganic Chemistry 2001, 29:156–163.
15. Ok S, Jeong W S. Optimization of Extraction Conditions for the 6-Shogaol-rich Extract from Ginger (Zingiber officinale Roscoe). Prev Nutr Food Sci 2012,17: 166-171

Brief objective of the project:

The objectives of the project:

- To evaluate the plants with high therapeutic potential in rheumatoid arthritis.
- To compare the constituents by conventional extraction and solvent free extraction methods.
- To evaluate analytically and optimize the extraction method with respect to the marker compound.
- To analyze the marker for its stability in the GI fluids in vitro.
- To design nanoparticulate formulation based on the results.

- To optimize the herbal composition in the final formulation using pharmacological and biotechnological screening.

Plan of Study

Methodology

- Literature survey, Procurement of plant materials, marker compounds and chemicals.
- Extraction of plant materials by conventional methods, and novel method of extraction.
- Standardization of the extracts using analytical techniques like HPLC and HPTLC with respect to the markers.
- Optimization of the extraction method on the basis of the concentration of markers in the extracts.
- Stability studies for the markers in physiological fluids using simulated gastric and intestinal fluids.
- Design of nano particulate formulation.
- Screening of the extracts for acute toxicity as per the OECD guidelines.
- Optimization of herbal composition by in vivo pharmacological evaluation and molecular mechanism.
- a) Freund's complete adjuvant induced arthritis in animal models.
- b) Evaluation of NF-kappa B, Interleukin, TNF-α inhibitory activities in FCA induced arthritis.

A. **Collection of plants sample and related markers.**

Eclipta alba, Zingiber officinale. were collected from Avsari Ghat area Pune in the month of Dec-Jan 2013.

- Wedelolactone - Natural Remedy, Bengaluru
- 6-Shagaol- Natural Remedy, Bengaluru
- Solvents – all other solvents used was of Merck analytical grade

B. **Authentication of plants**

Eclipta alba and *Zingiber officinale* were authenticated from Biological Survey of India, Pune.

C. **Preparation and characterization of extracts.**

Extraction by conventional method maceration:

Both the plants were shed dried and coarsely powdered. The powdered plants were extracted separately using ethanol. Each day the ethanol was filtered from the macerate and fresh ethanol was added till the filtered ethanol did not show any spot in TLC to achieve maximum extraction of the constituents.

The extract thus obtained was evaporated under reduced pressure using rota evaporator. The extract was quantified using HPLC.

D. **Novel Method of extraction**

The plants were shed dried and whole plant parts were coarsely powdered. The powdered plant was mixed with varying proportion of Tween 80 and oil Labrafil M2125 CS as follows and was vortexed for 30 minutes.

Sr . No.	Plant powder (mg)	Labrafil M2125 CS (ml)	Tween 80(ml)

1	500	1	0.25
2	500	1	0.50
3	500	1	0.75
4	500	1	1
5	500	1	1.25
6	500	1	1.5

Both plants were extracted by this method separately. After vortex 1 ml of water was added and again vortexed for 2-3 minutes and were centrifuged at 12,000 RM for 10 min to get clear supernatant. This supernatant was quantified using HPLC.

DETERMINATION OF ABSORBANCE MAXIMA (λ max) of for markers

Markers scanned in range of 200-800 nm by using UV-Visible spectrophotometer.

For determination of absorbance maxima. Absorption maxima for all three markers was found to be 243 nm.

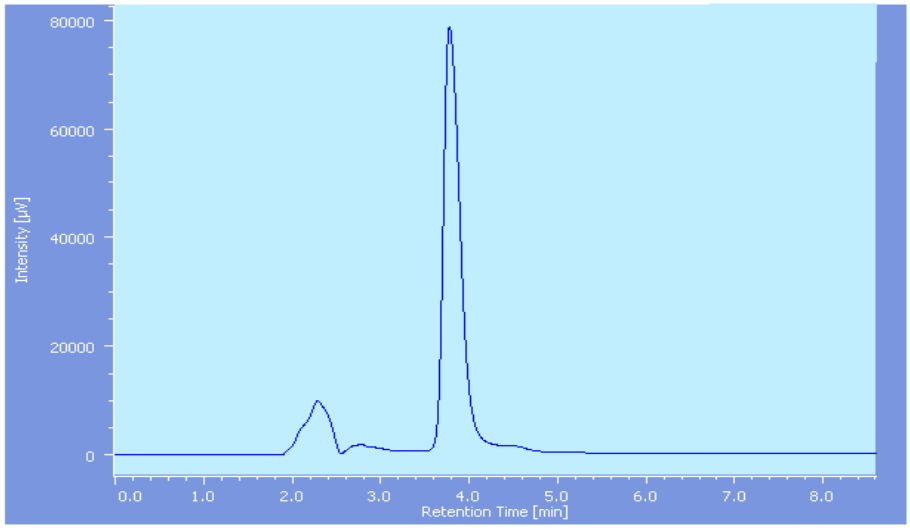
Development of simultaneous estimation method for Wedelolactone and 6-Shagaol

A suitable HPLC method was developed for simultaneous estimation method for Wedelolactone and 6-Shagaol

1. Column: Thermosil C_{18}, 250×4.6 mm, System: JASCO Preparative HPLC system, Detector: UV – at 243nm

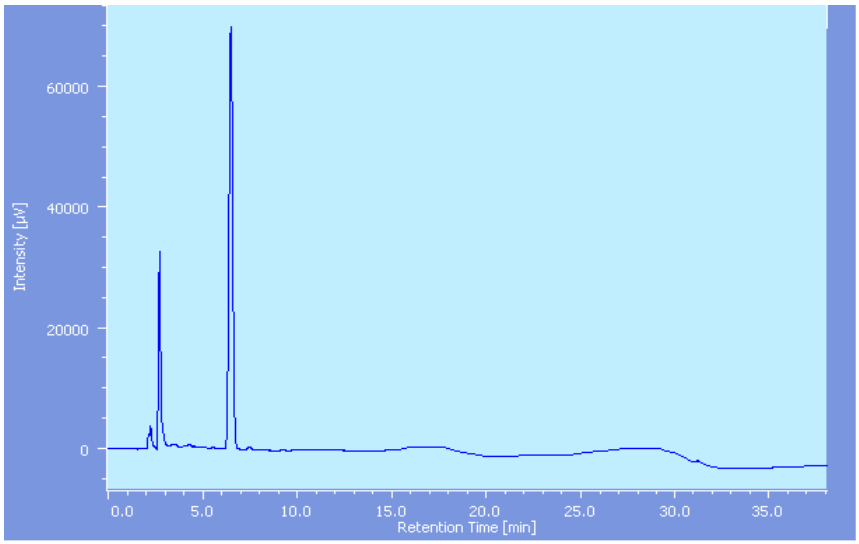
2. Mobile Phase: Acetonitrile: Water with 0.3 % glacial acetic acid in time gradient manner 55:45 for 6min and after 80:20 Flow 1 ml per minute
3. Injection volume 20ul

Wedelolactone 20 µg/ml



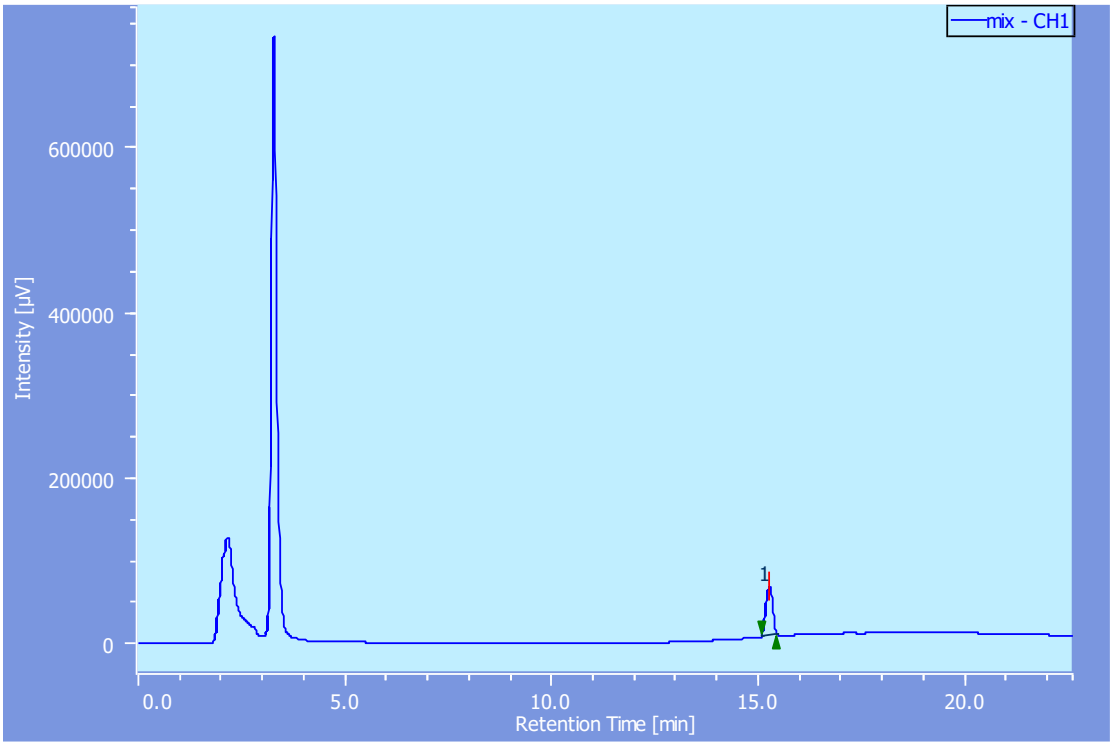
Retention Time – 3.4 min

- **6- Shagaol- 100 µg/ml**



Retention Time – 6.4 min

- **Wedelolactone and 6-shagaol by simultaneous estimation method-**



Retention time

Wdelolactone- 3.04 min 6-Shagaol-15.07min

The linearity and LOD and LOQ was determined

LOD And LOQ

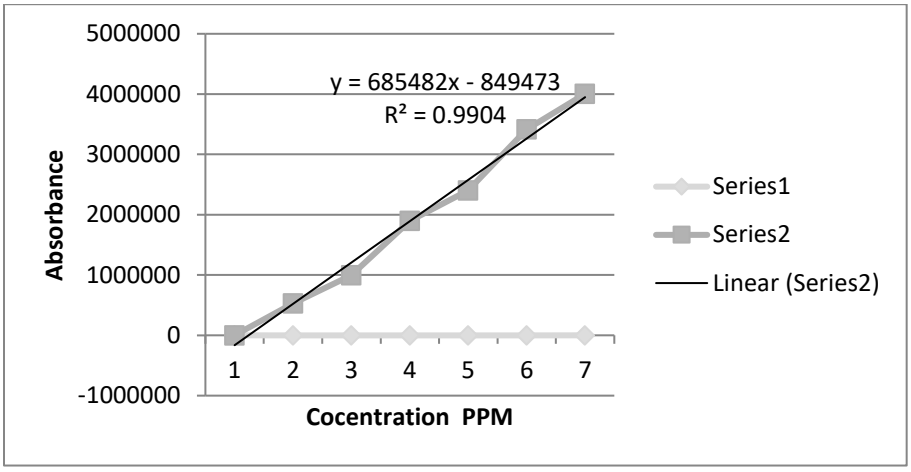
Wedelolactone-LOD for Wedelolactone was found to be **3 ppm**

6-Shagaol-LOD for 6-Shagaolwas found to be **50 ppm**

Corilagin-LOD for Corilagin was found to be **5 ppm**

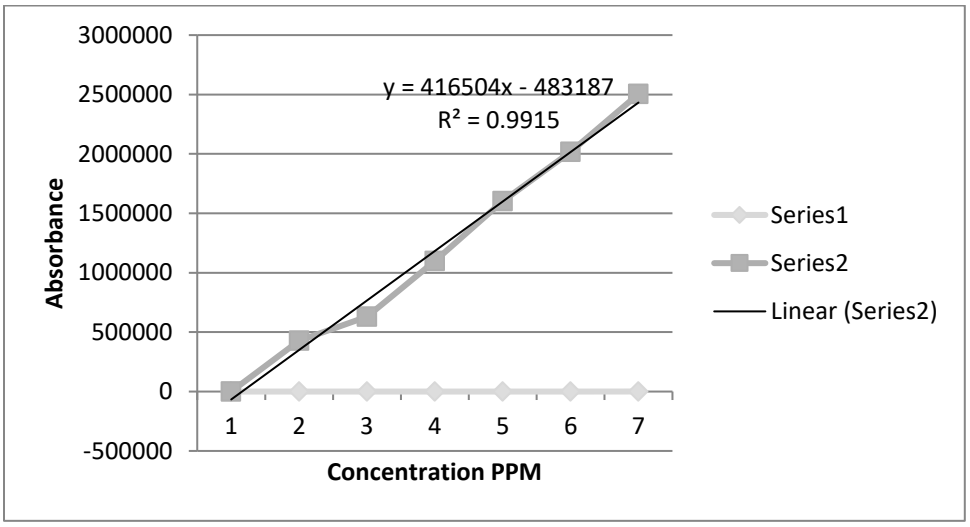
CALIBRATION CURVE

Wedelolactone



R^2=0.990

6-Shagaol



R^2=0.991

Characterization of extracts.

Sample preparation

Conventional extract

1mg of ethanolic extract was dissolved in 1 ml of methanol to get 1000ug/ml solution . from this 0.1 ml solution was again diluted to 1 ml to get 100ug/ml

Novel extract

The 20 µl of supernatant obtained was injected.

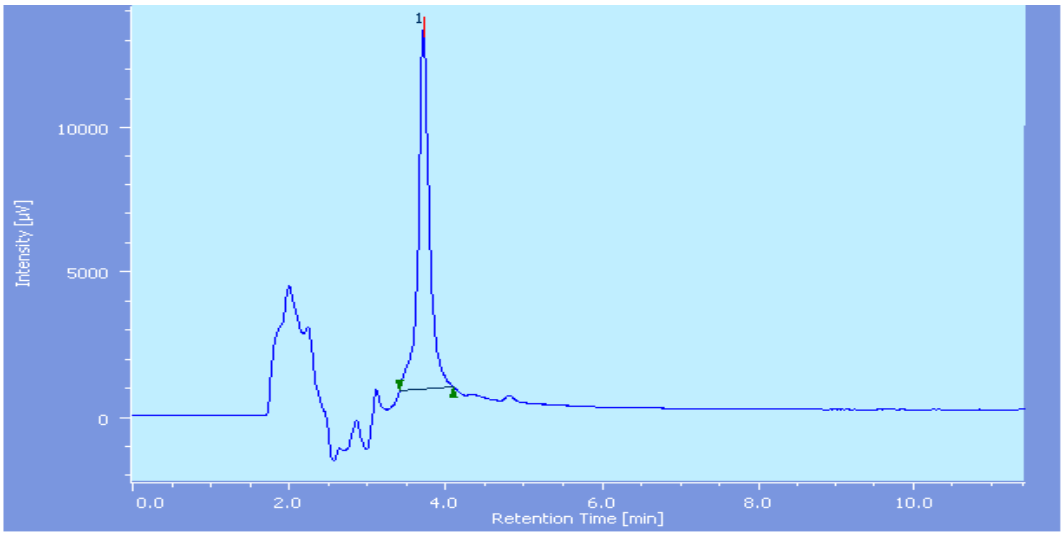
1. Column: Thermosil C_{18}, 250×4.6 mm, System: JASCO Preparative HPLC system, Detector: UV – at 243nm
2. Mobile Phase: Acetonitrile: Water with 0.1 % glacial acetic acid in time gradient manner 55:45 for 6min and after 80:20 Flow 1 ml per minute
3. Injection volume 20µl

1. Eclipta alba

HPLC mobile phase- Acetonitrile: water with 0.3% glacial Acetic acid 55:45

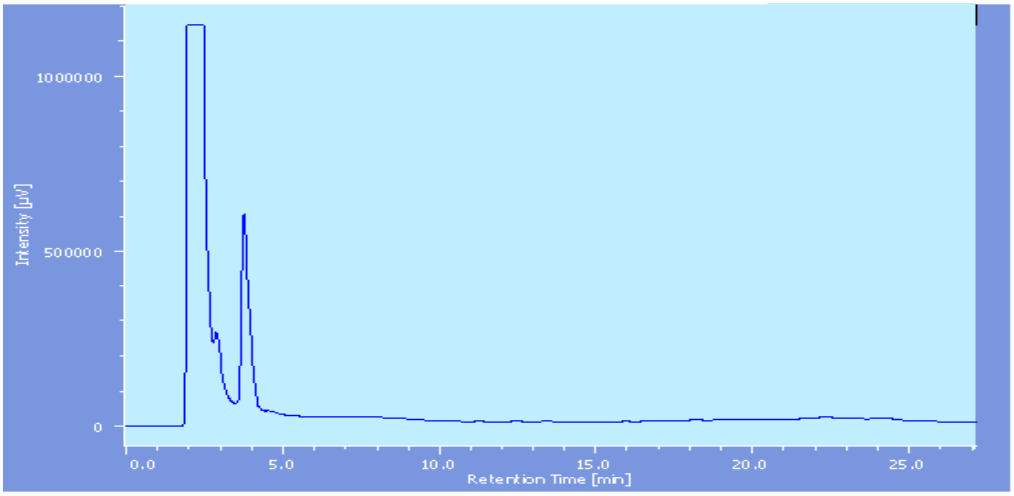
Conventional extract

Ethanolic extract (Maceration) 100 µg/ml-



Retention Time – 3.4

Novel extract



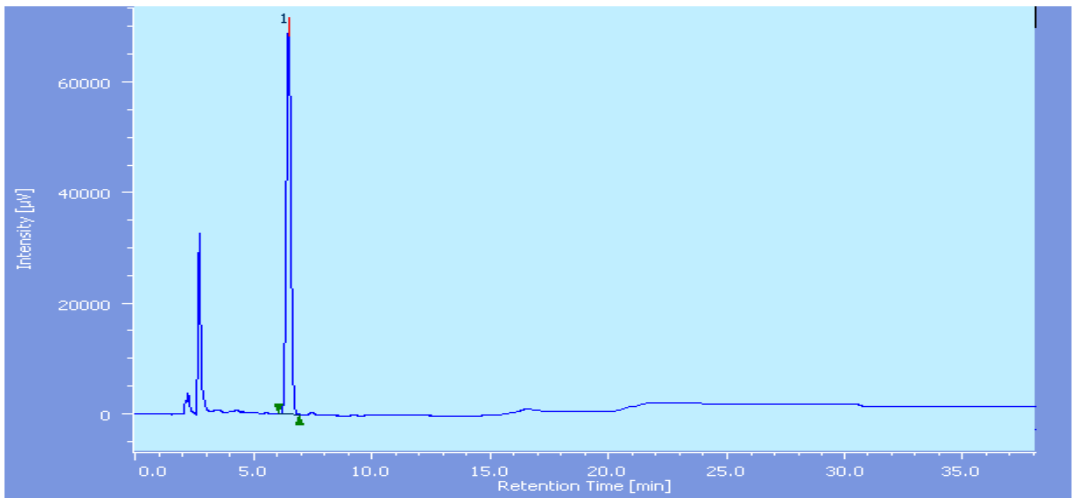
Retention Time- 3.4

Zingiber officinale

HPLC mobile phase- Acetonitrile: water with 0.3% glacial Acetic acid 80:20

Conventional extract

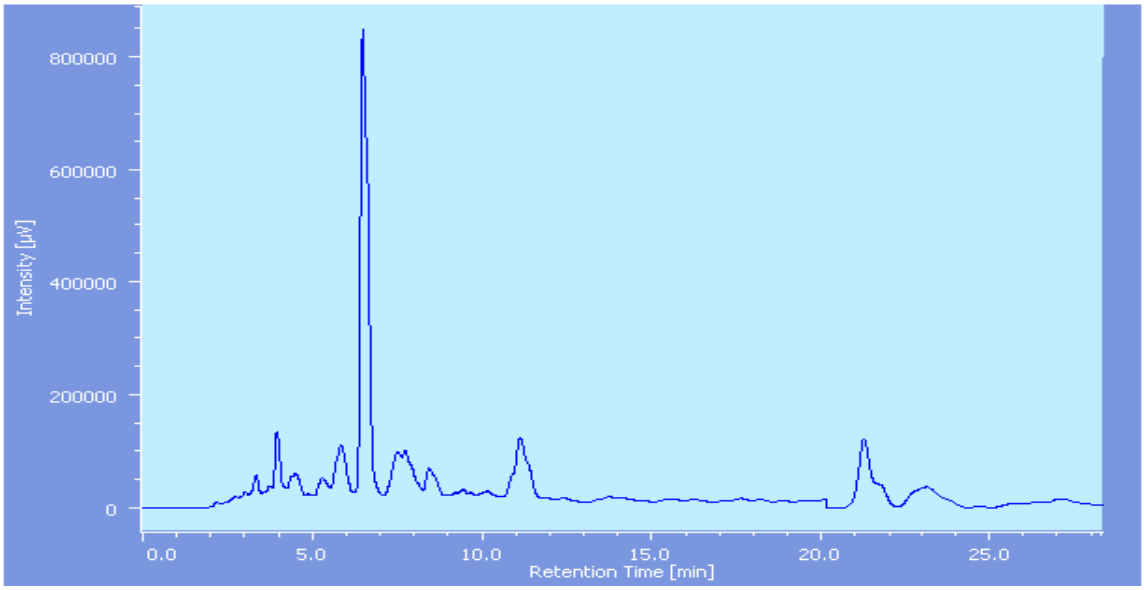
Ethanolic extract (Maceration) 100 µg/ml-



Retention Time – 6.4

6-shagaol

Novel extract



Retention Time- 6.4

Quantification of extracts

The quantification of extract resulted in enrichment of only ginger extract with 6-shagaol but not of *Eclipta alba* with wedelolactone. So further efforts were made to prepare wedelolactone enriched extract of *Eclipta alba.*

Accordingly *Eclipta alba.*was extracted as follows*;*

The aerial part of plant was shade dried, powdered and was subjected to Soxhlet extraction by ethanol and filtered to get ethanolic extract. This ethanolic extract was concentrated and washed with hot water. This was fractionated with equal volume of ethyl acetate in separating funnel [16]. The collected ethyl acetate fraction was concentrated by rota evaporator (Equitor Roteva, Medica Instruments Mfg Ltd.) to get wedelolactone rich fraction of *Eclipta alba* (WEA)

This extract was standardized and was found to enriched with wedelolactone so selected for further study.

Part II

Antiarthritic activity of wedelolactone rich fraction of *Eclipta alba.*

1. Introduction

Rheumatoid arthritis (RA) is one of the prime health predicaments worldwide, which is a foremost cause of disability and most common autoimmune disease in the world leading to premature death if not treated properly [1]. In RA, inflammation of synovial tissue lining the joint capsule results in invasion of the cartilage and bone leading to progressive joint dysfunction manifested as synovitis, synovial hyperplasia, stiffness and pain [2]. The extent of inflammation is determined by balance between proinflammatory and antiinflammatory cytokines [3]. Reactive oxygen species, addition to cytokines, play crucial role in development and progression of RA [4]. Both sexes are affected while females are more susceptible in the ratio 3:1. Conventional treatment with NSAIDs, DMARDs gives symptomatic relief and newer biologicals like tumor necrosis factor-α (TNF- α) antagonist brought therapeutic revolution by improving clinical, functional and radiographic outcomes. However, the adverse effects, toxicity and cost of the existing drugs appeal for a new alternative cost effective therapy which addresses the multiple targets in treatment of RA [5].

Herbs have been in use from the time immemorable as preventive and therapeutic medicine. Extensive research is going on to scientifically validate the potential medicinal value of such plants [6]. India has a rich collection of medicinal plants distributed in different geographical and ecological conditions widespread throughout the country. These plants endow with active principles and are proven to be valuable in treatment of various diseases which lead many to

study the phytoconstituents from plants [7, 8]. *Eclipta alba* (Linn.) Hassk, Family – Asteraceae is commonly known as "Bhringarajah". It is widely distributed throughout India, China, Thailand, and Brazil. The chemical constituents reported in the plant include wedelolactone, demethyl wedelolactone, thiophene-derivatives, steroids, triterpenes, luteolin, ecliptine and stigmasterol [9]. The herb is traditionally used to alleviate pain and inflammation [10] and reported to have anti-inflammatory [11], antinociceptive [12] and nootropic [13] activities.

Wedelolactone is reported to suppress LPS-induced caspase-11 expression (a key regulator of cytokine IL-1β maturation) and pathological apoptosis [14]. Wedelolactone has also been reported to inhibit activation of NF-κB, production of NO, PGE2, TNF-α, inducible Nitric Oxide Synthase and cyclooxygenase-2 [15]. However no attempts are reported to validate the antiarthritic potential of *Eclipta alba*. Considering the traditional analgesic and antiinflammatory use and *in vitro* effects of its principle constituent wedelolactone, in present study, effort was made to enrich the extract with wedelolactone. The extract was standardized using high performance liquid chromatography (HPLC) method and wedelolactone rich fraction of *Eclipta alba* (WEA) was subjected to preclinical evaluation in Freund's Complete Adjuvant (FCA) induced arthritis.

2. Materials and methods

2.1. Procurement and authentication of plant

Eclipta alba was collected in the month of January 2014, from Pune, Maharashtra, India. The plant was authenticated by Botanical Survey of India, Pune and voucher specimen (No BSI/WRC/Cert./2014/AS01) was deposited for future reference.

2.2. Drugs and chemicals

FCA (Sigma Aldrich, USA), etoricoxib (gift sample from Zydus Cadila, Gujarat), Wedelolactone (Natural Remedies, India), biochemical diagnostic kits (Accurex Biomedical Pvt. Ltd.) were used. ELISA kits were procured from Ray Biotech Lexington, KY. All other solvents and chemicals used for the study were of analytical grade from authentic vendors.

2.3 Animals

Female Wistar rats (180–220g) were obtained from National Toxicology Centre, Pune, India. The animals were maintained at 25±1º C temperature and 45 to 55 % relative humidity under 12 h light: 12 h dark cycle. The animals had free access to food pellets (Pranav Agro Industries Ltd, India) and water *ad libitum*. The experimental protocol was approved by Institutional Animal Ethics Committee constituted in accordance with the rules and guidelines of the Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA) (Registration No: 1703/PO/c/13/CPCSEA), India (Approval No CPCSEA/28/2014).

2.4 Extraction

The aerial part of plant was shade dried, powdered and was subjected to Soxhlet extraction by ethanol and filtered to get ethanolic extract. This ethanolic extract was concentrated and washed with hot water. This was fractionated with equal volume of ethyl acetate in separating funnel [16]. The collected ethyl acetate fraction was concentrated by rota evaporator (Equitor Roteva, Medica Instruments Mfg Ltd.) to get wedelolactone rich fraction of *Eclipta alba* (WEA)

2.5. Quantification of Wedelolactone using HPLC method

The wedelolactone content in ethanolic extract and WEA was quantified using HPLC method. The solutions of ethanolic extract (1000µg/ml), WEA (1000µg/ml) and wedelolactone (50µg/ml) were prepared in methanol. The HPLC system (Jasco Corporation, Japan) was equipped with dual pump Jasco PU- 2080 Plus, UV/Visible detector UV-2075 plus; Thermo Scientific Merck C18 reversed-phase column (I.D. 4.6mm×250mm, 5µm). Separation was achieved with a two pump linear gradient program for pump A (water containing 0.1% glacial acetic acid) and pump B (acetonitrile) in the ratio of 40:60 respectively at 243 nm. Flow rate and injection volume were 1.0 ml/min and 20µl, respectively. The chromatographic peak of the ethanolic extract and WEA was confirmed by comparing the retention time and UV spectra with the reference standard wedelolactone.

2.6. Acute toxicity studies

Acute toxicity studies were carried out for WEA following OECD guidelines No. 423[17]. The WEA was suspended in 2% w/v Tween 80 and administered orally at the dose of 5 mg/kg body weight to overnight-fasted, healthy female rats (n=3). The animals were observed individually for behavioral and autonomic profiles after dosing with special attention given during first 4 h, and daily thereafter, for a total period of 14 days. The test was repeated with doses of 50, 300 and 2000 mg/kg body weight.

2.7. FCA induced arthritis

The animals were divided into six groups of six animals each. Group I served as healthy control (2 % w/v Tween 80), Group II as arthritic control (2 % w/v Tween 80), Group III as standard

which received 10 mg/kg etoricoxib (p. o.), Group IV, V and VI received 100, 200 and 400 mg/kg WEA (p. o.), respectively. All animals except healthy control group were injected with 0.1 ml of FCA in the subplantar region of the left hind paw on day 0. The respective treatment started once signs of arthritis set in (day 12), orally once daily.

Body weight, paw volume, pain threshold, thermal and mechanical hyperalgesia and tactile allodynia were evaluated on day 0, 1, 4, 8, 12, 16, 20, 24 and day 28. On day 28, blood was withdrawn by retro orbital puncture under ether anesthesia for hematology; serum was separated for biochemical parameters and cytokine estimation [18].The animals were sacrificed by CO_2 euthanasia and spleen, thymus, liver and ankle joints were isolated.

2.7.1. Body weight

Body weight was recorded on all the above mentioned evaluation days using animal weighing balance [4].

2.7.2. Paw volume

Paw volume was measured using a Plethysmometer (UGO Basile, Italy). The change in paw volume was calculated as the difference between the final and initial paw volume [19].

2.7.3. Mechanical hyperalgesia (Paw withdrawal threshold)

It was measured as the paw withdrawal threshold of the animal in Randall-Selitto analgesiometer (UGO Basile, Italy). The hind paw was placed between the flat surface and blunt pointer and increasing pressure was applied. The pain threshold was determined when rat attempted to remove the hind paw from the apparatus. The cut-off pressure was 450g [18].

2.7.4. Thermal hyperalgesia (Paw withdrawal latency)

It was measured as paw withdrawal latency in radiant heat apparatus (UGO Basile, Italy). The paw was placed on the heat radiator with infrared intensity of lamp set at 40. A cut off latency of 15 sec was used to avoid tissue damage [18].

2.7.5. Tactile allodynia (Mechanical nociceptive threshold)

It was determined as mechanical nociceptive threshold by measuring paw withdrawal upon probing of the plantar surface with a series of calibrated fine filaments (von Frey hairs, Almemo, Germany) of increasing gauge (0.6 to 12.6g). The rats were allowed to acclimatize for 10 min in the perspex box. A series of three stimuli were applied to each paw with each hair in a period of 2–3sec. The lowest weight of von Frey hair to evoke a withdrawal from the three consecutive applications was considered as threshold. Lifting of the paw was recorded as a positive response [18].

2.7.6. Measurement of Cytokine levels

On day 28, serum Tumor necrosis factor- α (TNF-α), Interleukin 1β (IL-1β) and Interleukin-6 (IL-6) were determined using ELISA kit by sandwich method [18].

2.7.7. Hematological and biochemical parameters

On day 28, RBC count, hemoglobin (Hb), and platelet (PLT) count were determined by usual standardized laboratory methods [16]. Serum was used for the estimation of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total protein (TP) and C-reactive protein (CRP) [18].

2.7.8.Spleen and thymus weight

On day 28, rats were sacrificed by CO_2 euthanasia. The spleen and thymus of all the rats were removed and weighed [18]. The liver was isolated for antioxidant studies while the ankle joints were isolated for histopathology.

2.7.9. Antioxidant parameters

The liver isolated after sacrificing was washed in ice-cold saline and homogenated with 0.1M Tris-HCl buffer (pH 7.4). The supernatant was used to determine superoxide dismutase (SOD) [19], malondialdehyde (MDA) [20], and reduced glutathione (GSH) [21].

2.7.10. Histopathological analysis of ankle joints

The ankle joints separated from the hind paw were immersed in 10% buffered formalin and processed for paraffin embedding section at 5 µ thickness. The sections were stained with haematoxylin-eosin dye and evaluated under light microscope with 10X magnifications [18].

2.7.11. Statistical Analysis

The data was analyzed by one way ANOVA followed by Dunnett's test for biochemical analysis, two way ANOVA followed by Bonferroni's post hoc test for *in vivo* parameters. All statistical analyses were performed using Graph Pad Prism software (San Diego, CA). Data was considered statistically significant at $P<0.05$.

3. Results

3.1. Standardization of extract

The yield of the plant extract was found to be 8.3% w/w. The HPLC analysis of ethanolic extract and WEA confirmed the presence of wedelolactone (6.62 % w/w and 25.3 % w/w, respectively) (Fig 1A, 1B and 1C).

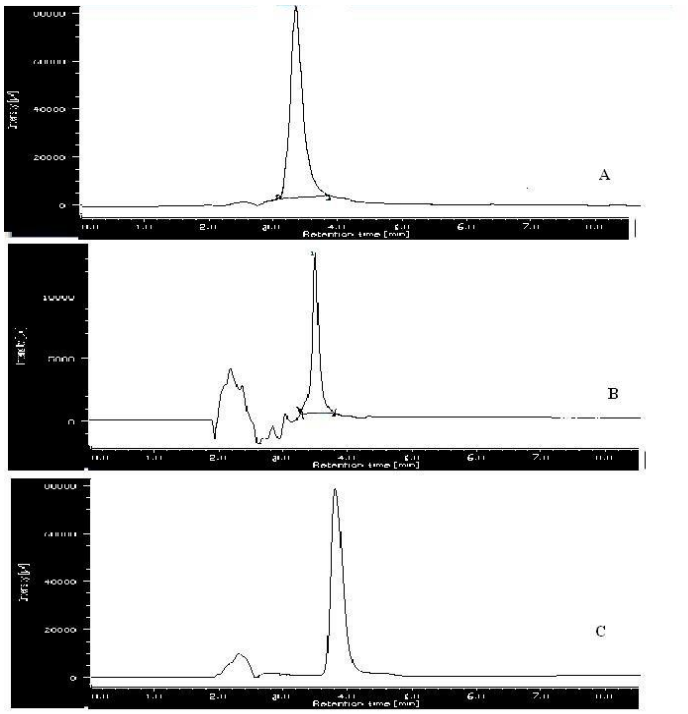


Fig. 1. Chromatograph

1A: Wedelolactone Standard 50 ppm retention time 3.4 minutes, **1B**: EEA 1000 ppm retention time 3.45 min,

1C: WEA 1000 ppm retention time 3.45 minutes

3.2.Acute toxicity studies

The test animals did not exhibit any change in autonomic, behavioral profile and survived beyond recommended duration of observation with 2000 mg/kg of WEA (OECD Guideline No. 423). Hence it was safe up to 2000 mg/kg.

3.3. Effect of WEA on body weight in FCA induced arthritis in rats

There was a significant decrease in body weight of all arthritic animals. On treatment with etoricoxib there was non-significant increase in body weight when compared with arthritic control group of animals. Furthermore, treatment with WEA non-significantly improved the body weight when compared to arthritic control group (Fig. 2).

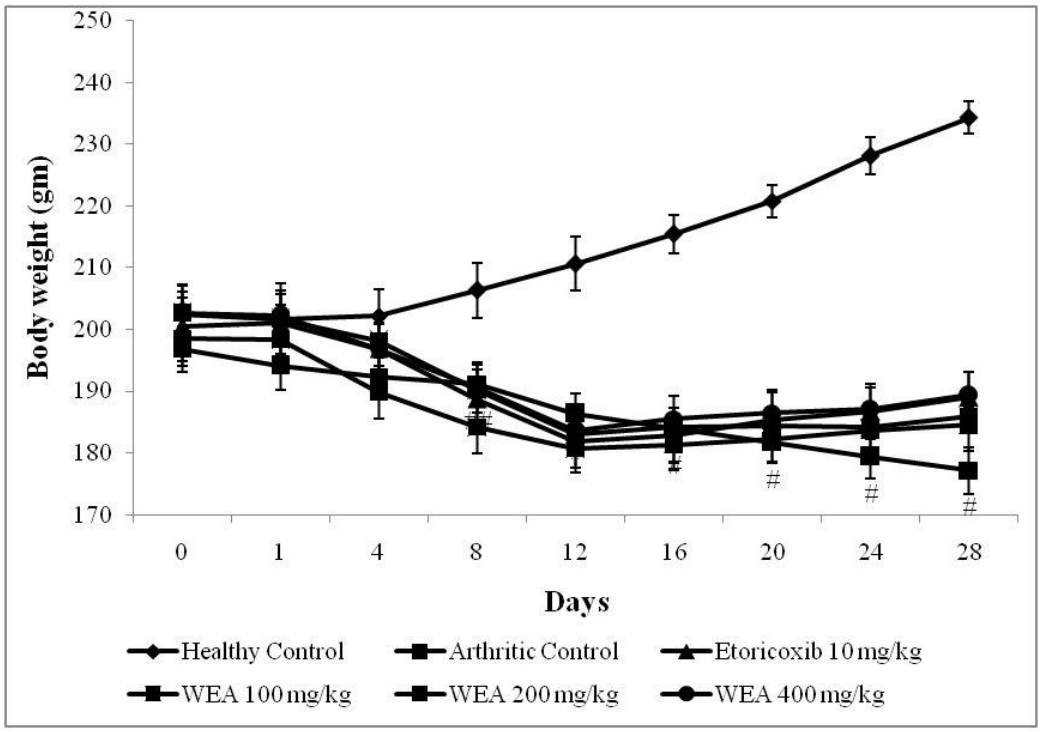


Fig. 2. Effect of WEA on body weight in FCA induced arthritis in rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared to healthy control

3.4. Effect of WEA on paw volume in FCA induced arthritis in rats

There was a significant increase in paw volume of all the rats in arthritic control group when compared to healthy control. Treatment with etoricoxib and WEA (200 and 400 mg/kg) significantly decreased the paw volume from day 16 and 24 onwards, respectively as compared to arthritic control group. WEA at the dose of 100 mg/kg was comparatively less effective and significantly decreased the paw volume on day 28 (Fig. 3).

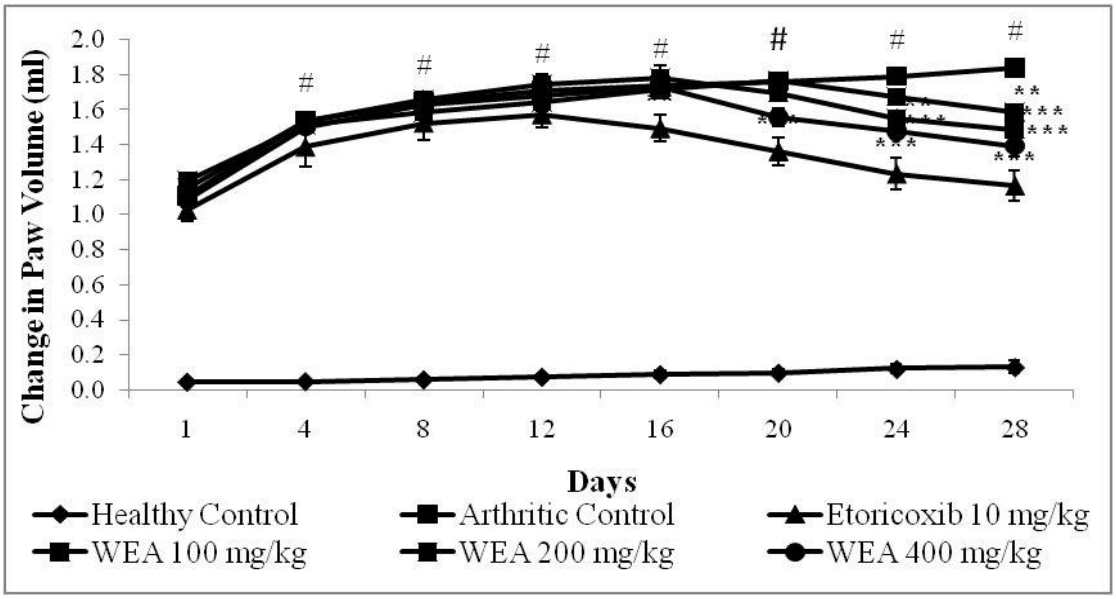


Fig. 3.Effect of WEA on paw volume in FCA induced arthritis in rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{**}P<0.01$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared to healthy control

3.5. Effect of WEA on Mechanical hyperalgesia (Paw withdrawal threshold) in FCA induced arthritis in rats

Paw withdrawal threshold in all arthritic animals decreased progressively till day 12 when compared to healthy control animals. The paw withdrawal threshold in arthritic control animals was significantly less compared to healthy control animals till the end of study. Treatment with etoricoxib and WEA (400 and 200 mg/kg) significantly increased the paw withdrawal threshold from day 20 and 24 (8 and 12 days of treatment respectively) onwards respectively. However, WEA (100 mg/kg) significantly increased pain threshold only on day 28 (16 days of treatment) (Fig. 4).

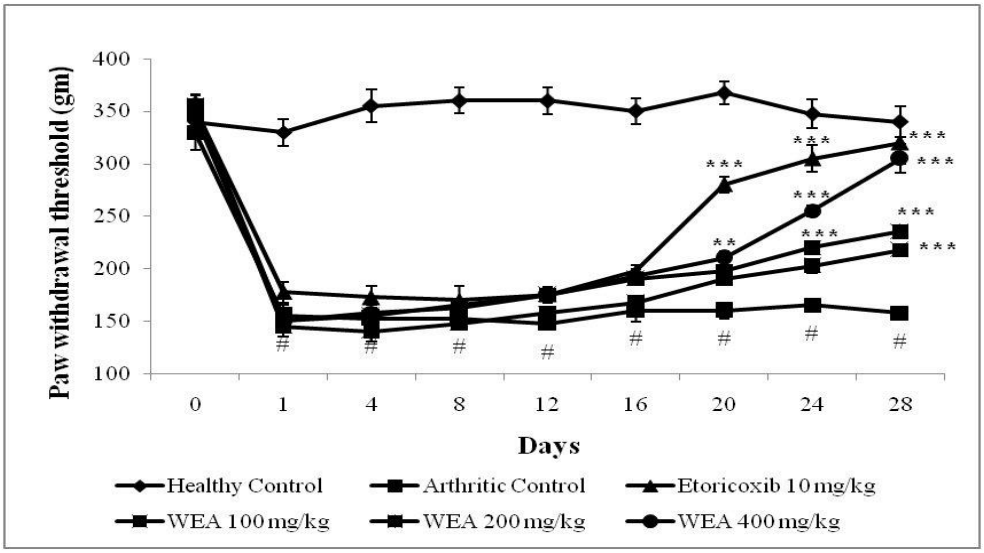


Fig.4 Effect of WEA on Mechanical hyperalgesia (Paw withdrawal threshold) in FCA induced arthritis in rats

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{**}P<0.01$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared to healthy control.

3.6.Effect of WEA on thermal hyperalgesia (paw withdrawal latency) in FCA induced arthritis in rats.

On induction of arthritis, there was a significant decrease in paw withdrawal latency of all arthritic animals till day 12 when compared to healthy control (Fig. 5). Treatment with etoricoxib and WEA (400 and 200 mg/kg) significantly increased the paw withdrawal latency from day 20. However, WEA 100 mg/kg significantly ($p< 0.001$) increased paw withdrawal latency only on day 28.

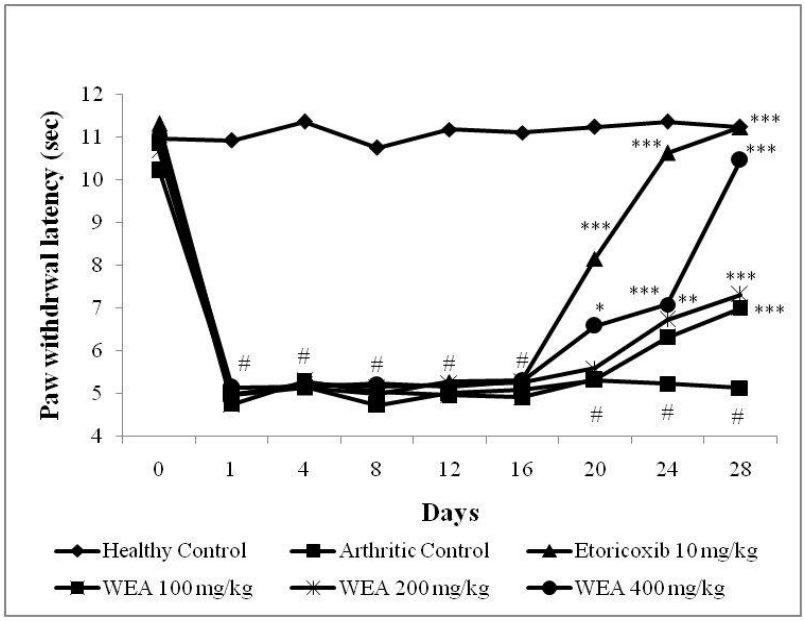


Fig.5 Effect of WEA on thermal hyperalgesia (paw withdrawal latency) in FCA induced arthritis in rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared to healthy control.

3.7. Effect of WEA on tactile allodynia (Mechanical nociceptive threshold) in FCA induced arthritis in rats

The mechanical nociceptive threshold was significantly decreased in arthritic animals till day 12. Administration of etoricoxib and WEA (200 and 400 mg/kg) from day 12 significantly improved the mechanical withdrawal threshold from day 20 onwards when compared to arthritic control while WEA 100 mg/kg significantly increased mechanical nociceptive threshold only on day 28 (Fig. 6).

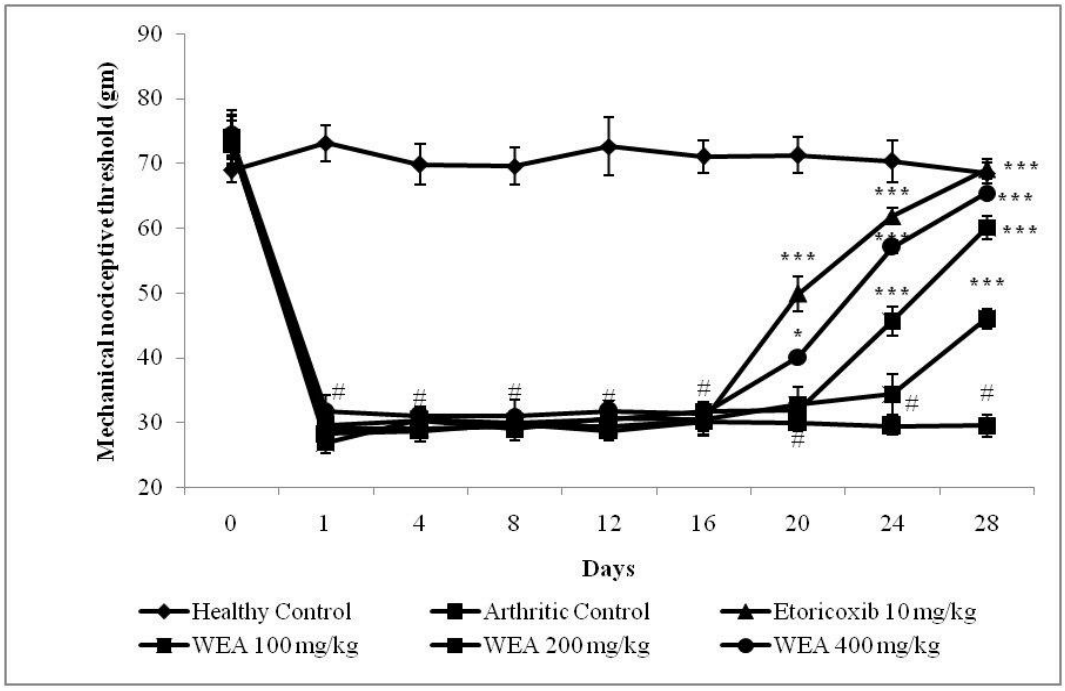


Fig. 6.Effect of WEA on tactile allodynia (Mechanical nociceptive threshold) in FCA induced arthritic rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{*}P<0.05$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared to healthy control

3.8. Effect of WEA on hematology and serum parameters in FCA induced arthritis in rats

There was a significant increase in platelet count, WBC count and ESR while decrease in RBC count and Hb level observed in arthritic control group when compared to healthy control group. These conditions reversed significantly and dose dependently on treatment with WEA (Table 1).

The serum CRP level was significantly increased in arthritic control group as compared to healthy control group. On treatment with etoricoxib and WEA (400 and 200 mg/kg), serum CRP level was found to be significantly decreased when compared with arthritic control group (Table 1).

The serum levels of AST, ALT and ALP were increased significantly in arthritic control group as compared to healthy control group. Treatment with etoricoxib and WEA (400 and 200 mg/kg) significantly decreased AST, ALT and ALP level whereas WEA 100 mg/kg non-significantly decreased these levels when compared with arthritic control group (Table 2).

The level of TP was significantly decreased in arthritic control group when compared to healthy control group. Etoricoxib and WEA (200 and 400 mg/kg) were found to be significant in restoring it when compared to arthritic control group. However, WEA 100 mg/kg non-significantly restored the TP level (Table 2).

3.9. Effect of WEA on serum cytokine levels in FCA induced arthritis in rats

The challenge with FCA caused significant increase in serum TNF-α, IL-1β and IL-6 levels, when compared to healthy control group. Treatment with WEA (200 and 400 mg/kg) caused significant decrease in the serum TNF-α, IL-1β and IL-6 levels as compared to arthritic control group (Table 2).

Table. 1. Effect of WEA on hematological parameters in FCA induced arthritis in rats.

Groups	RBC (10^3cells/mm^3)	WBC (10^3cells/mm^3)	Hb (gm/dL)	Platelet (10^3cells/$mm^{3)}$	ESR (mm/h)	CRP (mg/lit)
Healthy Control	6.932±0.105	7.625±0.211	14.48±0.240	9.157 ± 0.112	8.350± 0.189	1.592±0.033
Arthritic Control	3.312±0.065$^{\#}$	15.39±0.255$^{\#}$	8.782±0.151$^{\#}$	18.47 ±0.274$^{\#}$	15.40± 0.339$^{\#}$	6.928±0.229$^{\#}$
Etoricoxib 10 mg/kg	5.953±0.090***	12.85±0.451***	13.22±0.128***	13.95±0.245***	9.850± 0.375***	3.255±0.088***
WEA 100 mg/kg	3.183±0.043	15.33±0.058	9.050±0.134	18.04 ± 0.076	14.17± 0.125*	6.417±0.207
WEA 200 mg/kg	4.197±0.212**	13.75±0.421**	9.892±0.384**	17.28 ± 0.200**	13.63± 0.305**	5.948±0.237**
WEA 400 mg/kg	5.042±0.272***	13±0.128***	13.20±0.224***	15.24±0.293***	11.57± 0.416***	4.902±0.086***

WEA: wedelolactone rich extract of *Eclipta alba*; FCA: Freund's complete adjuvant, RBC: red blood cell, WBC: white blood cell, Hb: hemoglobin, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein.

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared with arthritic control group and $^{\#}P<0.001$when arthritic control group compared with healthy control group.

Table.2. Effect of WEA on biochemical parameters and cytokines in FCA induced arthritis in rats.

Groups	TP (gm/dL)	AST (U/L)	ALT (U/L)	ALP(U/L)	TNF- α(pg/ml)	IL - 1β (pg/ml)	IL – 6(pg/ml)
Healthy Control	7.21± 0.144	41.81 ±1.275	53.56±1.156	75.07±1.550	38.67 ± 1.022	120.5± 0.522	140.1± 1.104

Arthritic Control	5.583± 0.179 $^{\#}$	124.5±1.804$^{\#}$	186.9±1.433$^{\#}$	446.2±2.420$^{\#}$	120.2 ± 1.447$^{\#}$	426.6± 3.491$^{\#}$	407.0± 1.936$^{\#}$
Etoricoxib 10 mg/kg	7.433 ±0.128***	109.3±0.997***	172.8±3.688***	435.1±1.566***	114.3± 1.202*	413.9± 2.230*	398.7 ± 2.519*
WEA 100 mg/kg	5.767 ±0.185	117.3±2.321*	179.3±2.025*	441.8±1.204	111.5 ± 2.172**	408.0± 2.565**	400.7 ± 1.965
WEA 200 mg/kg	6.200±0.093*	115.7±1.437**	175.0±1.508**	436.7±2.463**	107.8 ± 1.424***	319.6± 3.599***	399.7 ± 1.295*
WEA 400 mg/kg	6.9±0.0912***	107.8±2.173***	169.4±0.870***	431.6±1.480***	97.33 ± 1.333***	268.7± 5.067***	394.6± 1.957***

WEA: wedelolactone rich extract of *Eclipta alba*; FCA: Freund's complete adjuvant, TP: Total protein, AST: Aspartate transaminase, ALT: Alanine transaminase, ALP: alkaline phosphatase, TNF- α: Tumor necrosis factor- α, IL - 1β: Interleukin- 1β, IL-6: Interleukin-6.

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test. *P<0.05, **P<0.01, ***P<0.001when compared with arthritic control group and $^{\#}$P<0.001when arthritic control group compared with healthy control group.

3.10. Effect of WEA on spleen and thymus weight in FCA induced arthritis in rats

There was significant increase in the spleen and thymus weight in FCA induced arthritic control animals when compared to healthy control group. Treatment with etoricoxib and WEA (400 and 200 mg/kg) significantly reduced the spleen and thymus weights when compared to arthritic control group. However, WEA 100 mg/kg did not change spleen and thymus weight when compared to arthritic control group (Table 3).

3.11. Effect of WEA on Antioxidant parameters in FCA induced arthritis in rats

There was a significant decrease in GSH and SOD levels while significant increase in MDA levels in arthritic control group observed when compared with healthy control group. Treatment with etoricoxib and WEA (400 and 200 mg/kg) significantly increased GSH and SOD levels while significantly decreased MDA level (Table 3).

Table.3. Effect of oral administration of WEA on spleen and thymus weight and antioxidant parameters in FCA induced arthritis in rats.

Groups	Spleen weight (gm)	Thymus weight (gm)	SOD (mU/mg protein)	MDA (nmole MDA/mg protein)	GSH (µmol/mg protein)
Healthy Control	0.5383±0.010	0.09483±0.017	4.470±0.033	1.992±0.013	70.56±0.598
Arthritic Control	0.8017±0.012$^{\#}$	0.22000±0.005$^{\#}$	2.433±0.028$^{\#}$	3.437±0.013$^{\#}$	44.33±0.829$^{\#}$
Etoricoxib 10 mg/kg	0.6517±0.012***	0.13500±0.004***	2.872±0.028***	2.957±0.018***	55.84±0.632***
WEA 100 mg/kg	0.7600±0.014	0.19170±0.006	2.418±0.029	3.387±0.024	46.76±0.693
WEA 200 mg/kg	0.7350±0.012**	0.17170±0.007**	2.617±0.061**	3.332±0.019**	48.45±0.515**
WEA 400 mg/kg	0.6733±0.008***	0.15000±0.003***	3.177±0.039***	2.788±0.030***	52.98±0.939***

WEA: wedelolactone rich extract of *Eclipta alba*; FCA: Freund's complete adjuvant, SOD: Superoxide dismutase, MDA: Malondialdehyde, GSH: Glutathione reductase. Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test. $^{**}P<0.01$, $^{***}P<0.001$ when compared with arthritic control group and $^{\#}P<0.001$when arthritic control group compared with healthy control group.

3.12. Effect of WEA on histopathological analysis of ankle joint in FCA induced arthritis in rats

Histopathology of ankle joint of healthy control rats showed no signs of inflammation, with intact synovial lining (Fig. 7A). Histopathology of ankle joint of arthritic control animals showed massive influx of inflammatory cells, chronic inflammation and disturbed synovial lining (Fig. 7B). In contrast to these, histopathology of ankle joint of animals treated with WEA (400mg/kg) and etoricoxib showed significant protection against synovial lining and low influx of inflammatory cells (Fig. 7F and Fig. 7C, respectively). WEA (200 mg/kg) treated rats showed moderate disturbance in synovial lining with little presence of inflammatory cells (Fig. 7E) and WEA (100 mg/kg) treated rats showed influx of inflammatory cells with evidence of disturbed synovial lining (Fig. 7D).

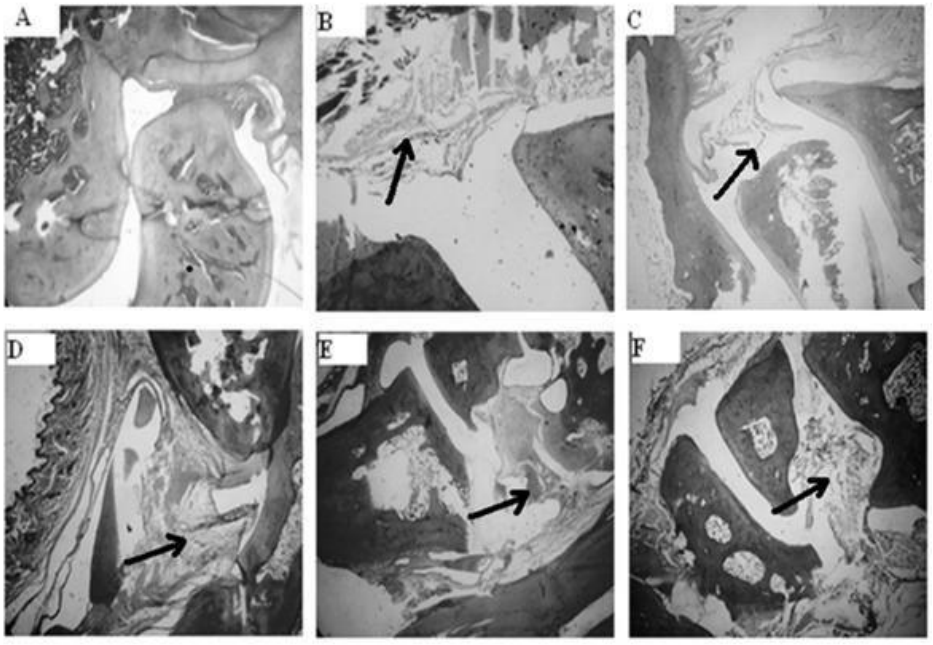


Fig. 6.Histopathology of ankle joint

6A: Healthy control: Joint- Joint Bone with no infiltration of inflammatory cells exudate in joint tissue H&E 10X**;** Thickness: 5µ; Magnification: 40X **6B: Arthritic Control: Joint-** Joint bone with maximum infiltration of inflammatory cells exudate in joint tissue**.** H&E 10X**;** Thickness: 5µ; Magnification: 40X **6C: Etoricoxib 10**

mg/kg: Joint- Joint Bone with no infiltration of inflammatory cells only minimal exudate in joint tissue H&E 10X**;** Thickness: 5µ; Magnification: 40X **6D: WEA 100mg/kg: Joint-** Joint Bone with Moderate infiltration of inflammatory cells and exudate in joint H&E 10X; Thickness: 5µ; Magnification: 40X **6E: WEA 200mg/kg: Joint-** Joint Bone with minimal infiltration of inflammatory cells and fibrous tissue H&E 10X; Thickness: 5µ; Magnification: 40X **6F: WEA 400mg/kg: Joint-** Joint Bone with no infiltration of inflammatory cells only minimal exudate in joint tissue H&E 10X; Thickness: 5µ; Magnification: 40X.

3. Discussion

RA is chronic autoimmune inflammatory disease. The existing drugs relieve pain and inflammation without much effect on disease progression. The use of biologicals is limited owing to the cost involved. Herbs are promising, inexpensive, highly tolerated and are vital source of new therapeutic agents for various diseases. Employing herbal medicines based on their traditional use for treatment of chronic diseases has increased in the last few decades [22]. The various bioactive phytoconstituents present in plants are more effective as they act on multiple sites and shows synergistic activity [23, 24]. *Eclipta alba* is traditionally claimed to alleviate pain and inflammation. Wedelolactone is a major constituent of *Eclipta alba* reported to inhibit cytokines and to posses antiinflammatory and immunomodulatory effect. These findings prompted us to evaluate the antiarthritic potential of wedelolactone rich fraction of *Eclipta alba* in FCA induced arthritis in rats.

Enrichment of active phytoconstituent leads to potentiation of pharmacological activity hence in present we prepared enriched extract of wedelolactone. The content of wedelolactone in ethanolic and enriched fraction was quantified by validated HPLC method. The enrichment was significantly high with 4 fold increase in the content of wedelolactone when compared to ethanolic extract.

FCA induced arthritis is a type of cell mediated immunity which results in synovial inflammation characterized with accumulation of T cells, plasma cells, macrophages, increased numbers of blood vessels and hyperplasia of the invasive intimal lining with intense immunological activity in synovial environment [25, 26]. The present study demonstrated that treatment with WEA dose dependently attenuated adjuvant induced arthritis and facilitated recovery as shown by decrease in paw volume, hyperalgesia, cytokine levels which are further substantiated by histopathology of ankle joints.

Body weight is considered as indirect index of health status and disease recovery. Proinflammatory cytokines causes acute increase in leptin level leading to weight loss by anorexia and elevated metabolic rate [27]. WEA prevented the weight loss indicating their ability to suppress these proinflammatory cytokines.

In arthritis increase in paw volume is due to inflammation caused by synovial destruction mediated through proinflammatory cytokines [26]. In the present study WEA significantly inhibited the inflammation which is evident by decreased paw volume.

Inflammation causes hyperalgesia, increased responses to noxious stimuli which are mediated by release of prostaglandins and other endogenous mediators [28]. This hyperalgesia limits ability of patient to do daily activities and therefore diminishes quality of life. In the present study, the pain threshold in arthritic control group significantly decreased while WEA treatment increased pain threshold in mechanical hyperalgesia, thermal hyperalgesia and tactile allodynia screening. This confirms the antiinflammatory and analgesic potential of WEA.

RA causes decrease in RBC and Hb while increase in ESR as presence of proinflammatory cytokines decreases iron absorption and transport, decreases erythropoiesis and causes premature destruction of RBCs while IL-1 stimulates a moderate rise in WBCs [29]. WEA significantly

increased RBCs count and Hb level while decreased ESR and WBC count. These findings confirmed the attenuation of inflammatory cytokines. Serum AST and ALT play important role in the formation of inflammatory mediators and hence serves as a simple and excellent tool to measure the antiarthritic activity. ALP levels are increased in case of bone erosion and periarticular osteopenia, a sensitive marker of cellular integrity and cellular toxicity induced by pathological conditions [30, 31]. Treatment with WEA caused decrease in AST, ALT and ALP levels which confirmed the decrease in inflammation and bone erosion.

TNF- α and IL-6 are proven therapeutic targets (biologicals) to treat RA. Cytokines mainly TNF-α, IL-1 β and IL-6 secreted by macrophages promotes induction of adhesion molecules and proteinase gene expression, which in turn plays important role in joint destruction. TNF- α mainly causes inflammation while IL-1 is responsible for cartilage and bone destruction, induction of acute phase proteins by hepatocytes, stimulation of prostaglandin and collagenase production by synovial cell [26]. Wedelolactone has been reported to have antinflammatory action by inhibiting release of cytokines especially IL-1 β. The observed decrease in the disease severity was associated with reduced levels of inflammatory cytokines TNF- α, IL-1 β and IL-6 when treated with WEA owing to the presence of wedelolactone.

Free radicals generated during RA causes phagocytosis of immune complexes leading to lipid peroxidation which increased MDA content, decreased GSH and SOD levels in arthritic animals as a consequence of their consumption during oxidative stress and cellular lysis [32]. In the present study, WEA administration reversed these effects which validated the antiarthritic potential of WEA.

Autoimmunity in arthritis leads to splenomegaly generalized lymphadenopathy and altered hepatic function [33]. Spleen and thymus weight was significantly increased in arthritic control

group as compared to healthy control group, while WEA decreased spleen and thymus weight. This indicated the inhibition of lymphocytes and decreased immunological response on WEA treatment.

Histological changes of ankle joint revealed the elevated number of inflammatory cells, chronic inflammation and disturbed synovial lining which are hallmarks of RA was found in arthritic control animals. WEA treatment was able to reverse the histological findings to normal. This effect of WEA on joints cartilage in arthritic rats is mediated by attenuation of inflammatory cytokines and antioxidant activity.

4. Conclusion

WEA was found to posses considerable antiarthritic potency as evident by alleviated paw volume, cytokine levels and other critical parameters associated with arthritis. This is attributed to presence of wedelolactone a proven antiinflammatory agent along with other phytoconstituents responsible for its effectiveness.

5. Acknowledgement

The authors acknowledge the grant received from University Grants Commission (UGC) for the project grant under Major Research Project (MRP).

6. References

1. Bais S, Abrol N, Prashar Y, Kumari R. Modulatory effect of standardised amentoflavone isolated from *Juniperus communis* L. against Freund's adjuvant induced arthritis in rats (histopathological and X Ray analysis). Biomedicine and Pharmacotherapy 2017;86:381–392.

2. Chitme HR, Patel NP. Antiarthritis activity of *Aristolochia Bracteata* extract in experimental animals. The Open Natural Products Journal, 2009; 2: 6-15.
3. Yu D, Ye X, Che R, Wu Q, Qi J, Song L, Guo X, Zhang S, Wu H, Ren G, Lia D. FGF21 exerts comparable pharmacological efficacy with Adalimumab in ameliorating collagen-induced rheumatoid arthritis by regulating systematic inflammatory response. Biomedicine & Pharmacotherapy 2017; 89:751–760.
4. Anantha DA, Rameshkumara A, Jeyadevia R, Aseervathama GSB, Sripriya J, Bose PC, Sivasudha T. Amelioratory effect of flavonoids rich *Pergularia daemia* extract against CFA induced arthritic rats. Biomedicine & Pharmacotherapy 2016;80: 244–252.
5. Datta P, Sarkar A, Biswas AK, Gomes A. Anti arthritic activity of aqueous extract of Indian black tea in experimental and clinical study. Oriental Pharmacy Experimental Medicine. 2012; 12:265–271.
6. Murugananthan G, Sudheer KG, Sathya CP, Mohan S. Anti-Arthritic and Anti-Inflammatory Constituents from Medicinal Plants. Journal of Applied Pharmaceutical Science 2013;3 (04):161-164.
7. Dixit PK, Mittal S. Herbal sources of anti-arthritic potential: A comprehensive review. International Journal of Pharmaceutical and Biomedical Research 2013; 4 (2): 88-92.
8. Akinnawo OO, Anyasor G N, Osilesi O. Aqueous fraction of *Alstonia boonei* de Wild leaves suppressed inflammatory responses in carrageenan and formaldehyde induced arthritic rats. Biomedicine & Pharmacotherapy 2017;86: 95–101.
9. Mansooralia KP, Prakasha T, Kotreshab D, Prabhu K, Rama RN. Cerebroprotective effect of *Eclipta alba* against global model of cerebral ischemia induced oxidative stress in rats. Phytomedicine 2012;19: 1108– 1116.

10. Gogte VM. Ayurvedic Pharmacology and Therapeutic uses of Medicinal Plants (Drvyagunvignyan), Published by Shri Ramakrishnan Bhartiya Vidya Bhavan Kulapati 1st edition 2000:452.
11. Yuan F, Chen J, Sun P, Guan S, Xu J. Wedelolactone inhibits LPS-induced pro-inflammation via NF-kappa B Pathway in RAW 264.7 cells. Journal of Biomedical Science 2013:20-84.
12. Sawant M, Isaac J C, Narayanan S. Analgesic studies on total alkaloids and alcohol extracts of *Eclipta alba* (Linn.) Hassk. Phytotherapy Research 2004;18(2): 111-13.
13. Thakur VD, Mengi SA. Neuropharmacological profile of *Eclipta alba* (Linn.) Hassk. Journal of Ethnopharmacology 2005;102: 23–31.
14. Kobori M, Yang Z, Gong D, Heissmeyer V, Zhu H, Jung Y-K, Gakidis MAM, Rao A, Sekine T, Ikegami F, Yuan C, Yuan J. Wedelolactone suppresses LPS-induced caspase-11 expression by directly inhibiting the IKK Complex. Cell Death and Differentiation 2004; 11:123–130.
15. Jahan R, Al-Nahain A, Majumder S, Rahmatullah M. Ethnopharmacological Significance of *Eclipta alba* (L.) Hassk. (Asteraceae). International Scholarly Research Notices. 2014; Article ID 385969:1-22.
16. Mehta A, Sethiya N, Mehta C, Shah G. Anti-arthritis activity of roots of *Hemidesmus indicus* R. Br. (Anantmul) in rats. Asian Pacific Journal Tropical Medicine 2012; 130–135.
17. OECD Guidelines for testing of chemicals, Acute oral toxicity, Acute toxic class method. 2012; 423, 1.
18. Bihani GV, Rojatkar SR, Bodhankar SL. Anti-arthritic activity of methanol extract of *Cyathocline purpurea* (whole plant) in Freund's complete adjuvant-induced arthritis in rats. Biomedicine & Aging Pathology 2014; 4: 197–206.

19. Mishra H, Fridocich. The role of superoxide anion in the autooxidation of epinephrine and a simple assay for SOD. Journal of Biological Chemistry 1972; 3170–3175.
20. Slater T, Sawyer B. The stimulatory effects of carbon tetrachloride and other halogenoalkanes or peroxidative reactions in rat liver fraction in vitro. Biochemical Journal 1971; 123:805–14.
21. Morgon M, Depirre J, Mannervik B. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochemica et Biophysica Acta 1979; 582: 67–78.
22. Chaoa J, Lua T, Liao J, Huang T, Leed M, Chenga H, Hoe L, Kuo C, Penga W. Analgesic and anti-inflammatory activities of ethanol root extract of *Mahonia oiwakensis* in mice. Journal of Ethnopharmacology 2009; 125: 297–303.
23. Kaur G, Sultana S. Evaluation of antiarthritic activity of isoeugenol in adjuvant induced arthritis in murine model. Food and Chemical Toxicology 2012; 50: 2689–2695.
24. Venkatesha SH, Astry B, Nanjundaiah SM, Kim HR, Rajaiah R, Yang Y, Tong L, Yu H, Berman BM, Moudgil KD. Control of autoimmune arthritis by herbal extracts and their bioactive components. Asian Journal of Pharmaceutical Sciences 2016; 11: 301–307.
25. Talwar SK, Nandakumar K, Nayak PG, Bansal P, Mudgal J, Mor V, Rao CM, Lobo. Anti-inflammatory activity of *Terminalia paniculata* barks extract against acute and chronic inflammation in rats. Journal of Ethnopharmacology 2011; 134: 323–328.
26. Chen Q, Wei W. Effects and mechanisms of glucosides of *Chaenomeles speciosa* on collagen-induced arthritis in rats. International Immunopharmacology 2003; 3: 593–608.
27. Barbier M, Cherbut C, Aubé AC, Blottière HM, Galmiche JP. Elevated plasma leptin concentrations in early stages of experimental intestinal inflammation in rats. Gut 1998; 43(6): 783–90.

28. Hans-Georg S. Nociceptive neurons detect cytokines in arthritis. Arthritis Research & Therapy 2014; 16:470.

29. Romanowska-Próchnicka K, Rzodkiewicz P, Olesińska M, Szukiewicz D, Maśliński S. Extraskeletal Manifestations in Rheumatoid Arthritis -Clinical Cases. Innovative Rheumatology, (7) 131-164. Available from: http://www.intechopen.com/books/innovative-rheumatology/extraskeletal-manifestations-in-rheumatoid-arthritis-clinical-cases

30. Babu PN, Pandikumar P, Ignacimuthu S. Anti-inflammatory activity of *Albizia lebbeck* Benth., an ethnomedicinal plant, in acute and chronic animal models of inflammation. Journal of Ethnopharmacology 2009; 125: 356–360.

31. Mishra NK, Biswal GS, Chowdary KA, Mishra G. Anti-arthritic activity of *Tribulus terrestris* studied in Freund's adjuvant induced arthritic rat. Journal of Pharmceutical Education and Research. 2013;4(1): 41-46.

32. Mythilypriya R, Shanthi P, Sachdanandam P. Salubrious effect of Kalpaamruthaa, a modified indigenous preparation in adjuvant-induced arthritis in rats–A biochemical approach. Chemico Biological Interactions 2008;173: 148–158.

33. Otari KV, Shete RV, Upasani CD, Adak VS, Bagade MY, Harpalani A N. Evaluation of anti-inflammatory and anti-arthritic activities of ethanolic extract of *Vernonia anthelmintica* seeds. Journal of Cell and Tissue Research 2010; 10(2): 2269-2280.

Part II Formulation development

Rheumatoid arthritis (RA), an autoimmune disease, has characteristic pathological changes of persistent synovitis, vascular proliferation, infiltration of inflammatory cells, as well as damage of cartilage and bone (Lee et al., 2009; Pan et al., 2009; Xu et al., 2013a). In spite of available treatment risk of morbidity and mortality still remains high. The current treatment strategies have changed from traditional one like NSAIDs along with steroids and DMARDs to biologicals like TNF alpha blockers but are out of favor because of side effects associated and cost involved. (Wang et al., 2016)

Eclipta alba and *Zingiber officinale* both plants and their active constituents viz. Wedelolactone and 6-shogaol are proven challenging for the treatment of various chronic inflammatory diseases. Although 6-shogaol has been reported to posses wide array of therapeutic effects it exhibits poor bioavailability on account of its minimal amount in systemic circulation. Recent studies have shown that, 6-shogaol undergoes biotransformation by glucorunic acid and sulfate conjugation which contributes to poor bioavailability (Nikam *et al*., 2013) Wedelolactone is also reported have poor absorption through intestine and hence poorly bioavailable (Upadhyay *et al*., 2012).

The literature illustrates the tremendous therapeutic potential of *Eclipta alba* as well as *Zingiber officinale* and their active phytoconstituents in treatment of various inflammatory diseases like rheumatoid arthritis but having poor solubility and bioavailability. Considering the poor oral absorption and poor water solubility of wedelolactone and 6-shogaol the present study was aimed to design and develop a self emulsifying nano drug delivery system (SNEDDS) to improve its solubility and to achieve greater bioavailability. Further to evaluate the developed SNEDDS for antiarthritic activity in Freund's Complete Adjuvant induced arthritis in rats.

SNEDDS is one of the lipid based drug delivery system in recent times employed for drugs having poor oral bioavailablity owing to their poor solubility. It is isotropic mixture of oil, surfactant and cosurfactant which forms fine oil in water emulsion upon simple agitation. It is preconcentrated drug solution which forms fine nanosized emulsion when get diluted in GI fluids. It offers advantages like high stability, large interfacial, surface area hence improvement in dissolution rate and absorption profile thereby reducing the dose and dosing frequency (Sermkaew *et al.,* 2013).

In the present study, we tried to enhance the absorption and efficacy by preparing the phytoconstituent enriched extracts to get target specific action and formulated it in approach to enhance its bioavailability to get the synergistic action.

Materials and methods

Materials

Transcutol, Labrafil, Labrafac, were obtained as gift sample from Gattefosse Canada Inc. Tween 20, Tween 60, Tween 80, Span 80 and PEG were purchased from S.D. Fine Chem. Ltd., Mumbai, India. All chemicals were of Analytical Grade. Freshly prepared distilled water was used throughout the study.

Methods

Extraction and enrichment

Ethanolic extraction

The dried aerial parts of *Eclipta alba* and dry rhizomes of *Zingiber officinale* were powdered coarsely separately and was subjected to Soxhlets extraction by ethanol and filtered. The filtrate was concentrated to get ethanolic extract of *Eclipta alba* and *Zingiber officinale* respectively

Ethyl acetate fraction of *Eclipta alba*

A part of ethanolic extract of *Eclipta alba* was washed with hot water and fractionated with equal volume of ethyl acetate in separating funnel (Mehta *et al*., 2012). The collected ethyl acetate fraction was concentrated to obtain powdered extract by Rota evaporator (Equitor Roteva, Medica Instruments Mfg Ltd.).

Extraction using oils

Powder of both plants was extracted using oils labrafac, labrfil and transcutol. To 100 mg of powdered plant material 1 ml of oil was added and was vortexed for 15 min and then centrifuged, supernatant was collected as extract. Briefly, 200 mg of extract was added to each of the eppendorf tubes containing 1 ml of different oils investigated in this study. The mixture was vortexed on a vortex mixer Remi Mumbai, India) for 20 min to facilitate proper mixing and extraction in the oil. The mixtures were then allowed to equilibriate at room temperature for 48 h [11]. The samples were then centrifuged at 12000 rpm for 20 min to separate the undissolved drug. The supernatant solution was taken and filtered through a Millipore membrane filter (0.45 µm) and then suitably diluted with methanol. The concentrations of 6-shogaol and wedelolactone were determined using validated HPLC method (Jasco Corporation, Japan) was equipped with dual pump Jasco PU- 2080 Plus, UV/Visible detector UV-2075 plus; Thermo Scientific Merck C18 reversed-phase column (I.D. 4.6mm×250mm, 5µm) at 243 nm.

All the extracts of *Eclipta alba* and *Zingiber officinale* were quantified for wedelolactone and 6-shogaol respectively by validated high performance liquid chromatography method.

Solubility study

Various commonly used oils were screened for their ability to dissolve maximum amount of extract. Briefly, 200 mg of extract was added to each of the eppendorf tubes containing 1 ml of different oils investigated in this study. The mixture was vortexed on a vortex mixer Remi Mumbai, India) for 20 min to facilitate proper mixing and extraction in the oil. The mixtures were then allowed to equilibriate at room temperature for 48 h. The samples were then centrifuged at 12000 rpm for 20 min to separate the undissolved drug. The supernatant solution was taken and filtered through a Millipore membrane filter (0.45 µm) and then suitably diluted with methanol. The concentrations of 6-shogaol and wedelolactone were determined using validated HPLC method (Jasco Corporation, Japan) was equipped with dual pump Jasco PU-2080 Plus, UV/Visible detector UV-2075 plus; Thermo Scientific Merck C18 reversed-phase column (I.D. 4.6mm×250mm, 5µm) at 243 nm (Sermkaew *et al.,* 2013).

Selection of surfactant

The solubility of extract was determined in Tween 20(16.7) and Tween 60(14.9) by following the same procedure as described for the selection of oil by substituting oils with the surfactants. Thereafter, the surfactants were screened based on their ability to emulsify the selected oil phase. To determine the emulsification ability, 50 µl of surfactant was added to 50 µl of the selected oily phase, mixed thoroughly and then 25 µl of this mixture was diluted to 25 ml with distilled water. From that surfactant showing maximum emulsification was chosen for further study. The selected surfactant in varying proportion was mixed with 50 ul of oil. The oil surfactant

composition showing maximum and stable emulsification was chosen for further study. The ease of formation of emulsions was monitored by the number inversions of volumetric flask required to produce a uniform emulsion (Sermkaew *et al.,* 2013).

Selection of co-surfactant

The solubility of extract was also determined in various cosurfactants Span 80, Span 20, PEG-200, PEG-400 and Propylene Glycol by following the same procedure as described previously. Co-surfactants were screened based on their efficacy to improve the nanoemulsification ability of the selected surfactants. For this, 60 µl of surfactant was mixed with 40 µl of the co-surfactant The selected oil (100 µl) was added to this mixture (oil:Smix* = 1:1) and the mixture was gently heated in a water bath to allow proper mixing. 25 µl of this mixture was diluted to 25 ml with distilled water and the ease of formation of emulsions was monitored by the number of inversions required to produce uniform emulsion. The emulsions were allowed to stand for 2 h (Sermkaew *et al.,* 2013).

Preparation of SNEDDS

The finalized ratio was selected for preparation of SNEDDS. 200 mg Zo powder was extracted using finalized volume of labrafil for the prpeparation of SNEDDS and to the same tube was added 200 mg WEA. Surfactant and co-surfactant were incorporated in their determined ratios into oil phase containing drug.

Finally homogeneous mixture was obtained by vortex mixing. The prepared SNEDDS was kept in a tightly closed bottle at 25◦C and from these the stable formulations were subjected to further studies i.e. dilution studies, droplet size analysis, self-emulsification time, particle size analysis, and invitro drug release (Sermkaew *et al.,* 2013).

Characterization of formulations

Self emulsification time

Self emulsification time is the time required by the preconcentrate to form a homogeneous mixture upon dilution, when disappearance of SNEDDS is observed visually. The efficiency of self emulsification of SNEDDS was assessed by using a standard USP II dissolution apparatus. One ml of each formulation was added dropwise to the medium (900 ml of water with a paddle speed of 100 rpm at 37.0 ± 0.5◦C) by a dropping pipette and the time required for the disappearance of the SNEDDS was recorded. The efficiency of self emulsification was visually assessed.

Robustness to dilution

Robustness of SNEDDS to dilution was studied by diluting it 50, 100 and 1000 times with various dissolution media i.e. water, buffer pH 1.2, buffer pH 3.0 and buffer pH 6.8. The diluted samples were stored for 12 h and observed for any signs of phase separation or precipitation .

Particle size analysis

The particle size of the selected formulation was determined by Malvern 2000 Sm; Malvern Instruments UK.

In vitro drug release study

The release profile study of 6-shogaol and wedelolactone from SNEDDS was performed using the dialysis bag method according to dissolution apparatus II in USP 24. SNEDDS was instilled in to the dialysis bag (Dialysis Membrane-110). This was firmly sealed with dialysis clamp and was placed in 250 ml, 1.2 pH simulated gastric fluid as the disso-lution medium at 37◦C. The revolution speed of the paddle was maintained at 100 rpm. The samples (5 ml) were drawn at predetermined time intervals, and replenished with the same volume of fresh dissolution

medium. The drug content in the samples was assayed using validated HPLC method (Jasco Corporation, Japan) was equipped with dual pump Jasco PU- 2080 Plus, UV/Visible detector UV-2075 plus; Thermo Scientific Merck C18 reversed-phase column (I.D. 4.6mm×250mm, 5µm) at 243 nm (Sermkaew *et al.*, 2013).

Results

Solubility study

The purpose of drug solubility in various excipients used in SNEDDS was to get self emulsifying preparation to form nanodroplet size to improve in vivo behavior. 2. Based on solubility profile of extract in oil, surfactants and cosurfactant; Labrafil, Tween-80 and Span 80 were selected as oil phase, surfactant and cosurfactant respectively for the development of optimal SNEDDS of extract. It was evident that extracts exhibited highest solubility in Labrafil (89.56 %) and least solubility was observed in labrasol. Labrafil was selected as an oil phase for further investigation due to its solubilizing potential of extracts. Among various surfactants screened, tween 80 exhibited highest solubility of extracts. Whereas various cosurfactants, span 80 exhibited good solubilization property of extracts. In essential oils, labrafil showed higher solubility. Surfactants (tween 80) and co-surfactants (span 80) showed more significance than other surfactants and co-surfactants respectively.

The optimized formulation was selected among the five based on particle size analysis (<200 nm) and they are further studied for self emulsification time, dilution studies, in vitro drug release study, preclinical evaluation in the treatment of arthritis.

Characterization of formulations

Self emulsification time

The rate of emulsification was a major index for assessment of the efficiency of self-emulsification. The SNEDDS should disperse completely and quickly when subjected to dilution under mild agitation. Formulation F4 showed very less emulsification time (<1 min) when compared to others.

Robustness to dilution

Uniform emulsion formation from SNEDDS is very important at different dilutions because drugs may precipitate at higher dilution in vivo which affects the drug absorption significantly .Different fold dilutions of selected formulations were exposed to different media to mimic the in vivo conditions where the formulation would encounter gradual dilution. Hence, each formulation was subjected to 50, 100, 1000 times dilution in water, pH 1.2, pH 3 and pH 6.6. The resulting emulsions (F1, F2, F3 and F4) were found to be in the acceptable nanoemulsion region (<200 nm), proving their robustness to dilution. This result will ensure the prospect of uniform drug release profile in vivo. Even after 24 h, formulations F4 showed no signs of precipitation, cloudiness or separation which ensured the stability of the reconstituted emulsion. On the contrary, the rest of the formulations showed phase separation and turbidity.

Particle size analysis

The selected SNEDDS formulation taken for particle size analysis. Smaller particle size of the emulsion droplets may lead to more rapid absorption and improve bioavailability. The particle size of the formulation was found to be 200 nm (d- 0.9) (Fig.).

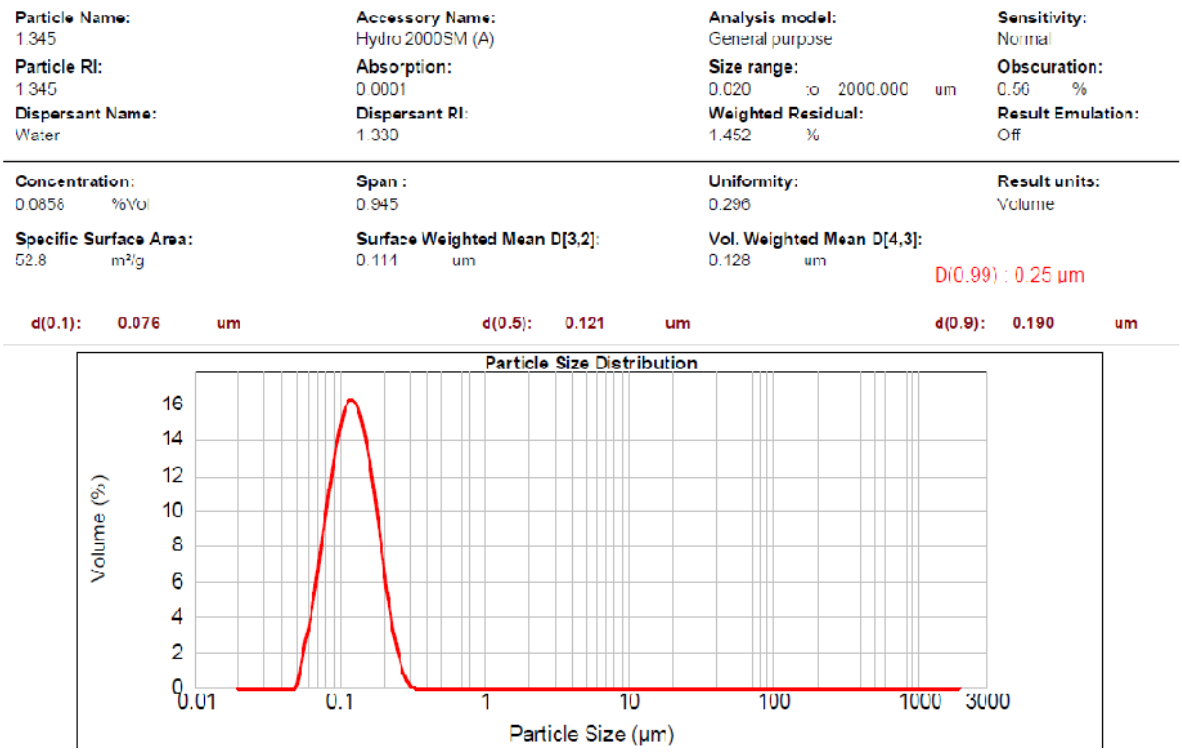


Fig. Particle size analysis of SNEDDS F4

In vitro drug release study

The in vitro drug release study of the optimized formulations was performed in 250 ml, at simulated gastric pH 1.2. The release pattern of SNEDDS reveals that the maximum drug release was observed with F4 formulation after 120 min. This could be due to proper composition of oil and surfactant in the system. The data indicates that the release rate of 6-shogaol from SNEDDS formulations was considerably faster when compared with standard 6-shogaol. The cumulative percentage drug release of 6-shagaol and wedelolactone from F was found to be 96.21% for 120min and 97.12% at 120 min respectively. Thus, in vitro results reveal that the prepared SNEDDS formulation showed improved solubility of extracts.

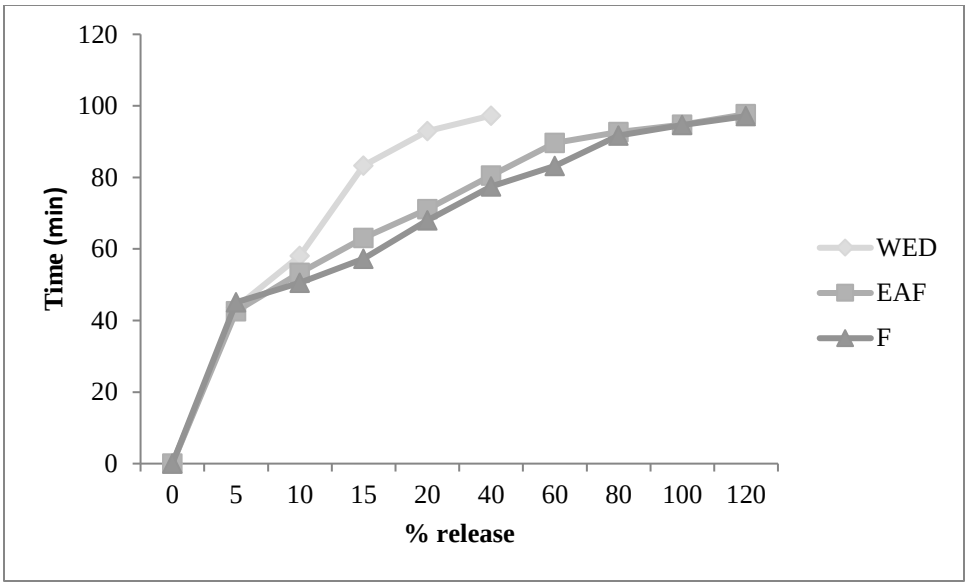


Fig. In vitro drug release study for formulation of EAF and F

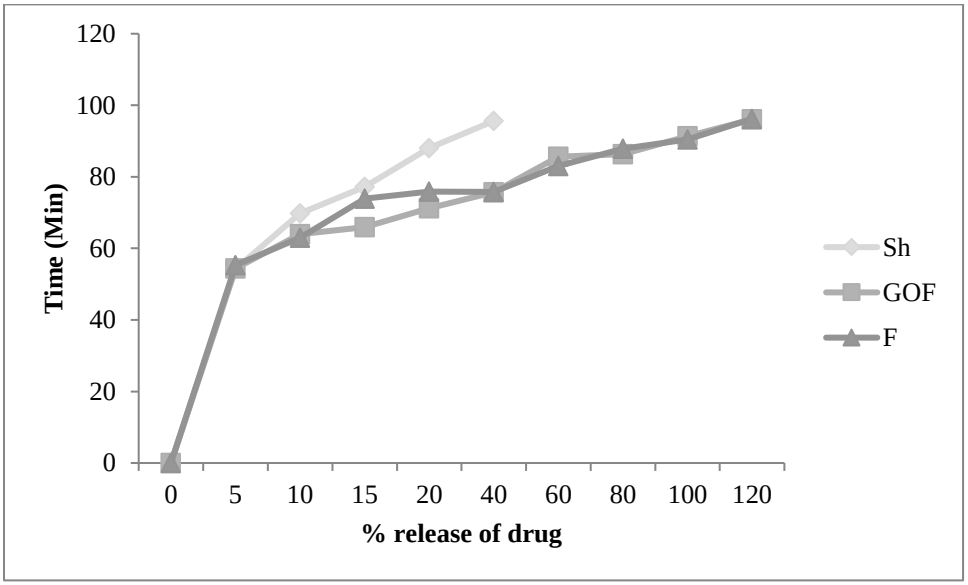


Fig. In vitro drug release study for formulation of GOF and F

Part III Antiarthritic evaluation of formulation

RA is associated with systemic inflammatory disorders involving multiple joints. It is an autoimmune disorder of unknown etiology that is characterized by progressive joint destruction, deformity, disability and premature death in most patients (Lee et al., 2009; Zhang et al., 2014). It is pathologically characterized by synovial hyperplasia, inflammatory cell infiltration and angiogenesis (Pan et al., 2009). CFA-induced secondary inflammation mimics sub-acute RA characterized by excessive immunologic activity in the synovium (Fan et al., 2005).

Plants are rich sources of pharmacologically active compounds. They can be thought of as natural biosynthetic factories for chemicals with small and large molecular weights. The existence of these chemicals has ensured the survival of the plant through millions of year evolution.

Natural products especially those derived from herbs, have since ancient times contributed to the development of modern therapeutic drugs. Herbal medicinal products target specific defined mediators of inflammation and pain. The major benefit of using herbs and other natural products lies in their limited or no undesirable side effects. Therefore, the interdisciplinary efforts of researchers are aimed at identifying new herbal products and defining their mechanisms of action has been reinforced. This has facilitated the discovery and development of safe and effective natural products for the treatment of chronic pain (Chpoade and Sayyed., 2013)

Due to its multidirectional promising aspects, the interest in natural product continues to this day

This phenomenon has been mirrored by an increasing attention to phytomedicines as a form of alternative therapy by the health professions; in many developing countries there is still a major reliance on crude drugs prepared from plants used in traditional medicines, for primary healthcare (Maitera *et al.*, 2011)

Eclipta alba and *Zingiber officinale* both plants and their active constituents viz. Wedelolactone and 6-shogaol are proven challenging for the treatment of various chronic inflammatory diseases. Although 6-shogaol has been reported to posses wide array of therapeutic effects it exhibits poor bioavailability on account of its minimal amount in systemic circulation. Recent studies have shown that, 6-shogaol undergoes biotransformation by glucorunic acid and sulfate conjugation which contributes to poor bioavailability (Nikam *et al*., 2013) Wedelolactone is also reported have poor absorption through intestine and hence poorly bioavailable (Upadhyay *et al*., 2012).

The literature illustrates the tremendous therapeutic potential of *Eclipta alba* as well as *Zingiber officinale* and their active phytoconstituents in treatment of various inflammatory diseases like rheumatoid arthritis but having poor solubility and bioavailability. Considering the poor oral absorption and poor water solubility of wedelolactone and 6-shogaol the present study was aimed to design and develop a self emulsifying nano drug delivery system (SNEDDS) to improve its solubility and to achieve greater bioavailability. Further to evaluate the developed SNEDDS for antiarthritic activity in Freund's Complete Adjuvant induced arthritis in rats.

SNEDDS is one of the lipid based drug delivery system in recent times employed for drugs having poor oral bioavailability owing to their poor solubility. It is isotropic mixture of oil, surfactant and cosurfactant which forms fine oil in water emulsion upon simple agitation. It is preconcentrated drug solution which forms fine nanosized emulsion when get diluted in GI fluids. It offers advantages like high stability, large interfacial, surface area hence improvement in dissolution rate and absorption profile thereby reducing the dose and dosing frequency. (Soliman *et al.*, 2016)

In the present study, we tried to enhance the absorption and efficacy by preparing the phytoconstituent enriched extracts to get target specific action and formulated it in approach to enhance its bioavailability to get the synergistic action.

Materials and methods

Chemicals

Reference standard 6-shogaol (99%) and wedelolactone (99%) was purchased from Natural Remedies Pvt. Ltd. Bangalore, Karnataka, India. Food grade Labrafil was a generous gift from Gattefosse, France. Freund's Complete Adjuvant was purchased from Sigma Aldrich, USA. Ethanol GR grade was purchased from Merck Chemicals (Mumbai, India), etoricoxib (gift sample from Zydus Cadila, Gujarat) All other solvents used for the study were of analytical grade.

Plant materials

The dried rhizomes of *Zingiber officinale* and *Eclipta alba* were purchased from local market Pune, India. The samples were authenticated at Botanical Survey of India (BSI), Pune, Maharashtra, India and voucher specimen (No BSI/WRC/Cert./2014/AS03) and (No BSI/WRC/Cert./2014/AS01)respectively was deposited for future reference. The shade-dried roots of *Eclipta alba* were ground, the resulting powder passed through 30 mesh screens and stored in an air-tight container at 15–20 °C until further use.

Extraction

Extraction and formulation developed as described in Part I and II respectively.

Acute toxicity studies

Acute toxicity studies were carried out for GOF following OECD guidelines No. 423. The extract was administered orally at the dose of 5 mg/kg body weight to overnight-fasted, healthy

female rats (n=3). The animals were observed individually for behavioral and autonomic profiles after dosing with special attention given during first 4 h, and daily thereafter, for a total period of 14 days. The test was repeated with doses of 50, 300 and 2000 mg/kg body weight.

FCA induced arthritis

The animals were divided into six groups of six animals each. Group I served as healthy control (2 % w/v Tween 80), Group II as arthritic control (treated with vehicle), Group III as standard which received 10 mg/kg etoricoxib (p. o.), Group IV, V and VI received 50, 100 and 200 mg/kg GOF (p. o.), respectively. All animals except healthy control group were injected with 0.1 ml of FCA in the subplantar region of the left hind paw on day 0. The respective treatment started once signs of arthritis set in (day 12), orally once daily.

Body weight, paw volume, pain threshold, thermal and mechanical hyperalgesia and tactile allodynia were evaluated on day 0, 1, 4, 8, 12, 16, 20, 24 and day 28. On day 28, blood was withdrawn by retro orbital puncture under ether anesthesia for hematology, serum was separated for biochemical parameters and cytokine estimation (Bihani *et al*., 2014).The animals were sacrificed by CO_2 euthanasia and spleen, thymus, liver and ankle joints were isolated.

Body weight

Body weight was recorded on all the above mentioned evaluation days using animal weighing balance (Ananth *et al*., 2016).

Paw volume

Paw volume was measured using a Plethysmometer (UGO Basile, Italy). The change in paw volume was calculated as the difference between the final and initial paw volume (Ananth *et al*., 2016).

Mechanical hyperalgesia

It was measured as the paw withdrawal threshold of the animal in Randall-Selitto analgesiometer (UGO Basile, Italy). The hind paw was placed between the flat surface and blunt pointer and increasing pressure was applied. The pain threshold was determined when rat attempted to remove the hind paw from the apparatus. The cut-off pressure was 450g (Bihani *et al*., 2014).

Thermal hyperalgesia

It was measured as paw withdrawal latency in radiant heat apparatus (UGO Basile, Italy). The paw was placed on the heat radiator with infrared intensity of lamp set at 40. A cut off latency of 15 sec was used to avoid tissue damage (Bihani *et al*., 2014).

Tactile allodynia

It was determined as mechanical nociceptive threshold by measuring paw withdrawal upon probing of the plantar surface with a series of calibrated fine filaments (von Frey hairs, Almemo, Germany) of increasing gauge (0.6 to 12.6g). The rats were allowed to acclimatize for 10 min in the perspex box. A series of three stimuli were applied to each paw with each hair in a period 2–3sec. The lowest weight of von Frey hair to evoke a withdrawal from the three consecutive applications was considered as threshold. Lifting of the paw was recorded as a positive response (Bihani *et al*., 2014).

Measurement of Cytokine levels

On day 28, serum TNF-α, Interleukin 1β (IL-1β) and Interleukin-6 (IL-6) were determined using ELISA kit (Bihani *et al*., 2014).

Hematological and biochemical parameters

On day 28, RBC count, hemoglobin (Hb), and platelets (PLT) count were determined by usual standardized laboratory methods (Mehta *et al*., 2012). Serum was used for the estimation of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total protein (TP) and C-reactive protein (CRP) (Bihani *et al*., 2014).

Spleen and thymus weight

On day 28, rats were sacrificed by CO_2 euthanasia. The spleen and thymus of all the rats were removed and weighed (Bihani *et al*., 2014). The liver was isolated for antioxidant studies while the ankle joints were isolated for histopathology.

Antioxidant parameters

The liver isolated after sacrificing was washed in ice-cold saline and homogenated with 0.1M Tris-HCl buffer (pH 7.4). The supernatant was used to determine superoxide dismutase (SOD) (Mishra and Fridovich 1972), malondialdehyde (MDA) (Slater and Sawyer 1971), and reduced glutathione (GSH) **(**Morgon *et al*., 1979).

Histopathological analysis of ankle joints

The ankle joints separated from the hind paw were immersed in 10% buffered formalin and processed for paraffin embedding section at 5 µ thickness. The sections were stained with haematoxylin-eosin dye and evaluated under light microscope with 10X magnifications.

Statistical Analysis

The data was analyzed by one way ANOVA followed by Dunnett's test for biochemical analysis, two way ANOVA followed by Bonferroni's post hoc test for in vivo parameters. All statistical analyses were performed using Graph Pad Prism software (San Diego, CA). Data was considered statistically significant at $P<0.05$.

References:

- Pan R, Dai Y, Gao X H, Xia Y F. 2009. Scopolin isolated from *Erycibe obtusifolia* Benth stems suppresses adjuvant-induced rat arthritis by inhibiting inflammation and angiogenesis. International Immunopharmacology 9, 859–869.
- Lee J S, Kim K A, Jeong S H, Lee S G, Park H J, Kim N J, Lim S. 2009. Antiinflammatory, anti-nociceptive and anti-psychiatric effects by the rhizomes of *Alpinia officinarum* on complete Freund's adjuvant-induced arthritis in rats. Journal of Ethnopharmacology 126, 258–264.
- Zhang CF, Zhang SL, He X, Yang XL, Wu HT, Lin BQ, Jiang CP, Wang J, Yu CH, Yang ZL, Wang CZ, Li P, Yuan CS. Antioxidant effects of Genkwa flos flavonoids on Freund's adjuvant-induced rheumatoid arthritis in rats. Journal of Ethnopharmacology 153 (2014) 793–800.
- Chen S. Natural Products Triggering Biological Targets- A Review of the Anti- Inflammatory Phytochemicals Targeting the Arachidonic Acid Pathway in Allergy Asthma and Rheumatoid Arthritis *Current Drug Targets,* 2011, *12,* 288-301 .
- Chopade AR, Sayyad FJ. Antinociceptive effect of *Phyllanthus fraternus* extract in complete Freund's adjuvant induced chronic pain in mice Biomedicine & Aging Pathology 3 (2013) 235–240

- O.N. Maitera, M. E. Khan* and T. F. James Phytochemical analysis and the chemotherapeutics of leaves and stembark of Nauclea latifolia grown in Hong, Adamawa State Nigeria. Asian Journal of Plant Science and Research, 2011, 1 (3):16-22

- Upadhyay K, Gupta NK, Dixit VK. Development and characterization of phyto-vesicles of wedelolactone for hepatoprotective activity. Drug Development and Industrial Pharmacy. 2012,38:1152-1158.

- Nikam AR, Sathiyanarayanan L, Mahadik KR. Mapping pharmacokinetic and tissue distribution profile of [6]-shogaol from ginger oleoresin International Journal of Pharmacy and Pharmaceutical Sciences 2013, 5, (1): 185-189.

- Sermkaew N, Ketjinda W, Boonme P, Phadoongsombut N, Wiwattanapatapee R. Liquid and solid self-microemulsifying drug delivery systems for improving the oral bioavailability of andrographolide from a crude extract of *Andrographis paniculata.* European Journal of Pharmaceutical Sciences 50 (2013) 459–466.

- Bihani GV, Rojatkar SR, Bodhankar SL. Anti-arthritic activity of methanol extract of *Cyathocline purpurea* (whole plant) in Freund's complete adjuvant-induced arthritis in rats. Biomedicine & Aging Pathology 2014; 4: 197–206.

Results of antiarthritic activity of GOF in FCA induced arthritis in rats

Acute toxicity studies

The test animals did not exhibit any change in autonomic, behavioral profile and survived beyond recommended duration of observation with 2000 mg/kg of GOF (OECD Guideline No. 423). Hence it was safe up to 2000 mg/kg.

Effect of GOF on body weight in FCA induced arthritis in rats

There was a significant decrease in body weight of all the arthritic animals. On treatment with etoricoxib there was nonsignificant increase in body weight when compared with arthritic control group of animals. On treatment with GOF 50 mg/kg significantly improved body weight from day 24 onwards when compared to arthritic control. However, treatment with GOF (200 mg/kg and 100 mg/kg) significantly improved the body weight day 20 onwards as compared to arthritic control group (Fig. 2).

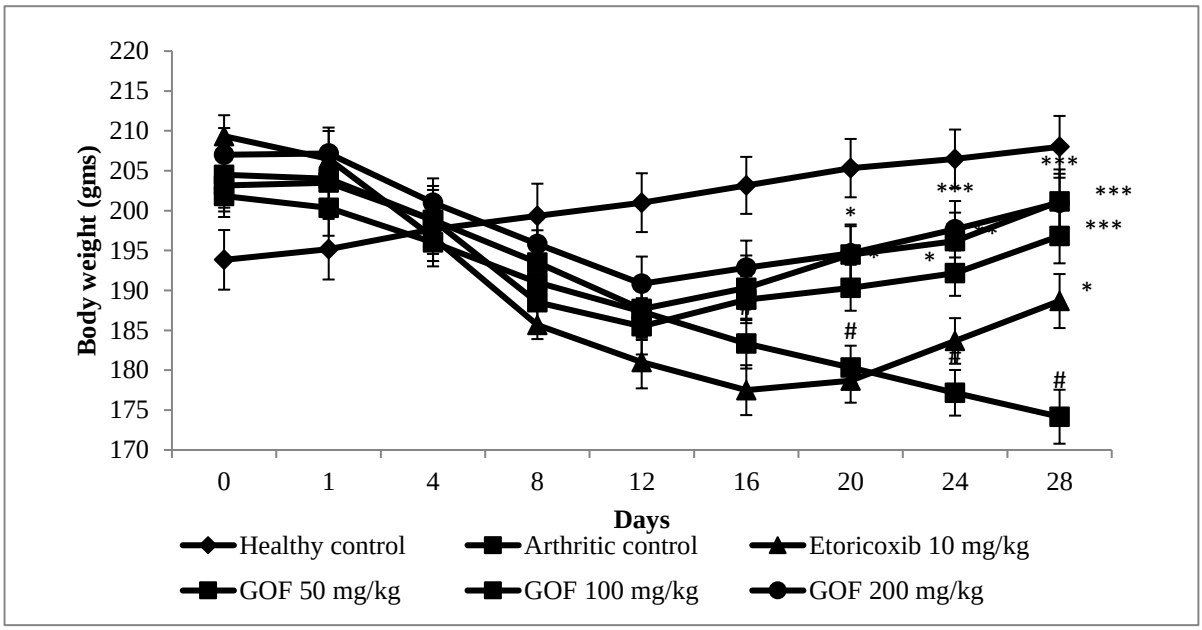


Fig. 2. Effect of GOF on body weight in FCA induced arthritis in rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared to healthy control

Effect of GOF on paw volume in FCA induced arthritis in rats

There was a significant increase in paw volume of all the rats in arthritic control group when compared to healthy control. Treatment with etoricoxib and GOF (200 and 100 mg/kg) significantly decreased the paw volume from day 16 onwards, respectively as compared to arthritic control group. GOF at the dose of 50 mg/kg was comparatively less effective and decreased the paw volume day from 20 onwards (Fig. 3).

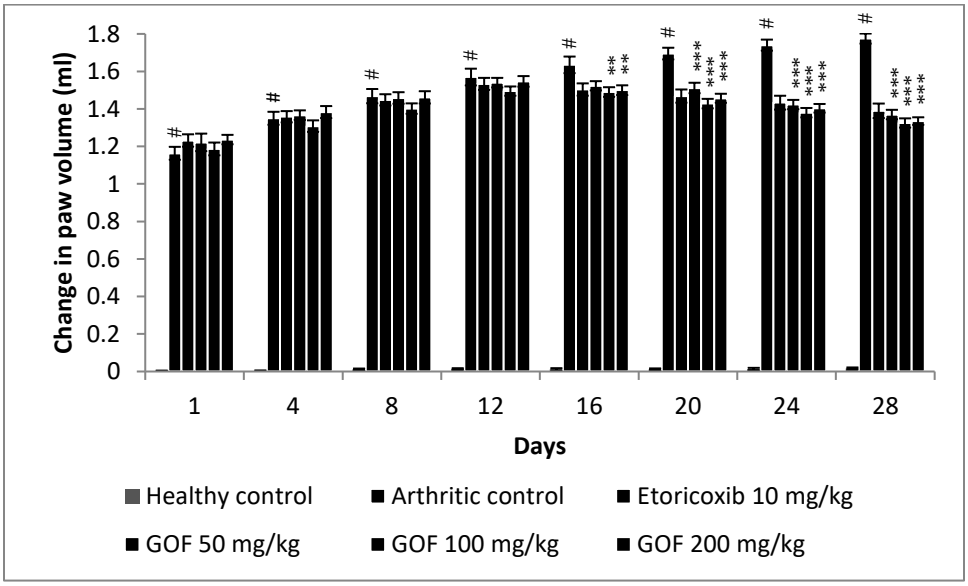


Fig. 3.Effect of GOF on paw volume in FCA induced arthritis in rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{**}P<0.01$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared to healthy control

Effect of GOF on Mechanical hyperalgesia in FCA induced arthritis in rats

Paw withdrawal threshold in all arthritic animals decreased progressively till day 12 when compared to healthy control animals. The paw withdrawal threshold in arthritic control animals was significantly less compared to the healthy control animals till the end of study. Treatment with Etoricoxib, GOF from day 12 onwards, significantly increased the pain threshold from day 16 onwards respectively at all dose levels. (Fig. 4).

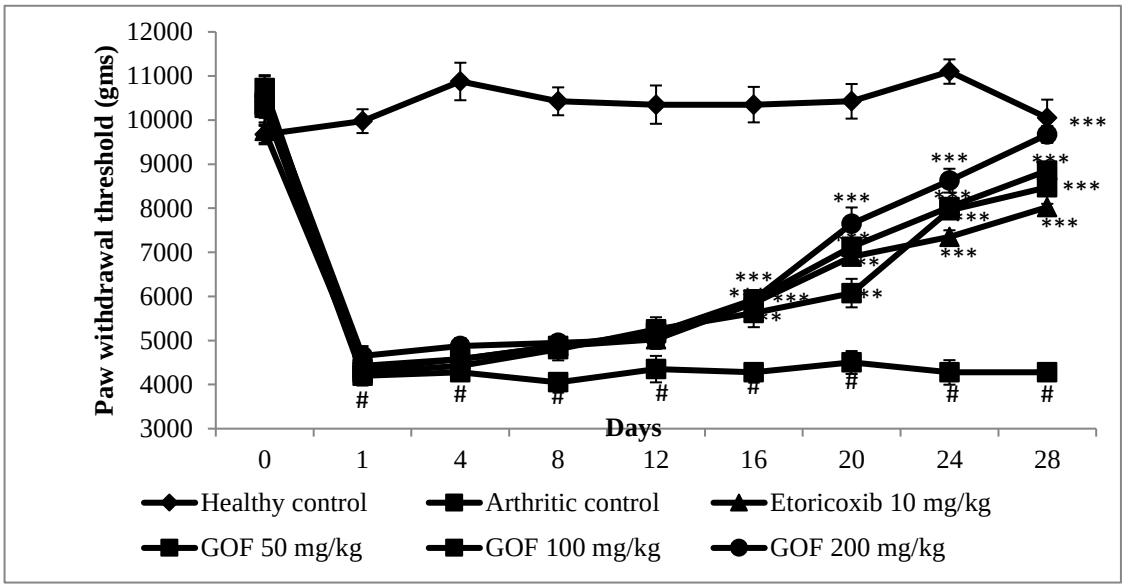


Fig.4 Effect of GOF on Mechanical hyperalgesia in FCA induced arthritis in rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{**}P<0.01$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared with healthy control.

Effect of GOF on Thermal Hyperalgesia in FCA induced arthritis in rats.

On induction of arthritis, there was a significant decrease in paw withdrawal latency of all arthritic animals till day 12 when compared to healthy control (Fig. 5). Treatment with etoricoxib and GOF (200 and 100 mg/kg) from day 12 significantly increased the paw withdrawal latency from day 16 onwards. However, GOF 50 mg/kg significantly ($p < 0.001$) increased in paw withdrawal latency from day 20.

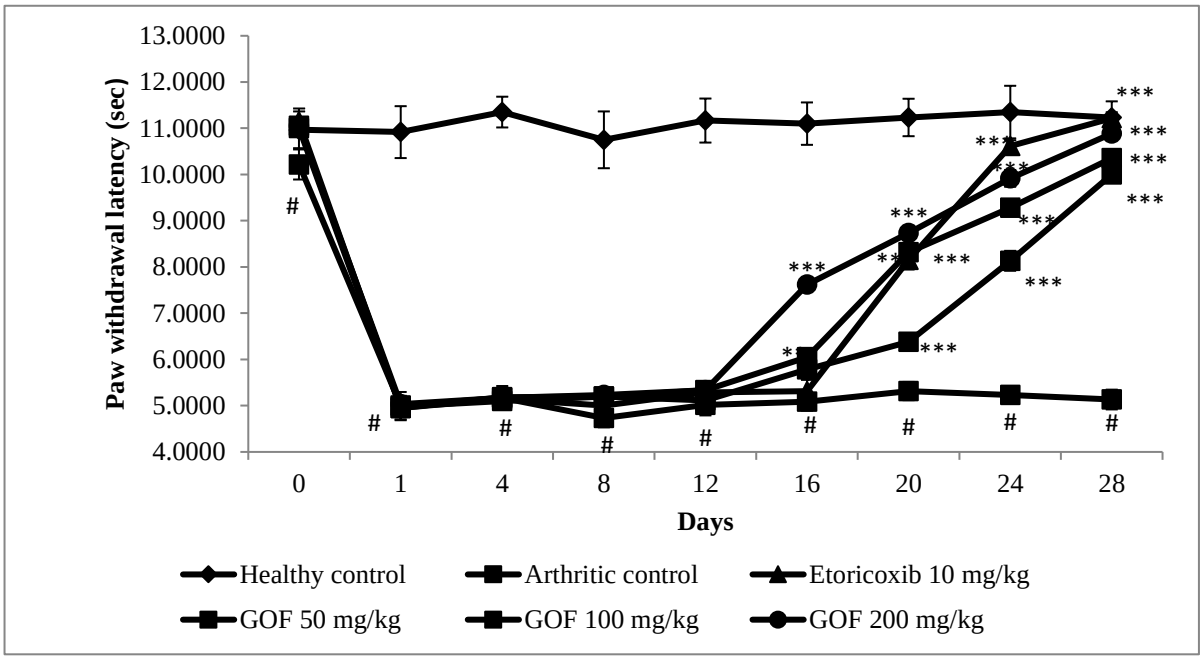


Fig.5 Effect of GOF on thermal hyperalgesia in FCA induced arthritis in rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared to healthy control

Effect of GOF on Tactile allodynia in FCA induced arthritis in rats

The mechanical threshold was significantly decreased in arthritic animals till day 12. Administration of etoricoxib and GOF (200 and 100 mg/kg) from day 12 significantly improved the mechanical withdrawal threshold from day 20 and 24 onwards respectively when compared with arthritic control while GOF 50 mg/kg significantly increased mechanical withdrawal threshold only on day 28 (Fig. 6). However there was no change in tactile allodynia in arthritic control group of animals.

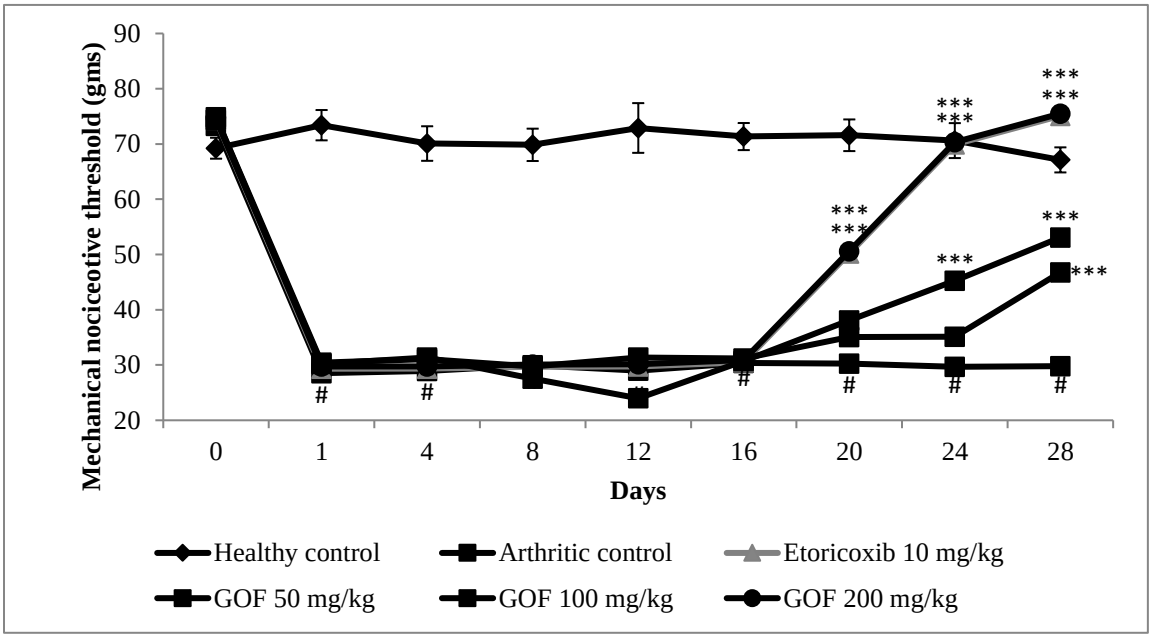


Fig. 6.Effect of GOF on Tactile allodynia in FCA induced arthritic rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{*}P<0.05$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared to healthy control

Effect of GOF on hematology and serum parameters in FCA induced arthritis in rats

There was significant increase in platelet count, WBC count and ESR while decrease in RBC count and Hb level observed in arthritic control group when compared to healthy control group. These conditions were reversed significantly and dose dependently on treatment with GOF (Table 1).

The serum CRP level was significantly increased in arthritic control group as compared to healthy control group. On treatment with etoricoxib and GOF, serum CRP level was found to be significantly decreased when compared with arthritic control group (Table 1).

The serum levels of AST, ALT and ALP were increased significantly in arthritic control group as compared to healthy control group. Treatment with etoricoxib and GOF significantly decreased AST, ALT and ALP level (Table 2).

The level of total protein was significantly decreased in arthritic control group when compared with healthy control group. Etoricoxib and GOF were found to be significant in restoring it when compared with arthritic control group (Table 2).

Effect of GOF on serum cytokine levels in FCA induced arthritis in rats

The challenge with FCA caused significant increase in serum TNF-α, IL-1β and IL-6 levels, when compared to healthy control group. Treatment with GOF caused significant and dose dependant decrease in the serum IL-1β, TNF-α and IL-6 levels as compared to arthritic control group (Table 2).

Table. 1. Effect of GOF on hematological parameters in FCA induced arthritis in rats.

Groups	RBC (10^3cells/mm^3)	WBC (10^3cells/mm^3)	Hb (gm/dL)	Platelet (10^3cells/$mm^{3)}$	ESR (mm/h)	CRP (mg/lit)
Healthy Control	6.845±0.1132	7.625±0.2118	14.38±0.3221	9.238±0.1322	8.325±0.2869	1.602±0.034
Arthritic Control	3.265±0.07377$^{\#}$	15.39±0.255$^{\#}$	8.9±0.1817$^{\#}$	18.46±0.3516$^{\#}$	15.45±0.5331$^{\#}$	7.177±0.166$^{\#}$
Etoricoxib 10 mg/kg	6.033±0.1188***	13.1±0.3058***	13.21±0.1519***	13.93±0.3861***	9.925±0.536***	3.682±0.1408***
GOF 50 mg/kg	4.325±0.2079**	14.18±0.1959*	10.75±0.407**	16.45±0.3978**	13.35±0.3708**	5.580±0.049***
GOF 100 mg/kg	4.573±0.2123***	12.19±0.0601***	11.04±0.4858***	14.91±0.2889***	12.1±0.2273***	4.823±0.016***
GOF 200 mg/kg	5.325±0.0817***	11.28±0.0607***	12.49±0.3269***	13.35±0.3804***	11.08±0.2496***	3.940±0.059***

GOF: *Zingiber officinale* formulation; FCA: Freund's complete adjuvant, RBC: red blood cell, WBC: white blood cell, Hb: hemoglobin, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein.
Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared with arthritic control group and $^{\#}P<0.001$when arthritic control group compared with healthy control group.

Table.2. Effect of GOF on biochemical parameters and cytokines in FCA induced arthritis in rats.

Groups	TP (gm/dL)	AST (U/L)	ALT (U/L)	ALP(U/L)	TNF- α (pg/ml)	IL - 1β (pg/ml)	IL – 6 (pg/ml)
Healthy Control	7.45±0.06455	41.17±1.457	52.34±1.534	74.94±2.076	38.33 ± 0.9888	120.5± 0.7801	142.2± 1.185
Arthritic Control	5.625±0.2287$^{\#}$	126.9±1.557$^{\#}$	188.6±1.73$^{\#}$	451.6±2.514$^{\#}$	121.2 ± 1.558$^{\#}$	428.9± 1.657$^{\#}$	407.3± 1.899$^{\#}$
Etoricoxib 10 mg/kg	6.525±0.1652***	106.1±2.349***	172.2±2.923***	438.8±1.438***	115.8± 0.7032**	410.5± 0.9717*	397.2 ± 2.128**
GOF 50 mg/kg	6.3±0.108*	118.8±1.203**	170.3±0.8255***	419.6±1.341***	105.0 ± 1.826***	347.1± 4.872***	374.1 ± 1.813***
GOF 100 mg/kg	6.75±0.1555***	111.5±0.6057***	152.9±1.074***	402.1±1.446***	98.67 ± 1.256***	297.2± 2.929***	339.2 ± 1.576***
GOF 200 mg/kg	6.975±0.08539***	105.9±0.8149***	140.1±1.138***	392.8±0.948***	91.5 ± 0.5627***	244.4± 3.392***	315.5± 1.38***

GOF: *Zingiber officinale* formulation; FCA: Freund's complete adjuvant, TP: Total protein, AST: Aspartate transaminase, ALT: Alanine transaminase, ALP: alkaline phosphatase, TNF- α: Tumor necrosis factor- α, IL - 1β: Interleukin- 1β, IL-6: Interleukin-6.
Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test. *$P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$when compared with arthritic control group and $^{\#}P<0.001$when arthritic control group compared with healthy control group.

Effect of GOF on spleen and thymus weight in FCA induced arthritis in rats

There was significant increase in the spleen and thymus weight in FCA induced arthritic control animals when compared to healthy control group. Treatment with etoricoxib and GOF significantly reduced the spleen and thymus weights when compared to arthritic control group (Table 3).

Effect of GOF on Antioxidant parameters in FCA induced arthritis in rats

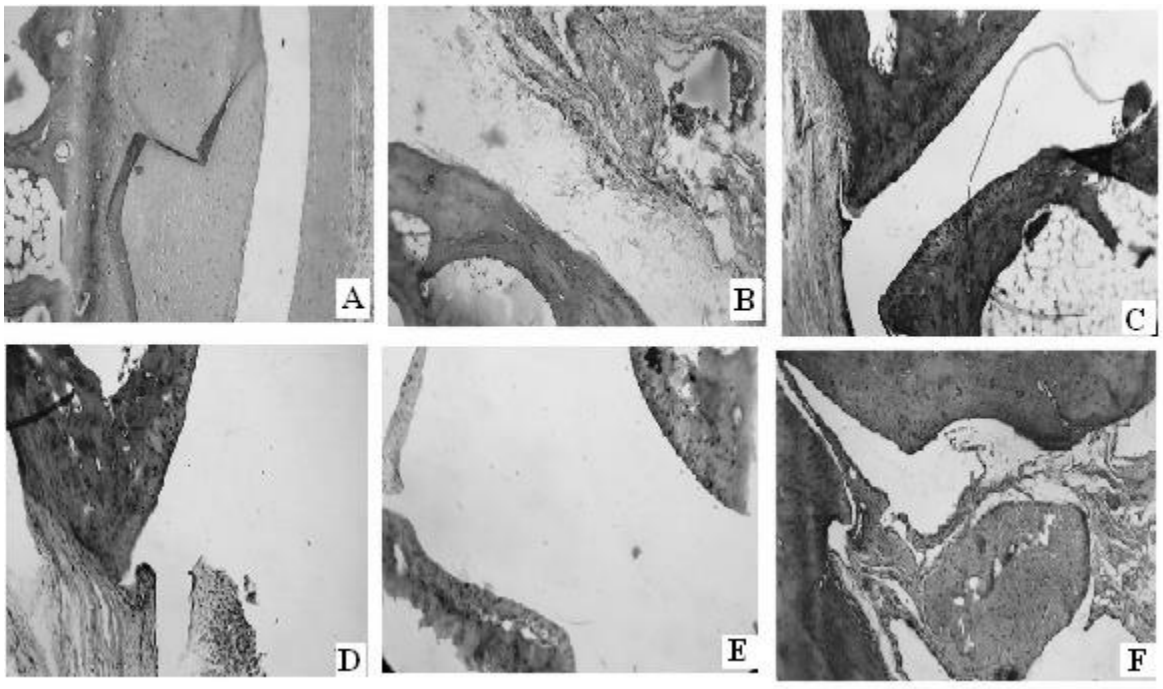
There was a significant decrease in GSH and SOD levels while significant increase in MDA levels in arthritic control group observed when compared with healthy control group. Treatment with etoricoxib and GOF significantly increased GSH and SOD levels while significantly decreased MDA level (Table 3).

Table.3. Effect of oral administration of GOF on spleen and thymus weight and antioxidant parameters in FCA induced arthritis in rats.

Groups	Spleen weight (gm)	Thymus weight (gm)	SOD (mU/mg protein)	MDA (nmole MDA/mg protein)	GSH (µmol/mg protein)
Healthy Control	0.5383±0.007923	0.0965±0.01799	4.508±0.02469	1.998±0.0296	71.01±0.4215
Arthritic Control	0.8417±0.01701$^{\#}$	0.2217±0.00654$^{\#}$	2.392±0.03135$^{\#}$	3.447±0.01563$^{\#}$	45.16±0.7465$^{\#}$
Etoricoxib 10 mg/kg	0.7083±0.02151***	0.1517±0.008333***	2.532±0.01447**	2.922±0.02272***	50.23±0.9446***
GOF 50 mg/kg	0.7783±0.01167***	0.1767±0.008433**	2.525±0.01384**	3.315±0.03294**	49.15±0.6988**
GOF 100 mg/kg	0.6283±0.0094***	0.1617±0.006009***	3.223±0.01926***	2.703±0.02667***	55.19±0.9851***
GOF 200 mg/kg	0.5917±0.01014***	0.155±0.004282***	3.35±0.0216***	2.518±0.01447***	62.58±0.3166***

GOF: *Zingiber officinale* formulation; FCA: Freund's complete adjuvant, SOD: Superoxide dismutase, MDA: Malondialdehyde, GSH: Glutathione reductase. Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test. $^{**}P<0.01$, $^{***}P<0.001$ when compared with arthritic control group and $^{\#}P<0.001$when arthritic control group compared with healthy control group.

Effect of GOF on histopathological analysis of ankle joint in FCA induced arthritis in rats



Histopathology of ankle joint of healthy control rats showed no signs of inflammation, with intact synovial lining (Fig. 7A). Histopathology of ankle joint of arthritic control animals showed massive influx of inflammatory cells, chronic inflammation and disturbed synovial lining (Fig. 7B). In contrast to these, histopathology of ankle joint of animals treated with GOF (200mg/kg and 100 mg/kg) and etoricoxib showed significant protection against synovial lining and no influx of inflammatory cells (Fig. 7F, 7E and Fig. 7C, respectively). GOF (50 mg/kg) treated rats minimal infiltration of inflammatory cells in joint (Fig. 7D).

Results of antiarthritic activity of EAF in FCA induced arthritis in rats

Acute toxicity studies

The test animals did not exhibit any change in autonomic, behavioral profile and survived beyond recommended duration of observation with 2000 mg/kg of EAF (OECD Guideline No. 423). Hence it was safe up to 2000 mg/kg.

Effect of EAF on body weight in FCA induced arthritis in rats

There was a significant decrease in body weight of all the arthritic animals. On treatment with etoricoxib there was nonsignificant increase in body weight when compared with arthritic control group of animals. On treatment with EAF 50 mg/kg significantly improved body weight only on day 28 when compared to arthritic control. However, treatment with EOF (200 mg/kg and 100 mg/kg) significantly improved the body weight day 24 onwards as compared to arthritic control group (Fig. 2).

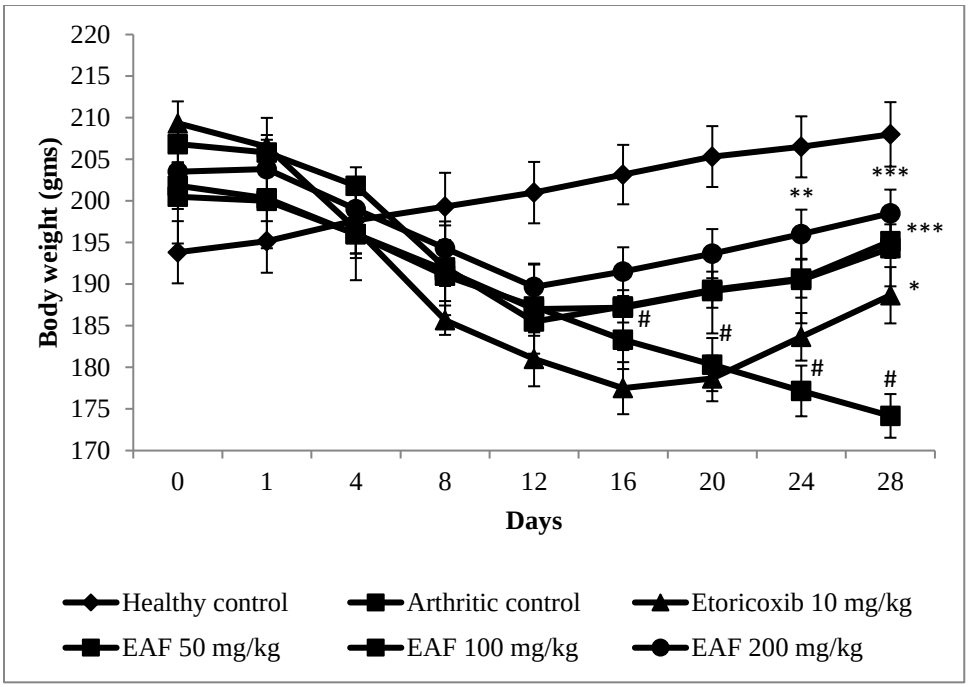


Fig. 2. Effect of EAF on body weight in FCA induced arthritis in rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared to healthy control

Effect of EAF on paw volume in FCA induced arthritis in rats

There was a significant increase in paw volume of all the rats in arthritic control group when compared to healthy control. Treatment with etoricoxib and EAF (200 and 100 mg/kg) significantly and dose dependently decreased the paw volume from day 16 onwards, respectively as compared to arthritic control group. EAF at the dose of 50 mg/kg was comparatively less effective and decreased the paw volume day from 20 onwards (Fig. 3).

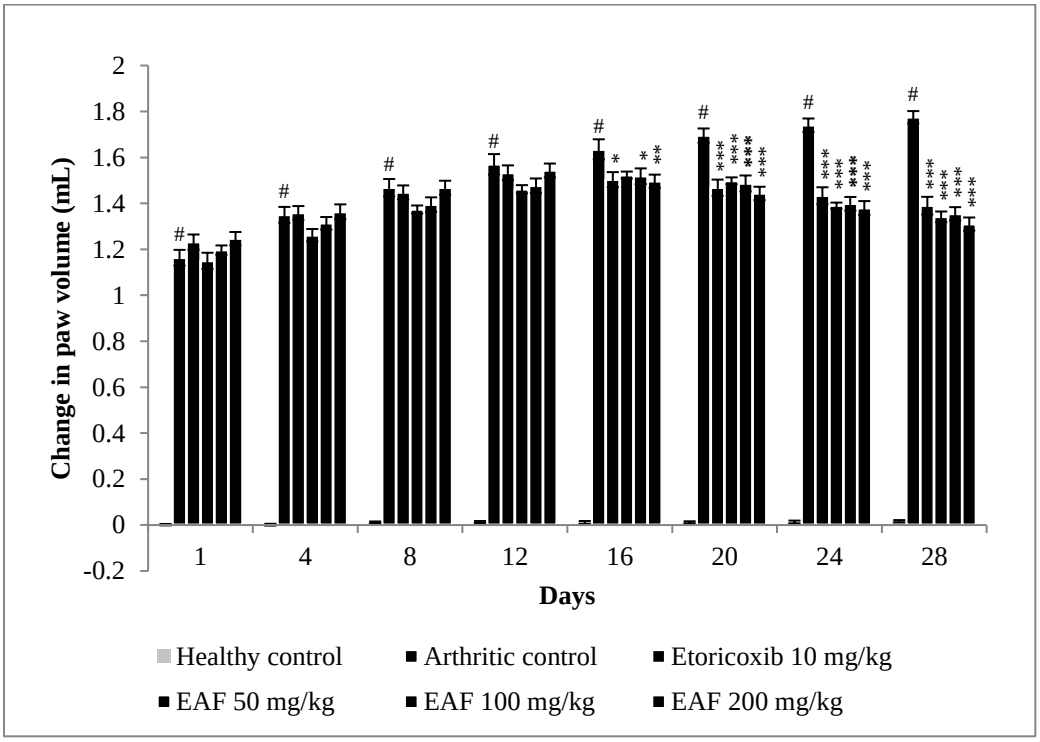


Fig. 3.Effect of GOF on paw volume in FCA induced arthritis in rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{**}P<0.01$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared to healthy control

Effect of GOF on Mechanical hyperalgesia in FCA induced arthritis in rats

Paw withdrawal threshold in all arthritic animals decreased progressively till day 12 when compared to healthy control animals. The paw withdrawal threshold in arthritic control animals was significantly less compared to the healthy control animals till the end of study. Treatment with Etoricoxib, GOF from day 12 onwards, significantly and dose dependently increased the pain threshold from day 16 onwards respectively. (Fig. 4).

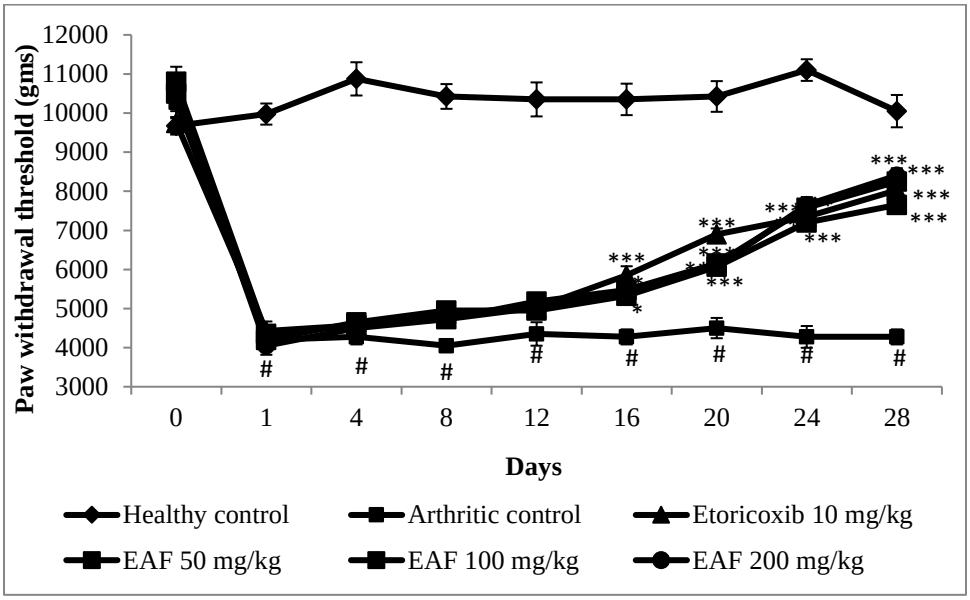


Fig.4 Effect of EAF on Mechanical hyperalgesia in FCA induced arthritis in rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{**}P<0.01$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared with healthy control.

Effect of EAF on Thermal Hyperalgesia in FCA induced arthritis in rats.

On induction of arthritis, there was a significant decrease in paw withdrawal latency of all arthritic animals till day 12 when compared to healthy control (Fig. 5). Treatment with etoricoxib and EAF (200 and 100 mg/kg) from day 12 significantly increased the paw withdrawal latency from day 16 and day 20 onwards respectively. However, EAF 50 mg/kg significantly ($p < 0.001$) increased in paw withdrawal latency from day 24.

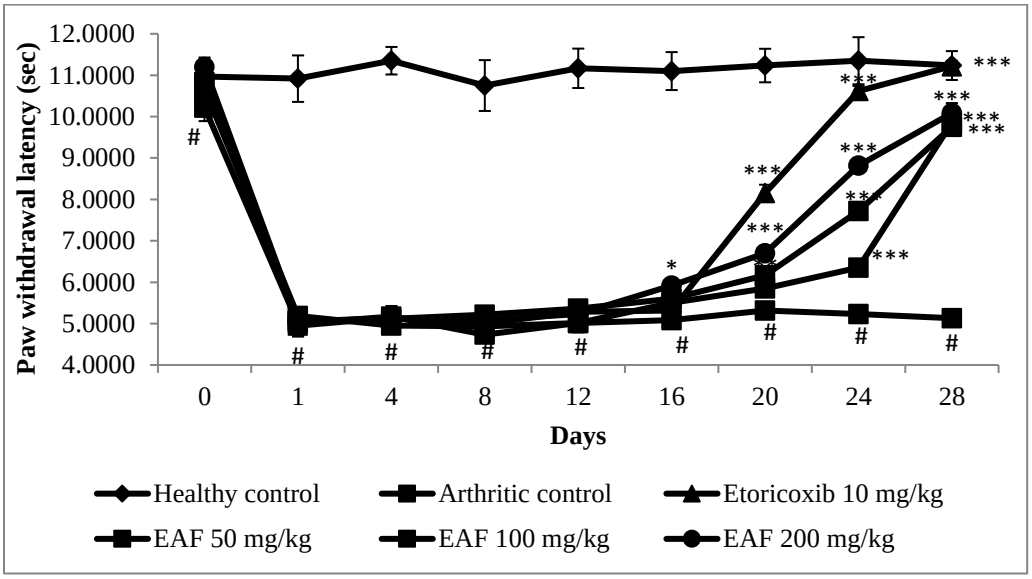


Fig.5 Effect of EAF on thermal hyperalgesia in FCA induced arthritis in rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared to healthy control

Effect of EAF on Tactile allodynia in FCA induced arthritis in rats

The mechanical threshold was significantly decreased in arthritic animals till day 12. Administration of etoricoxib and EAF (200 and 100 mg/kg) from day 12 significantly improved the mechanical withdrawal threshold from day 20 and 24 onwards respectively when compared with arthritic control while GOF 50 mg/kg significantly increased mechanical withdrawal threshold only on day 28 (Fig. 6). However there was no change in tactile allodynia in arthritic control group of animals.

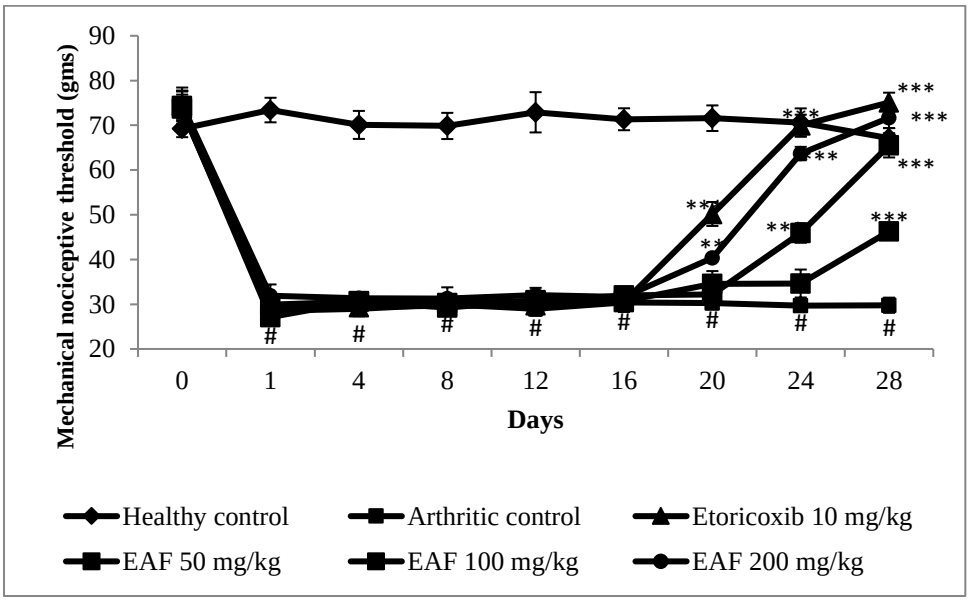


Fig. 6.Effect of EAF on Tactile allodynia in FCA induced arthritic rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{*}P<0.05$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared to healthy control

Effect of EAF on hematology and serum parameters in FCA induced arthritis in rats

There was significant increase in platelet count, WBC count and ESR while decrease in RBC count and Hb level observed in arthritic control group when compared to healthy control group. These conditions were reversed significantly and dose dependently on treatment with EAF (Table 1).

The serum CRP level was significantly increased in arthritic control group as compared to healthy control group. On treatment with etoricoxib and EAF, serum CRP level was found to be significantly decreased when compared with arthritic control group (Table 1).

The serum levels of AST, ALT and ALP were increased significantly in arthritic control group as compared to healthy control group. Treatment with etoricoxib and EAF significantly decreased AST, ALT and ALP level (Table 2).

The level of total protein was significantly decreased in arthritic control group when compared with healthy control group. Etoricoxib and EAF were found to be significant in restoring it when compared with arthritic control group (Table 2).

Effect of EAF on serum cytokine levels in FCA induced arthritis in rats

The challenge with FCA caused significant increase in serum TNF-α, IL-1β and IL-6 levels, when compared to healthy control group. Treatment with GOF caused significant and dose dependant decrease in the serum IL-1β, TNF-α and IL-6 levels as compared to arthritic control group (Table 2).

Table. 1. Effect of EAF on hematological parameters in FCA induced arthritis in rats.

Groups	RBC (10^3cells/mm^3)	WBC (10^3cells/mm^3)	Hb (gm/dL)	Platelet (10^3cells/$mm^{3)}$	ESR (mm/h)	CRP (mg/lit)
Healthy Control	6.845±0.1132	7.625±0.2118	14.38±0.3221	9.238±0.1322	8.325±0.2869	1.602±0.034
Arthritic Control	3.265±0.07377$^{\#}$	15.39±0.255$^{\#}$	8.9±0.1817$^{\#}$	18.46±0.3516$^{\#}$	15.45±0.5331$^{\#}$	7.177±0.166$^{\#}$
Etoricoxib 10 mg/kg	6.033±0.1188***	13.1±0.3058***	13.21±0.1519***	13.93±0.3861***	9.925±0.536***	3.682±0.1408***
EAF 50 mg/kg	4.138±0.0740*	14.23±0.4204*	10.50±0.1601*	16.77±0.1538*	14.00±0.1958*	6.547±0.1537***
EAF 100 mg/kg	4.25±0.2945**	14.00±0.360**	11.04±0.278***	16.48±0.4396**	13.13±0.2016***	5.718±0.1849***
EAF 200 mg/kg	4.893±0.4039***	12.34±0.3266***	11.59±0.5266***	15.37±0.4332***	11.25±0.5635***	4.353±0.052***

EAF: *Eclipta alba* formulation; FCA: Freund's complete adjuvant, RBC: red blood cell, WBC: white blood cell, Hb: hemoglobin, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein.

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared with arthritic control group and $^{\#}P<0.001$when arthritic control group compared with healthy control group.

Table.2. Effect of EAF on biochemical parameters and cytokines in FCA induced arthritis in rats.

Groups	TP (gm/dL)	AST (U/L)	ALT (U/L)	ALP(U/L)	TNF- α (pg/ml)	IL - 1β (pg/ml)	IL – 6 (pg/ml)
Healthy Control	7.45±0.06455	41.17±1.457	52.34±1.534	74.94±2.076	38.33 ± 0.9888	120.5± 0.7801	142.2± 1.185
Arthritic Control	5.625±0.2287$^{\#}$	126.9±1.557$^{\#}$	188.6±1.73$^{\#}$	451.6±2.514$^{\#}$	121.2 ± 1.558$^{\#}$	428.9± 1.657$^{\#}$	407.3± 1.899$^{\#}$
Etoricoxib 10 mg/kg	6.525±0.1652***	106.1±2.349***	172.2±2.923***	438.8±1.438***	115.8± 0.7032**	410.5± 0.9717*	397.2 ± 2.128**
EAF 50 mg/kg	6.25±0.0.064*	118.5±3.141**	180.6±0.6324**	441.00±0.713**	112.5 ± 0.7638***	392.1± 3.191***	397.3 ± 2.024**
EAF 100 mg/kg	6.625±0.0.085***	116.8±1.211***	164.1±2.558***	422.2±2.247***	108.3 ± 0.666***	323± 4.376***	380.8 ±1.771***
EAF 200 mg/kg	6.60±0.2799***	113.00±0.6094***	155.9±1.55***	400.3±2.205***	101.3 ± 0.7149***	264± 3.491***	354.6± 1.829***

EAF: *Eclipta alba* formulation; FCA: Freund's complete adjuvant, TP: Total protein, AST: Aspartate transaminase, ALT: Alanine transaminase, ALP: alkaline phosphatase, TNF- α: Tumor necrosis factor- α, IL - 1β: Interleukin- 1β, IL-6: Interleukin-6.

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$when compared with arthritic control group and $^{\#}P<0.001$when arthritic control group compared with healthy control group.

Effect of GOF on spleen and thymus weight in FCA induced arthritis in rats

There was significant increase in the spleen and thymus weight in FCA induced arthritic control animals when compared to healthy control group. Treatment with etoricoxib and GOF significantly reduced the spleen and thymus weights when compared to arthritic control group (Table 3).

Effect of GOF on Antioxidant parameters in FCA induced arthritis in rats

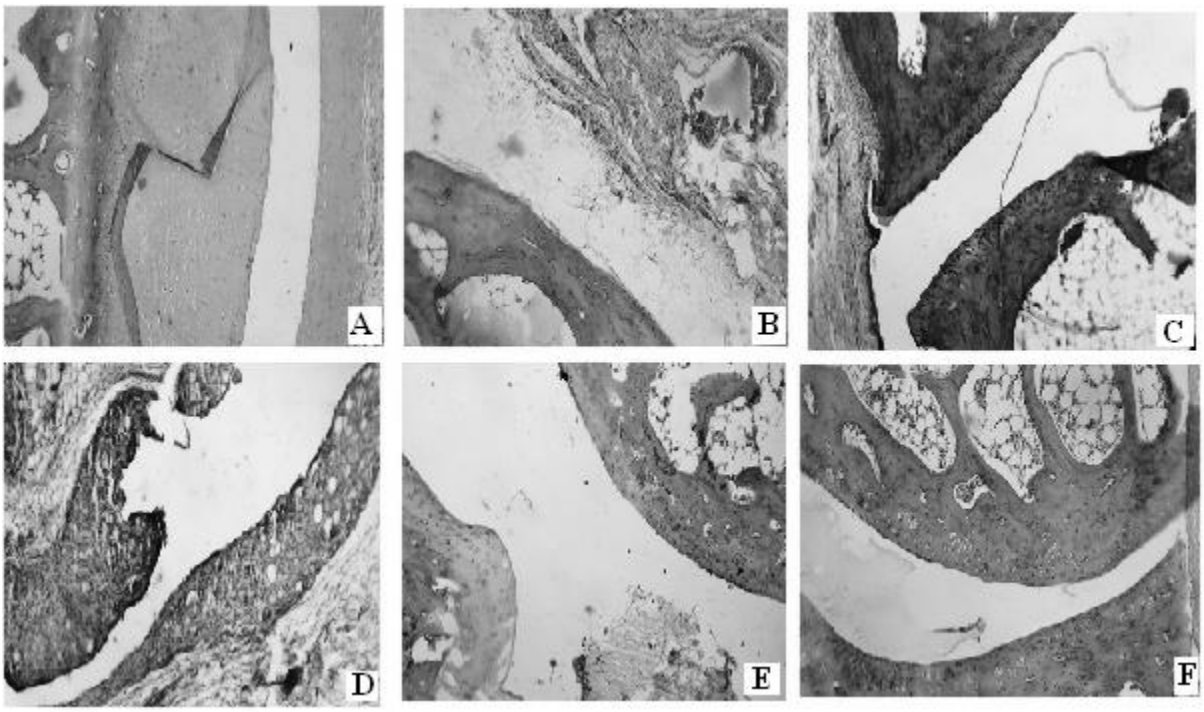
There was a significant decrease in GSH and SOD levels while significant increase in MDA levels in arthritic control group observed when compared with healthy control group. Treatment with etoricoxib and GOF significantly increased GSH and SOD levels while significantly decreased MDA level (Table 3).

Table.3. Effect of oral administration of EAF on spleen and thymus weight and antioxidant parameters in FCA induced arthritis in rats.

Groups	Spleen weight (gm)	Thymus weight (gm)	SOD (mU/mg protein)	MDA (nmole MDA/mg protein)	GSH (µmol/mg protein)
Healthy Control	0.5383±0.007923	0.0965±0.01799	4.508±0.02469	1.998±0.0296	71.01±0.4215
Arthritic Control	0.8417±0.01701$^{\#}$	0.2217±0.00654$^{\#}$	2.392±0.03135$^{\#}$	3.447±0.01563$^{\#}$	45.16±0.7465$^{\#}$
Etoricoxib 10 mg/kg	0.7083±0.02151***	0.1517±0.008333***	2.532±0.01447**	2.922±0.02272***	50.23±0.9446***
EAF 50 mg/kg	0.775±0.0108**	0.1917±0.0060	2.543±0.0066**	3.327±0.0190*	48.81±0.3485**
EAF 100 mg/kg	0.735±0.0125***	0.1717±0.0070***	3.038±0.0445***	3.29±0.02708***	51.81±0.4569***
EAF 200 mg/kg	0.6733±0.0133***	0.1433±0.0071***	3.188±0.0275***	2.793±0.0320***	61.96±1.2***

EAF: *Eclipta alba* formulation; FCA: Freund's complete adjuvant, SOD: Superoxide dismutase, MDA: Malondialdehyde, GSH: Glutathione reductase. Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test. $^{**}P<0.01$, $^{***}P<0.001$ when compared with arthritic control group and $^{\#}P<0.001$when arthritic control group compared with healthy control group.

Effect of EAF on histopathological analysis of ankle joint in FCA induced arthritis in rats



Histopathology of ankle joint of healthy control rats showed no signs of inflammation, with intact synovial lining (Fig. 7A). Histopathology of ankle joint of arthritic control animals showed massive influx of inflammatory cells, chronic inflammation and disturbed synovial lining (Fig. 7B). In contrast to these, histopathology of ankle joint of animals treated with EAF (200mg/kg) and etoricoxib showed significant protection against synovial lining and no influx of inflammatory cells (Fig. 7F, Fig. 7C, respectively). EAF (100 mg/kg) treated rats showed minimal infiltration of inflammatory cells in joint (Fig. 7E) EAF (50 mg/kg) treated rats mild infiltration of inflammatory cells in joint and synovial lining is disrupted (Fig. 7D).

References:

- Lee J S, Kim K A, Jeong S H, Lee S G, Park H J, Kim N J, Lim S. 2009. Antiinflammatory, anti-nociceptive and anti-psychiatric effects by the rhizomes of *Alpinia officinarum* on complete Freund's adjuvant-induced arthritis in rats. Journal of Ethnopharmacology 126, 258–264.
- Zhang CF, Zhang SL, He X, Yang XL, Wu HT, Lin BQ, Jiang CP, Wang J, Yu CH, Yang ZL, Wang CZ, Li P, Yuan CS. Antioxidant effects of Genkwa flos flavonoids on Freund's adjuvant-induced rheumatoid arthritis in rats. Journal of Ethnopharmacology 153 (2014) 793–800.
- Pan R, Dai Y, Gao X H, Xia Y F. 2009. Scopolin isolated from *Erycibe obtusifolia* Benth stems suppresses adjuvant-induced rat arthritis by inhibiting inflammation and angiogenesis. International Immunopharmacology 9, 859–869.
- Fan A Y, Lao L,. Zhang R X, Zhou A N, Wang L B, Moudgil K D, Lee D Y W Ma Z Z, Zhang W Y, Bermana B M Effects of an acetone extract of *Boswellia carterii* Birdw. (Burseraceae) gum resin on adjuvant-induced arthritis in lewis rats. Journal of Ethnopharmacology 101 (2005) 104–109
- Chopade AR, Sayyad FJ. Antinociceptive effect of *Phyllanthus fraternus* extract in complete Freund's adjuvant induced chronic pain in mice Biomedicine & Aging Pathology 3 (2013) 235–240
- Maitera O N, Khan M E, James T F. Phytochemical analysis and the chemotherapeutics of leaves and stembark of Nauclea latifolia grown in Hong, Adamawa State Nigeria. Asian Journal of Plant Science and Research, 2011, 1 (3):16-22
- Upadhyay K, Gupta NK, Dixit VK. Development and characterization of phyto-vesicles of wedelolactone for hepatoprotective activity. Drug Development and Industrial Pharmacy. 2012,38:1152-1158.
- Nikam AR, Sathiyanarayanan L, Mahadik KR. Mapping pharmacokinetic and tissue distribution profile of [6]-shogaol from ginger oleoresin International Journal of Pharmacy and Pharmaceutical Sciences 2013, 5, (1): 185-189.

- Soliman KA, Ibrahim HK, Ghorab MM. Formulation of avanafil in a solid self-nanoemulsifying drug delivery system for enhanced oral delivery. European Journal of Pharmaceutical Sciences 93 (2016) 447–455.
- Chen S. Natural Products Triggering Biological Targets- A Review of the Anti-Inflammatory Phytochemicals Targeting the Arachidonic Acid Pathway in Allergy Asthma and Rheumatoid Arthritis *Current Drug Targets*, 2011, *12*, 288-301 .
- Mehta A, Sethiya N, Mehta C, Shah G. Anti-arthritis activity of roots of *Hemidesmus indicus* R. Br. (Anantmul) in rats. Asian Pacific Journal Tropical Medicine 2012; 130–135.
- OECD Guidelines for testing of chemicals, Acute oral toxicity, Acute toxic class method. 2012; 423, 1.
- Bihani GV, Rojatkar SR, Bodhankar SL. Anti-arthritic activity of methanol extract of *Cyathocline purpurea* (whole plant) in Freund's complete adjuvant-induced arthritis in rats. Biomedicine & Aging Pathology 2014; 4: 197–206.
- Mishra H, Fridocich. The role of superoxide anion in the autooxidation of epinephrine and a simple assay for SOD. Journal of Biological Chemistry 1972; 3170–3175.
- Slater T, Sawyer B. The stimulatory effects of carbon tetrachloride and other halogenoalkanes or peroxidative reactions in rat liver fraction in vitro. Biochemical Journal 1971; 123:805–14.
- Morgon M, Depirre J, Mannervik B. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochemica et Biophysica Acta 1979; 582: 67–78.

Results of antiarthritic activity of F in FCA induced arthritis in rats

Acute toxicity studies

The test animals did not exhibit any change in autonomic, behavioral profile and survived beyond recommended duration of observation with 2000 mg/kg of F (OECD Guideline No. 423). Hence it was safe up to 2000 mg/kg.

Effect of F on body weight in FCA induced arthritis in rats

There was a significant decrease in body weight of all the arthritic animals. On treatment with etoricoxib there was nonsignificant increase in body weight when compared with arthritic control group of animals. On treatment with F 50 mg/kg significantly improved body weight from day 24 onwards when compared to arthritic control. However, treatment with F (200 mg/kg and 100 mg/kg) significantly improved the body weight day 20 onwards as compared to arthritic control group (Fig. 2).

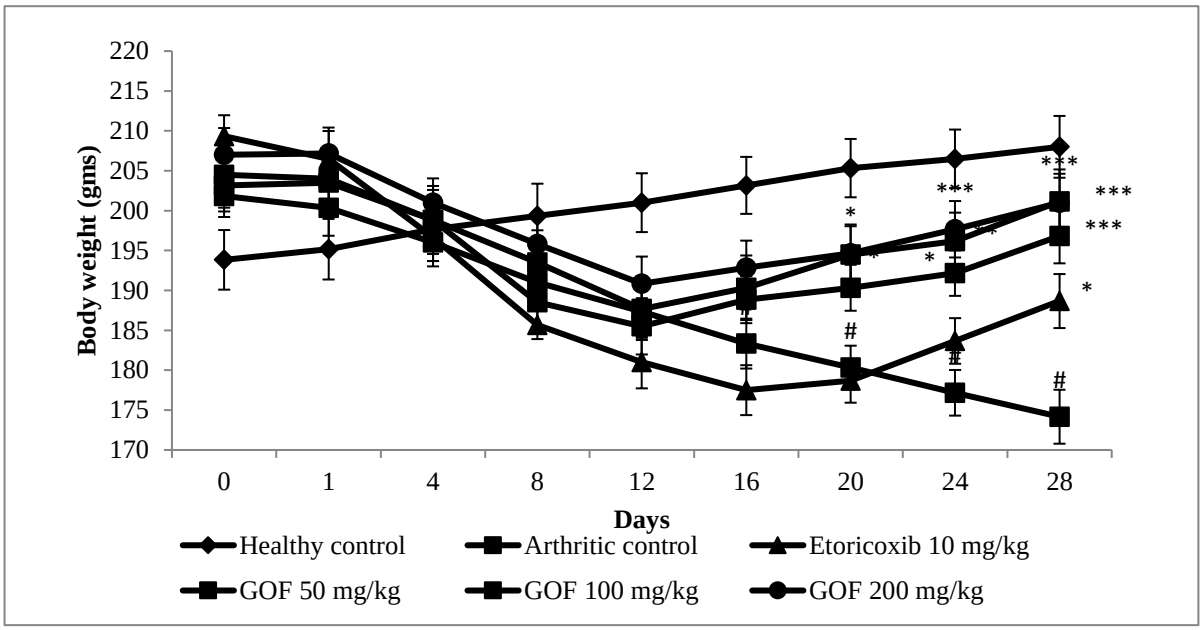


Fig. 2. Effect of GOF on body weight in FCA induced arthritis in rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared with Arthritic control and # P<0.001 when arthritic control compared to healthy control.

Effect of F on paw volume in FCA induced arthritis in rats

There was a significant increase in paw volume of all the rats in arthritic control group when compared to healthy control. Treatment with etoricoxib and GOF significantly decreased the paw volume from day 16 onwards, respectively as compared to arthritic control group. (Fig. 3).

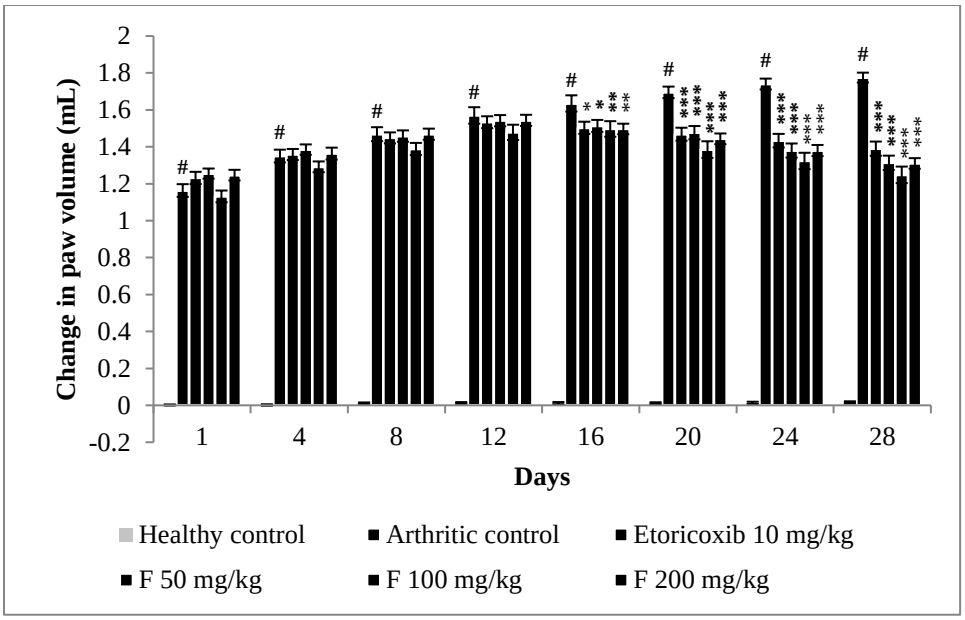


Fig. 3.Effect of F on paw volume in FCA induced arthritis in rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{**}P<0.01$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared to healthy control

Effect of F on Mechanical hyperalgesia in FCA induced arthritis in rats

Paw withdrawal threshold in all arthritic animals decreased progressively till day 12 when compared to healthy control animals. The paw withdrawal threshold in arthritic control animals was significantly less compared to the healthy control animals till the end of study. Treatment with Etoricoxib, GOF from day 12 onwards, significantly increased the pain threshold from day 16 onwards respectively at all dose levels (Fig. 4).

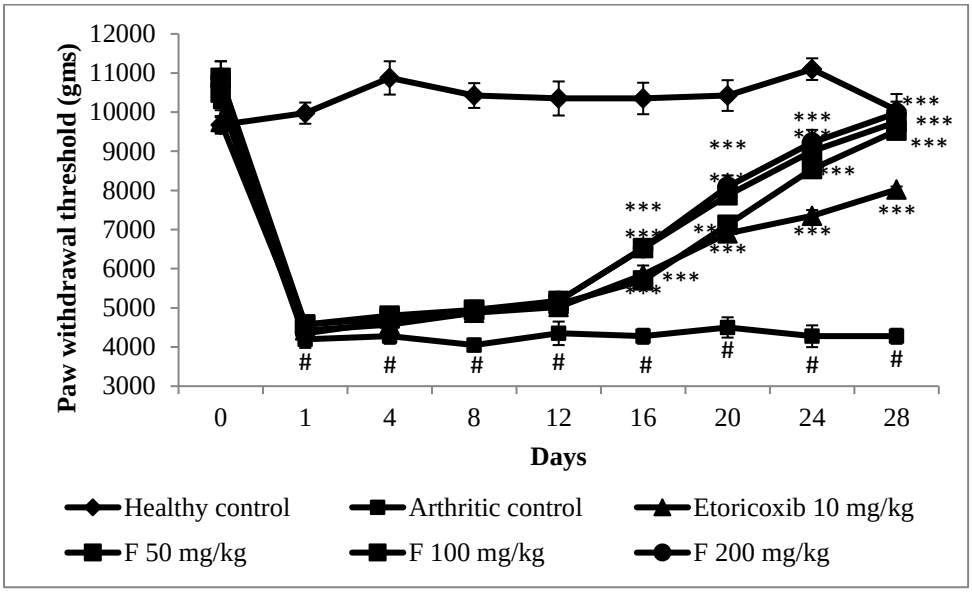


Fig.4 Effect of F on Mechanical hyperalgesia in FCA induced arthritis in rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{**}P<0.01$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared with healthy control.

Effect of F on Thermal Hyperalgesia in FCA induced arthritis in rats.

On induction of arthritis, there was a significant decrease in paw withdrawal latency of all arthritic animals till day 12 when compared to healthy control (Fig. 5). Treatment with etoricoxib and GOF from day 12 significantly increased the paw withdrawal latency from day 16 onwards.

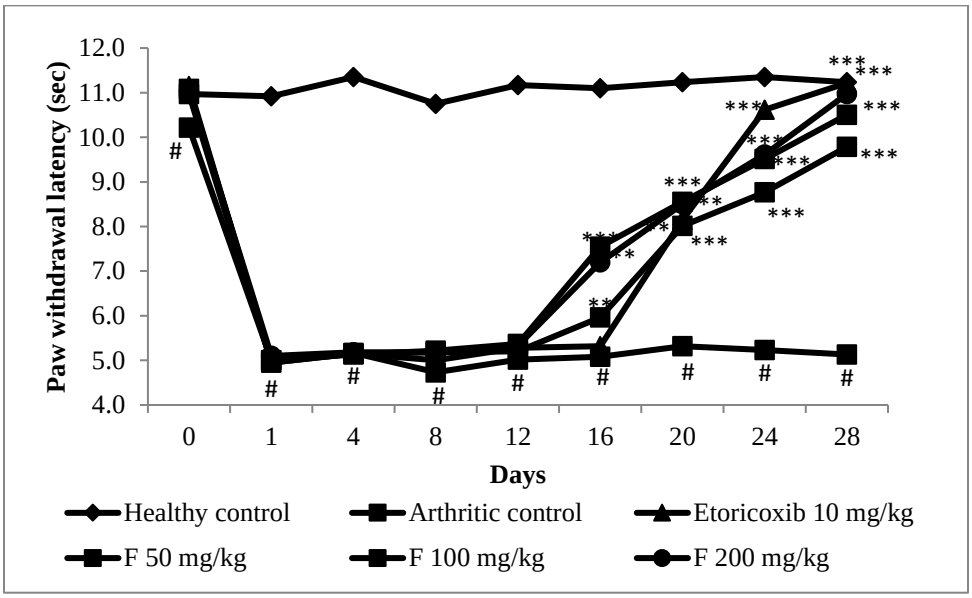


Fig.5 Effect of F on thermal hyperalgesia in FCA induced arthritis in rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared to healthy control

Effect of F on Tactile allodynia in FCA induced arthritis in rats

The mechanical threshold was significantly decreased in arthritic animals till day 12. Administration of etoricoxib and F (200 and 100 mg/kg) from day 12 significantly improved the mechanical withdrawal threshold from day 16 onwards respectively when compared with arthritic control while F 50 mg/kg significantly increased mechanical withdrawal threshold from day 20 onwards (Fig. 6). However there was no change in tactile allodynia in arthritic control group of animals.

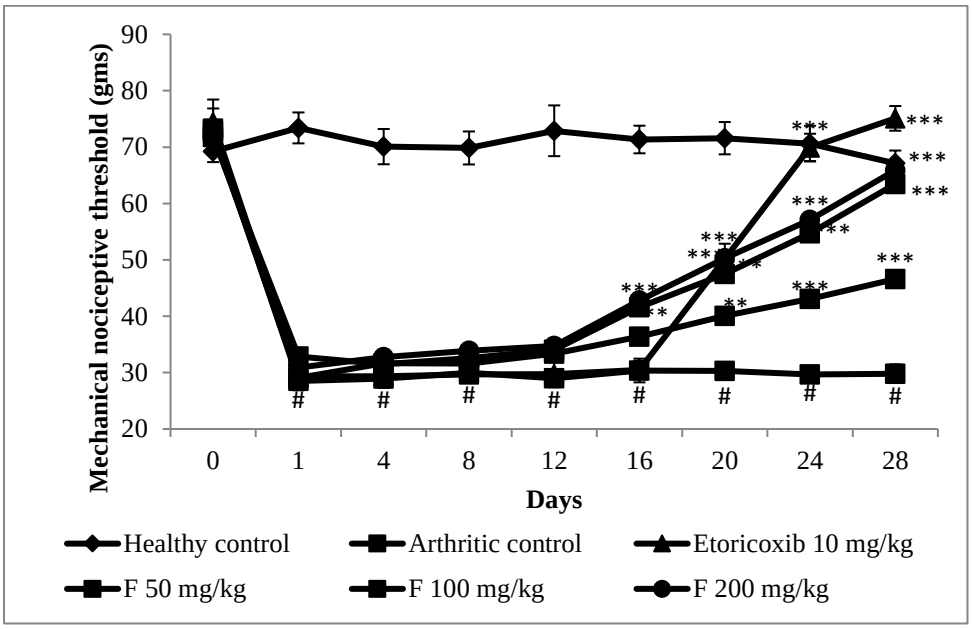


Fig. 6.Effect of GOF on Tactile allodynia in FCA induced arthritic rats.

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. $^{*}P<0.05$, $^{***}P<0.001$ when compared with Arthritic control and # $P<0.001$ when arthritic control compared to healthy control

Effect of F on hematology and serum parameters in FCA induced arthritis in rats

There was significant increase in platelet count, WBC count and ESR while decrease in RBC count and Hb level observed in arthritic control group when compared to healthy control group. These conditions were reversed significantly and dose dependently on treatment with F (Table 1).

The serum CRP level was significantly increased in arthritic control group as compared to healthy control group. On treatment with etoricoxib and F, serum CRP level was found to be significantly decreased when compared with arthritic control group (Table 1).

The serum levels of AST, ALT and ALP were increased significantly in arthritic control group as compared to healthy control group. Treatment with etoricoxib and F significantly decreased AST, ALT and ALP level (Table 2).

The level of total protein was significantly decreased in arthritic control group when compared with healthy control group. Etoricoxib and F were found to be significant in restoring it when compared with arthritic control group (Table 2).

Effect of F on serum cytokine levels in FCA induced arthritis in rats

The challenge with FCA caused significant increase in serum TNF-α, IL-1β and IL-6 levels, when compared to healthy control group. Treatment with F caused significant and dose dependant decrease in the serum IL-1β, TNF-α and IL-6 levels as compared to arthritic control group (Table 2).

Table. 1. Effect of F on hematological parameters in FCA induced arthritis in rats.

Groups	RBC (10^3cells/mm^3)	WBC (10^3cells/mm^3)	Hb (gm/dL)	Platelet (10^3cells/$mm^{3)}$	ESR (mm/h)	CRP (mg/lit)
Healthy Control	6.845±0.1132	7.625±0.2118	14.38±0.3221	9.238±0.1322	8.325±0.2869	1.602±0.034
Arthritic Control	3.265±0.07377$^{\#}$	15.39±0.255$^{\#}$	8.9±0.1817$^{\#}$	18.46±0.3516$^{\#}$	15.45±0.5331$^{\#}$	7.177±0.166$^{\#}$
Etoricoxib 10 mg/kg	6.033±0.1188***	13.1±0.3058***	13.21±0.1519***	13.93±0.3861***	9.925±0.536***	3.682±0.1408***
F 50 mg/kg	4.635±0.0658***	13.13±0.0359***	12.19±0.3269***	16.07±0.1938***	12.75±0.1848***	5.477±0.04978***
F 100 mg/kg	5.215±0.0800***	12.07±0.1495***	12.59±0.3269***	13.25±0.5307***	10.90±0.1915 ***	4.807±0.0281***
F 200 mg/kg	5.663±0.0934***	10.86±0.1041***	13.19±0.3269***	10.69±0.2875***	9.95±0.2466***	3.7370±0.0346 ***

F: formulation; FCA: Freund's complete adjuvant, RBC: red blood cell, WBC: white blood cell, Hb: hemoglobin, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein.

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared with arthritic control group and $^{\#}P<0.001$when arthritic control group compared with healthy control group.

Table.2. Effect of F on biochemical parameters and cytokines in FCA induced arthritis in rats.

Groups	TP (gm/dL)	AST (U/L)	ALT (U/L)	ALP(U/L)	TNF- α (pg/ml)	IL - 1β (pg/ml)	IL – 6 (pg/ml)
Healthy Control	7.45±0.06455	41.17±1.457	52.34±1.534	74.94±2.076	38.33 ± 0.9888	120.5± 0.7801	142.2± 1.185
Arthritic Control	5.625±0.2287$^{\#}$	126.9±1.557$^{\#}$	188.6±1.73$^{\#}$	451.6±2.514$^{\#}$	121.2 ± 1.558$^{\#}$	428.9± 1.657$^{\#}$	407.3± 1.899$^{\#}$

Etoricoxib 10 mg/kg	6.525±0.1652***	106.1±2.349***	172.2±2.923***	438.8±1.438***	115.8± 0.7032**	410.5± 0.9717*	397.2 ± 2.128**
F 50 mg/kg	6.375±0.0.0853**	117.3±1.783***	162±1.454***	403.0±1.976***	98.83 ± 1.014***	321.7± 3.392***	345.9 ± 2.566***
F 100 mg/kg	6.60±0.0.108***	110.5±0.381***	154.2±1.092***	383.8±2.157***	91.67 ± 0.666***	280.7± 3.011***	317.2 ±2.042***
F 200 mg/kg	6.975±0.0629***	104.0±1.008***	133.3±1.138***	345.7±2.006***	87.5 ± 0.7638***	225.5± 7.97***	299.6± 0.9397***

F: formulation; FCA: Freund's complete adjuvant, TP: Total protein, AST: Aspartate transaminase, ALT: Alanine transaminase, ALP: alkaline phosphatase, TNF- α: Tumor necrosis factor- α, IL - 1β: Interleukin- 1β, IL-6: Interleukin-6.

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test. *P<0.05, **P<0.01, ***P<0.001when compared with arthritic control group and #P<0.001when arthritic control group compared with healthy control group.

Effect of F on spleen and thymus weight in FCA induced arthritis in rats

There was significant increase in the spleen and thymus weight in FCA induced arthritic control animals when compared to healthy control group. Treatment with etoricoxib and F significantly reduced the spleen and thymus weights when compared to arthritic control group (Table 3).

Effect of F on Antioxidant parameters in FCA induced arthritis in rats

There was a significant decrease in GSH and SOD levels while significant increase in MDA levels in arthritic control group observed when compared with healthy control group. Treatment with etoricoxib and F significantly increased GSH and SOD levels while significantly decreased MDA level (Table 3).

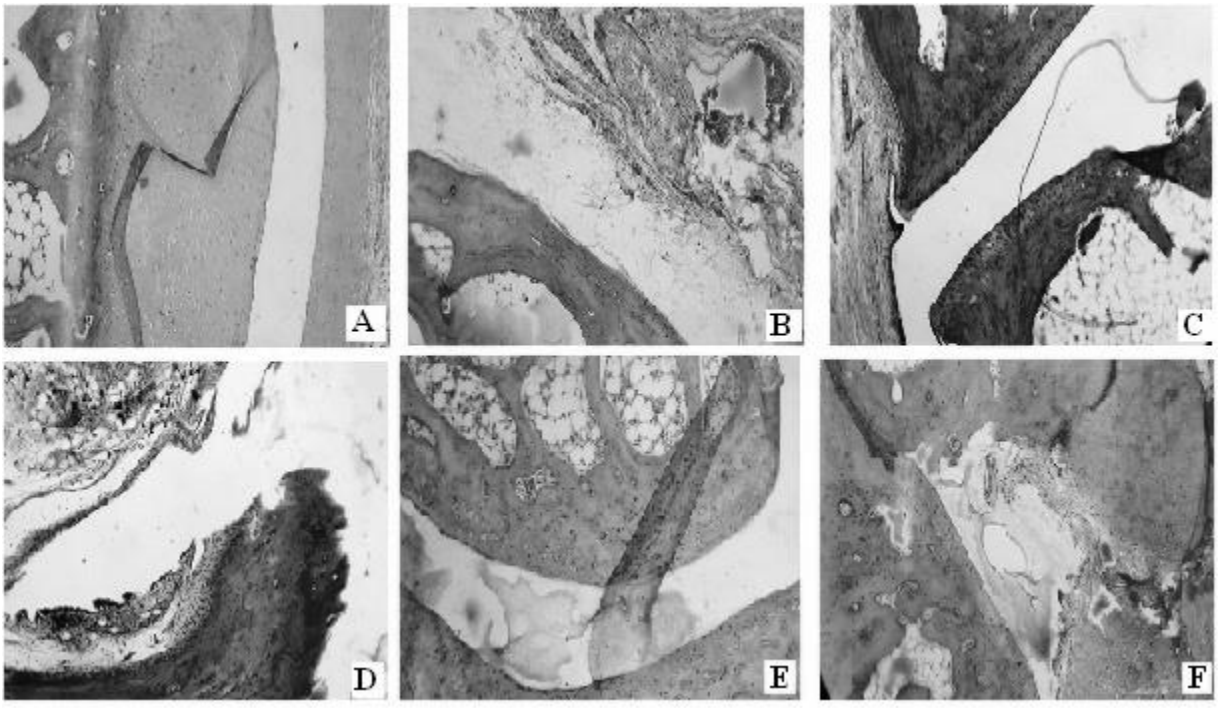
Table.3. Effect of oral administration of F on spleen and thymus weight and antioxidant parameters in FCA induced arthritis in rats.

Groups	Spleen weight (gm)	Thymus weight (gm)	SOD (mU/mg protein)	MDA (nmole MDA/mg protein)	GSH (µmol/mg protein)
Healthy Control	0.5383±0.007923	0.0965±0.01799	4.508±0.02469	1.998±0.0296	71.01±0.4215
Arthritic Control	0.8417±0.01701$^{\#}$	0.2217±0.00654$^{\#}$	2.392±0.03135$^{\#}$	3.447±0.01563$^{\#}$	45.16±0.7465$^{\#}$
Etoricoxib 10 mg/kg	0.7083±0.02151***	0.1517±0.008333***	2.532±0.01447**	2.922±0.02272***	50.23±0.9446***
F 50 mg/kg	0.6017±0.0079**	0.1667±0.0071***	3.078±0.0407***	2.98±0.0428***	53.93±0.3637***
F 100 mg/kg	0.5767±0.0088***	0.1583±0.0060***	3.383±0.0223***	2.45±0.01612***	59.32±0.7565***
F 200 mg/kg	0.5550±0.0105***	0.1483±0.0060***	3.5±0.0139***	2.3±0.0129***	64.16±0.2543***

F: formulation; FCA: Freund's complete adjuvant, SOD: Superoxide dismutase, MDA: Malondialdehyde, GSH: Glutathione reductase. Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test. $^{**}P<0.01$, $^{***}P<0.001$ when compared with arthritic control group and $^{\#}P<0.001$when arthritic control group compared with healthy control group.

Effect of F on histopathological analysis of ankle joint in FCA induced arthritis in rats

Histopathology of ankle joint of healthy control rats showed no signs of inflammation, with intact synovial lining (Fig. 7A). Histopathology of ankle joint of arthritic control animals showed massive influx of inflammatory cells, chronic inflammation and disturbed synovial lining (Fig. 7B). In contrast to these, histopathology of ankle joint of animals treated with F (200 mg/kg and 100 mg/kg) and etoricoxib showed significant protection against synovial lining and no influx of inflammatory cells (Fig. 7F, Fig. 7E, Fig. 7C, respectively). F (50 mg/kg) treated rats minimal infiltration of inflammatory cells in joint and synovial lining is intact (Fig. 7D).



Discussion

Herbal agents also are pleiotropic, i.e. they act on multiple loci to thwart arthritis. Ginger a well known and reported antiarthritic and Bhringraj traditionally claimed and reported antiinflammatory herb were selected for to evaluate the effect in treatment of RA. The therapeutic effect is directly proportional to bioavailability of active constituent of at target site. As reported in earlier pharmacokinetic studies, 6-shogaol and wedelolactone are poorly bioavailable, present study improved the solubility of enriched extracts and hence their therapeutic efficacy in RA.

SNEDDS is novel formulation approach specially designed to improve the aqueous solubility and oral absorption of poorly water soluble lipophilic drugs. SNEDDS is isotropic mixture of oil surfactant and co surfactant which is preconcentrate when added in aqueous media readily disperses with simple agitation to form nanoemulsion and is thermo stable (Sermkaew *et al.*, 2013).

RA is a chronic cytokine-mediated inflammatory disease, characterized by joint swelling, arthrodynia and impaired joint function (Chen *et al.*,2009).

Conventional treatments with non-steroidalanti-inflammatory drugs, disease modifying anti rheumatoid drugs act by reducing the inflammation. However, they are not satisfactory in controlling the disease progression. Newer therapies involving biologicals like Tumor Necrosis factor-α (TNF-α) antagonists have shown some success in reducing the inflammation and joint destruction but are very expensive and unaffordable. Hence, there is a need for cost effective alternative therapy which can control disease progression with effect on biological targets like TNF-α Interlukins and cytokines for treatment of rheumatoid arthritis. (Datta *et al*., 2012).

Non-ionic surfactants with high HLB values Tween 80 (HLB = 15) which is well miscible with labrafil, were chosen for the present formulation. Being less toxic and less affected by pH and ionic changes in the dispersion medium, nonionic surfactants are preferred to ionic surfactants (Constantinides, 1995).

Taking into consideration the pharmacological profile, the low oral bioavailability and rapid metabolism of active phytoconstituents of *Eclipta alba* and *Zingiber officinale* viz. wedelolactone and 6-shogaol, the present study proposes a novel herbal composition and improvement of oral bioavailability and efficacy by design of suitable formulation.
(Datta *et al*., 2012).

Maximum drug solubility is a crucial parameter in pharmaceutical practice which ensures dose be administered. In the present study solubility screening revealed *Zingiber officinale* and EAEA showed maximum solubility in labrafil.

Adjuvant arthritis in rats is a widely used experimental model sharing several features with rheumatoid arthritis in humans. Its pathophysiology involves initial acute periarticular inflammation with synovial mononuclear infiltration, following gradually by synovial hyperplasia and damage to periarticular bone and cartilage just as in the case of arthritis in human (Kaur G).

In present study we demonstrated that treatment with F can ameliorate the severity of RA and inhibit the progression of FCA induced arthritis. Orally administration of F for consecutive twenty days from the day 21, effectively alleviated clinical symptoms of rat evidenced by the notable reduction in inflammatory swelling and erythema in the paws, and also reversed the body weight loss along with the disease progression.

Decrease in body weight of the animals injected with FCA was used as markers for evaluating the secondary lesions in arthritic rats and therapeutic effects of treatment. The loss of body weight in the arthritic animals could be due to reduced absorption of glucose and leucine in rat intestine in arthritic condition (Geetha and Varalakshmi, 1998). Treatment with F restored body weight loss of animals suggesting its antiinflammatory activity.

Measurement of paw volume is one of the important, quick and simple parameter of evaluation and assessment of antiinflammatory activity (Wang *et al.,* 2016)

In FCA induced arthritis the initial edema and soft tissue thickening is due to irritant action of FCA while late phase arthritis is due to immunologic reactions (Jalalpure *et al.,* 2011). In present study treatment with F significantly and dose dependently decreased the paw volume suggesting antinflammatory activity.

FCA induced arthritis is one of the validated model for study of chronic pain. (Colpaert et al., 1982). The injection of FCA causes induces release of various inflammatory cytokines (Billiau and Matthys, 2001). Interlukin-1 β (IL-1β) is a potent mechanical and thermal hyperalgesic agent when injected into any number of peripheral tissues. Intraplantar injection of inflammatory agents, such as carrageenan, lipopolysaccharide (LPS), bacterial endotoxin or Freund's complete adjuvant (FCA) produce mechanical or thermal hyperalgesia associated with an upregulation of IL-1β and other inflammatory cytokines in the inflamed tissue and in the dorsal root ganglia (DRG) (Ren et al., 2009).

FCA induced arthritis leads to peripheral pain and hyperalgesia and functional impairment. The neurons in the injected paw become sensitive to allodynia and hyperalgesia. The hyperalgesia associated with arthritis has been reported to involve prostaglandin synthesis (Portanova et al., 1996). In the present study treatment with F significantly increased the pain threshold in arthritic

animals. The 6-shagaol and wedelolactone synergistically acting against hyperalgesia and various cytokines may be responsible for the efficacy.

In the present study, increase in serum AST, ALT, ALP levels and decrease in total protein level was observed in arthritic rats (Cawthorne et al., 1976).

In arthritis changes in plasma protein level with an increase in the globulin fraction and decrease in albumin fraction were well documented. General reduction of liver protein synthesis can be assessed by measuring albumin levels, because levels of this protein are lowered during inflammation and further, it was also reported that albumin synthesis was reduced by IL-1 (Lewis et al., 1998).

Moreover the mediators released such as histamine, bradykinin and prostaglandins during inflammation increase the permeability of vascular tissues to albumin leading to reduction in its serum levels. Assessment of the serum levels of AST, ALT and ALP provides an excellent and simple tool to measure the antiarthritic activity of the target drug (Kataoka et al., 2002).

The activities of aminotransferases and ALP were significantly increased in arthritic rats, since these are good indices of liver impairment, which are also considered as the features of adjuvant arthritis (Rainsford et al., 1882).

Serum AST and ALT have been reported to play a vital role in the formation of biologically active chemical mediators such as bradykinin in inflammatory process confirmed a positive correlation between the increased activity of serum AST and ALT and the disease activity in RA (Niino-Nanke et al., 1998).

In the present investigation, GOF, EAF and F were able to reduce the elevated level of AST, ALT, ALP and TP in the animals in a dose dependent manner.

C - reactive protein (CRP) is one of the acute phase proteins that is increased during systemic inflammation and used as a marker of inflammation (Ridker et al., 2002).

After the GOF, EAF and F treatment, decrease in CRP level indicated anti-inflammatory effect.

According to present study a decrease in Hb and RBC count, increase in the WBC and platelets count in arthritic rats was seen. Anemia is the most common extracellular manifestation in RA and a moderate hypochromic; normocytic anemia due to reduction in the RBC count with a modest reduction in the mean corpuscular haemoglobin count (MCHC) is a common feature of RA (Hochberg et al., 1988).

The increase in both WBC and platelet counts might be due to the stimulation of immune system against the invading pathogenic microorganism (Maria et al., 1983). This is evident by the infiltration of inflammatory mononuclear cells in the joints of arthritic rats. In the present study, the level of Hb and RBC was significantly increased, while the level of WBC and platelets was significantly reduced by GOF, EAF and F treatment.

The role of oxidative stress in arthritis is not surprising since reactive oxygen species serve as mediators of tissue damage. As a result circulating immune cells infiltrate into the inflamed tissue and upon activation they release pro-inflammatory cytokines and further reactive species in the surrounding tissues (Wong et al, 2008; Bauerova and Bezek, 1999; Feldmann et al, 2001 and Knight, 2000).

GOF, EAF and F treatment significantly reduces MDA levels while increased GSH and SOD levels suggesting its strong antioxidant potential.

pro-inflammatory cytokines TNF-α , IL-1ß and IL-6 are pivotal pro-inflammatory cytokines that have been shown to contribute to the clinical manifestations of RA, and play a primary role in

mediating the pathophysiological processes underlying inflammation and tissue destruction in RA (Gravallese and Goldring, 2000; Dayer, 2003).

Histological analysis showed that this compound dramatically no the infiltration of cells into the joint cavity, no synovial hyperplasia and pannus formation, and prevented bone and cartilage from erosion at 100 and 200 mg.kg of GOF, EAF and F. These findings support a view that 6-shagaol and wedelolactone and other pharmacologically active phytoconstituents from extract may be responsible for its beneficial effects for RA remedy (Luo *et al.*,2010).

Conclusion

The present study revealed that enrichment of extract with active phytoconstituents and its formulation development in order to increase its bioavailability and synergistic activity of these constituent was found to effective treating RA and its progression. The probable mechanism of action could be pleiotropic action of 6-shagaol and wedelolactone and other active constituents present. The present formulation could be better drug and adjuvant in the treatment of arthritis.

Rreferences

- Sermkaew N, Ketjinda W, Boonme P, Phadoongsombut N, Wiwattanapatapee R. Liquid and solid self-microemulsifying drug delivery systems for improving the oral bioavailability of andrographolide from a crude extract of *Andrographis paniculata.* European Journal of Pharmaceutical Sciences 50 (2013) 459–466.
- Chen S. Natural Products Triggering Biological Targets- A Review of the Anti-Inflammatory Phytochemicals Targeting the Arachidonic Acid Pathway in Allergy Asthma and Rheumatoid Arthritis *Current Drug Targets,* 2011, *12,* 288-301 .
- Datta P, Sarkar A, Biswas AK, Gomes A. Anti arthritic activity of aqueous extract of Indian black tea in experimental and clinical study Oriental Pharmacy and Experimental Medicine 2012,12:265–271.

- Constantinides P P. Lipid microemulsions for improving drug dissolution and oral absorption: Physical and biopharmaceutical aspects.Pharmaceutical research, 1995;12(11):1561-1572.
- Kaur G, Sultana S. Evaluation of antiarthritic activity of isoeugenol in adjuvant induced arthritis in murine model. Food and Chemical Toxicology 2012; 50: 2689–2695.
- Geetha T, Varalakshmi P. Antiinflammatory activity of lupeol linoleate in adjuvant induced arthritis. Fitoterapia 1998; 69: 13–19.
- Wang S, Wang Y, Liu X, Guan L, Yu L, Zhang X. Anti-inflammatory and anti-arthritic effects of taraxasterol on adjuvant-induced arthritis in rats Journal of Ethnopharmacology 187 (2016) 42–48.
- Jalalpurea S S, Mandavkar Y D , Khalure P R, Shinde G S, Shelar P A, Shah A S. Antiarthritic activity of various extracts of Mesua ferrea Linn. Seed Journal of Ethnopharmacology 138 (2011) 700– 704.
- Colpaert FC, Meert T, Witte P, Schmitt P. Further evidence validating adjuvant arthritis as an experimental model of chronic pain in the rat. Life Sciences 1982; 31: 67–75.
- Billiau A, Matthys P. Mode of action of Freund's adjuvant in experimental model of autoimmune disease. Journal of Leukocyte Biology 2001; 70: 849–860.
- Ren and Richard Torres. Role of interleukin 1β during pain and inflammation. Brain Research Reviews 2009; 60: 57-64.
- Portanova JP, Zhang Y, Anderson GD. Selective neutralization of prostaglandin E2 blocks inflammation, hyperalgesia, and interleukin 6 production *in vivo*. Journal of Experimental Medicine 1996; 184: 883–891.
- Portanova JP, Zhang Y, Anderson GD. Selective neutralization of prostaglandin E2 blocks inflammation, hyperalgesia, and interleukin 6 production *in vivo*. Journalof Experimental Medicine 1996; 184: 883–891.
- Cawthorne MA, Palmer ED, Green J. Adjuvant-induced arthritis and drug-metabolizing enzymes. Biochemical Pharmacology 1976; 25: 2683–2688.
- Lewis EJ, Bishop J, Aspinall SJ. A simple inflammation model that distinguishes between the actions of anti-inflammatory and antirheumatic drugs. Inflammation Research 1998; 47: 26–35.

- Kataoka H, Horiyama S, Yamaki. Anti-inflammatory and anti-allergic activities of hydroxylamine and related compounds. Biological and Pharmaceutical Bulletin 2002; 25: 1436–1441.
- Rainsford KD. Adjuvant polyarthritis in rats: Is this a satisfactory model for screening anti-arthritic drugs? Agents Actions 1982; 12: 452–458.
- Niino-Nanke Y, Akama H, Hara M, Kashiwazaki S, Alkaline phasphatase (ALP) activity in rheumatoid arthritis-Its clinical significance and synthesis of ALP in RA synovium. Ryumachi 1998; 38: 581–588.
- Ridker PM, Rifai N, Rose L, Buring JE, Cook NR. Comparison of C reactive protein and low density lipoprotein cholesterol levels in the prediction of first cardiovascular events. New England Journal of Medicine 2002; 347(20):1557-65.
- Hochberg MC, Arnold CM, Hogans BB, Spivak JL. Serum immunoreactive erythropoietin in rheumatoid arthritis: impaired response to anemia. Arthritis and Rheumatology 1988; 31: 1318–1321.
- Wong YF, Zhou H, Wang JR, Xie Y, Xu HX, Liu L. Anti-inflammatory and analgesic effects and molecular mechanisms of JCICM-6, a purified extract derived from an anti-arthritic Chinese herbal formula. Phytomedicine 2008; 15(6):416–26.
- Bauerova K, Bezek A. Role of reactive oxygen and nitrogen species in etiopathogenesis of rheumatoid arthritis. General Physiology and Biophysics 1999; 18: 15–20.
- Feldmann M, Brennan FM, Foxwell BM, Maini RN. The role of TNF-alpha and IL-1 in rheumatoid arthritis. Current Directions in Autoimmunity 2001; 3: 188–199.
- Knight JA. Free radicals, antioxidants, and the immune system. Annals of Clinical and Laboratory Science 2000; 30: 145–158.
- Gravallese, E.M., Goldring, S.R., 2000. Cellular mechanism and the role of cytokines in bone erosions in rheumatoid arthritis. Arthritis Rheumatol. 43, 2143–2151.
- Dayer, J.M., 2003. The pivotal role of interleukin-1 in the clinical manifestations of rheumatoid arthritis. Rheumatology 42, ii3–ii10.
- Luo Y, Liu M, Xia Y, Dai Y, Chou G, Wang Z. Therapeutic effect of norisoboldine, an alkaloid isolated from Radix Linderae, on collagen-induced arthritis in mice. Phytomedicine 17 (2010) 726–731.