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# Exploring the Therapeutic Efficacy of a 6-Shogaol-Enriched Formulation in Attenuating Freund's Complete Adjuvant-Induced Arthritis in Rats: A Comprehensive Study on Ameliorative and Antiarthritic Effects

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Background Zingiber Officinale (Family-Zingiberaceae) is a herb commonly used in traditional ayurvedic medicine for the treatment of arthritis, inflammation, pain, and wounds. In the view of its limited bioavailability of active constituent 6-shogaol, present study is aimed to enrich the extract with 6-shogaol and formulate it in order to increase its bioavailability and hence its effectiveness. On day 0, Arthritis was induced by injection of FCA and from day 12 to day 28 animals of test groups were treated with formulation of 6-shagaol rich extract of Zingiber Officinale (GOF) at 50, 100, and 200 mg/kg.
Results: The results suggests that GOF has promising anti-arthritic properties as it has significantly modulated joint inflammation, associated pain parameters, cytokine levels, oxidative stress, and other relevant clinical symptoms. The dose-dependent response and the observed changes in multiple parameters strengthen the evidence for GOF's potential as a treatment for arthritis.
Conclusion: Indeed, these findings clearly showcased the antiarthritic properties of GOF in combating experimental arthritis. The enhanced bioavailability of 6-

shagaol played a crucial role in inhibiting proinflammatory cytokines, thereby contributing to the efficacy of GOF.
**Keywords:** Rheumatoid arthritis, 6-shagaol, cytokines, Zingiber Officinale, bioavailability.

## 1. Introduction

Rheumatoid arthritis (RA) is a prevalent global health issue, serving as a significant contributor to disability and being the most prevalent autoimmune disease worldwide. If left untreated, it can result in premature death. (Bais et al., 2017). In RA, the synovial tissue inflammation leading to the invasion of cartilage and bone, resulting in progressive joint dysfunction characterized by synovitis, synovial hyperplasia, stiffness, and pain (Chitme and Patel, 2009). The extent of inflammation is dictated by the equilibrium between proinflammatory and anti-inflammatory cytokines (Huanghe et al., 2019, Anantha et al., 2016). Both males and females are affected by RA, but females are at high risk with a ratio of 3:1.It can occur at any age but more common in middle age.

Conventional treatment options include Non-Steroidal Anti-inflammatory Drugs, Disease Modifying Anti-Rheumatoid Drugs, and newer biologicals such as tumor necrosis factor-α (TNF-α) antagonists. These therapies have demonstrated efficacy in alleviating symptoms and ushered in a therapeutic revolution by enhancing clinical, functional, and radiographic results (Tyagi et al.2020).

However, these drugs have demonstrated a broad range of potentially fatal side effects. These adverse effects are mainly attributed to the drugs' widespread distribution throughout the body and their accumulation in unintended areas.

Developing safer and more effective therapies for RA remains a key goal in rheumatology. Herbal remedies, with their bioactive phytoconstituents, are being explored as adjuncts to conventional treatments due to their safety and personalized therapeutic potential. These compounds can interact with multiple pathways, offering versatile benefits for managing arthritis and other health conditions. Consequently, herbal medicines present a promising alternative for effective RA management.

Zingiber officinale Roscoe, (ZO) a member of the Zingiberaceae family, is widely valued as both a spice and a traditional remedy. Its rhizome is rich in bioactive phytochemicals, including gingerols, shogaols, and paradols, which contribute to its therapeutic properties. Historically used to treat ailments like colds, pain, and digestive issues, ginger offers diverse health benefits. Its nutritional and medicinal significance highlights its role in promoting overall well-being (Chrubasik et al., 2005; Ali et al., 2008; Baliga et al., 2015; Singh et al., 2022).

Ginger's therapeutic use is hindered by low bioavailability, poor solubility, instability and side effects, while conventional RA treatments face compliance and efficacy challenges (Yang et al 2020). A novel oral dosage form is essential to improve ginger's stability and effectiveness.

## 2. Materials and methods

### 2.1. Procurement and authentication of plant

Dried powder of ZO rhizomes was purchased from Pune, Maharashtra, India. An authentication was carried out by Botanical Survey of India, Pune and voucher specimen (No BSI/WRC/Cert./2014/AS03) was placed for future reference.

### 2.2. Drugs and chemicals

FCA (Sigma Aldrich, USA), etoricoxib was obtained as gift sample from Zydus Cadila, Gujarat, 6-shogaol was purchased from Natural Remedies, India, Diagnostic kits for biochemical analysis were purchased from Accurex Biomedical Pvt. Ltd. ELISA kits were obtained from Ray Biotech Lexington, KY. All other solvents and chemicals utilized for study were of analytical grade from reliable vendors.

### 2.3 Animals

Female Wistar rats weighing between 180 to 220 grams were procured from the National Toxicology Centre in Pune, India. These rats were housed in a controlled environment at a temperature of 25±1º C and a relative humidity of 45 to 55%, following a 12-hour light: 12-hour dark cycle. They were provided with free access to food pellets from Pranav Agro Industries Ltd, India, and water ad libitum. The experimental procedures conducted on the animals were in accordance with ethical standards and were approved by the Institutional Animal Ethics Committee. This committee was established following the guidelines of the Committee for the Purpose of Control and Supervision on Experimental Animals in India, with a registration number (1703/PO/c/13/CPCSEA) and protocol approval number (CPCSEA/28/2014).

### 2.4 Extraction

Dried powder of ZO rhizomes was extracted using lipids such as labrafac, labrafil and labrasol to get 6-shogaol enriched ZO extract (GOE). For this dried powder of ZO rhizomes (500 mg) was added to 1 ml of lipid and vortexed (Remi Mumbai, India) for 20 min to allow proper mixing and extraction in the lipids. The mixtures were allowed to equilibrate at room temperature for 48 h. The mixture was centrifuged at 12000 rpm for 20 min to separate the undissolved drug.

### 2.5. Quantification of 6-shogaol using HPLC method

Quantification of 6-shogaol in ZO respectively in ethanolic and enriched extracts were determined using validated HPLC method. The solutions of 6-shogaol (50µg/ml) and (6-shogaol enriched extract of ZO) GOE (1000µg/ml) were prepared in methanol. The HPLC system (Jasco Corporation, Japan) was configured with a dual pump (Jasco PU- 2080 Plus), a UV/Visible detector (UV-2075 Plus), and a Thermo Scientific Merck C18 reversed-phase column (I.D. 4.6mm×250mm, 5µm). Separation was achieved using a two-pump linear gradient program with pump A (water containing 0.1% glacial acetic acid) and pump B (acetonitrile) in a 40:60 ratio, monitored at 243 nm. The flow rate and injection volume were set at 1.0 ml/min and 20µl, respectively. To confirm the chromatographic peak of the extract, retention time and UV spectra were compared with reference standards.

## 2.6. Formulation

### Selection of surfactant

Tween 20 (Polyoxyethylene sorbitan monolaurate HLB=16.7), Tween 60 (Polyoxyethylenesorbitan monostearate HLB=14.9) and Tween 80 (Polyoxyethylene sorbitan monooleate HLB=15) surfactants were screened based on their ability to emulsify the selected oil phase. Various surfactant volumes (0.2–0.6 ml) were mixed with 1 ml of oil, diluted (25 µl to 25 ml water), and assessed for emulsification efficiency. The best-performing surfactant was tested with 50 µl oil in different ratios, measuring ease of emulsification by inversion count and stability after 2 hours. The most stable emulsion was identified visually.

### Selection of co-surfactant

Various co-surfactants such as Span 80 (Sorbitan monooleate HLB=4.3), Span 20 (Sorbitan monolaurate HLB=8.6 and propylene glycol (HLB=3.4) were screened based on their efficacy to improve the nanoemulsification ability of the selected surfactants. To 1 ml of lipid, 0.6 ml surfactant was mixed with varying co-surfactant volumes (0.2, 0.4, 0.6 ml), and 25 µl of the mixture was diluted to 25 ml with water. Emulsion formation ease was evaluated by the number of inversions required for uniformity, followed by 2-hour stability observation. The most effective combination was identified visually.

### Preparation of SNEDDS

The finalized ratio 1:0.6:0.4 of labrafil: Tween 80: propylene glycol was selected for preparation of SNEDDS.

## 2.7. Acute toxicity studies

Acute toxicity study was conducted for GOF in accordance to OECD guidelines No. 423. An overnight-fasted and healthy Female rats (n=3) were orally administered GOF (5 mg/kg body weight). Behavioral and autonomic profiles were monitored intensively for the first 4 hours post-dosing, with daily observations continuing for 14 days. The study was conducted using doses of 50, 300, and 2000 mg/kg body weight.

## 2.8. FCA induced arthritis

There were six group of animals each containing six animals. Group I was healthy control (2 % w/v Tween 80), Group II was arthritic control (2 % w/v Tween 80), Group III was standard and were administered 10 mg/kg etoricoxib (p.o.), Group IV, V and VI received 50, 100 and 200mg/kg GOF (p.o.), respectively. Animals in Groups II to VI received a subplantar injection of 0.1 ml of FCA into the left hind paw on day 0. The corresponding treatments commenced orally once daily upon the alleviation of arthritis symptoms on day 12. Body weight, paw volume, thermal and mechanical hyperalgesia pain threshold, tactile and allodynia were assessed on day 0, 1, 4, 8, 12, 16, 20, 24 and day 28. On day 28, blood was collected via retro-orbital puncture under ether anesthesia for hematological analysis, while serum was separated for evaluating biochemical parameters and cytokine levels as described by Atre et al. 2020. Subsequently, the animals were humanely euthanized using CO2, and organs including the spleen, liver, and ankle joints were isolated.

2.8.1. Body weight

On all evaluation days mentioned above, body weight was recorded using animal weighing balance (Atre et al., 2020, Anantha et al., 2016).

2.8.2. Paw volume

A Plethysmometer (UGO Basile, Italy) was used for measuring the paw volume.The difference between the final and initial paw volume is taken as change in paw. (Atre et al., 2020)

2.8.3. Mechanical hyperalgesia (Paw withdrawal threshold)

A mechanical hyperalgesia was assessed by Randall-Selitto analgesiometer (UGO Basile, Italy). The hind paw of animal was positioned between the flat surface and blunt pointer, and pressure was increased gradually. The paw withdrawal threshold of the animal was taken as response. The time point at which rat attempts to withdraw the paw from device was considered as pain threshold. The cut-off pressure was 450g (Atre et al., 2020).

2.8.4. Thermal hyperalgesia (Paw withdrawal latency)

A radient heat device (UGO Basile, Italy) was used to assess thermal hyperalgesia. The response was recorded as paw withdrawal latency. The infrared intensity of the lamp set at 40 and paw was placed on it. To avoid the any damage to tissue a cut off latency of 15 sec was set (Atre et al., 2020).

2.8.5. Tactile allodynia (Mechanical nociceptive threshold)

A mechanical nociceptive threshold was assessed as paw withdrawal when stimulus was given with probe to the plantar surface with a series of calibrated fine filaments (von Frey hairs, Almemo, Germany) of increasing gauge (0.6 to 12.6g). For this rats were previously acclimatized for 10 min in the perspex box. Three consecutive stimuli within the period of 2-3 sec were applied in to each paw with each hair.

Withdrawal of paw by animal caused by lowest weight of von Frey hair from the three consecutive applications was recorded as threshold. Lifting the paw was recorded as a affirmative response (Atre et al., 2020).

2.8.6. Measurement of Cytokine levels

On day 28, serum cytokine measurement such as Tumor necrosis factor-α (TNF-α), Interleukin 1β (IL-1β) and Interleukin-6 (IL-6) were done using ELISA kit by sandwich method (Atre et al., 2020).

2.8.7. Haematological and biochemical parameters

On day 28, haematological parameters like RBC count, haemoglobin (Hb), and platelet (PLT) count were evaluated by standardized laboratory methods (Mehta et al., 2012). Serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total protein (TP) and C-reactive protein (CRP) was measured (Atre et al., 2020).

2.8.8. Antioxidant parameters

On day 28, animals were sacrificed and liver was isolated. The isolated liver was washed in ice-cold saline and subsequently homogenated using 0.1M Tris-HCl buffer (pH 7.4). The

supernatant collected was used for estimation of superoxide dismutase (SOD) (Mishra and Fridovich 1972), malondialdehyde (MDA) (Slater and Sawyer 1971), and reduced glutathione (GSH) (Morgon et al., 1979).

2.8.9. Histopathological analysis of ankle joints

On day 28, animals were sacrificed and the ankle joint was isolated from the hind paw. The isolated ankle joint was then immersed in 10% buffered formalin and processed further for paraffin embedding section at 5 µ thickness. The sections were stainned with haematoxylin-eosin dye and observed under a light microscope with 10X magnifications (Atre et al., 2020).

2.8.10. Statistical Analysis

Statistical analysis of data were done by Graph Pad Prism software (San Diego, CA). For biochemical analysis oneway ANOVA followed by Dunnett's test was applied and for in vivo parameters, two way ANOVA followed by Bonferroni's post hoc test was applied. Data were considered statistically significant at $p<0.05$.

## 3. Results

3.1 Formulation

Among various surfactants screened, ZO exhibited highest solubility in Tween 80, whereas good emulsification in surfactant Tween 80 and cosurfactant Span 80 at the ratio of 1:0.6:0.4.

Self emulsification time

The time taken to form uniform emulsion was 45 sec. The formulation showed uniform and stable emulsion even after 2 hrs when observed visually.

Robustness to dilution

The formulation was diluted with water (50, 100, 1000 times), showing no precipitation, cloudiness, or separation after 24 hours, confirming emulsion stability. Labrafil demonstrated the highest lipid solubility (via HPLC), while Tween 80 and Span 80 showed the best solubility and emulsification, respectively, making them the chosen surfactant and co-surfactant. The final formulation combined 500 g of ZO rhizome powder with 1 ml labrafil, 0.6 ml Tween 80, and 0.4 ml Span 80.

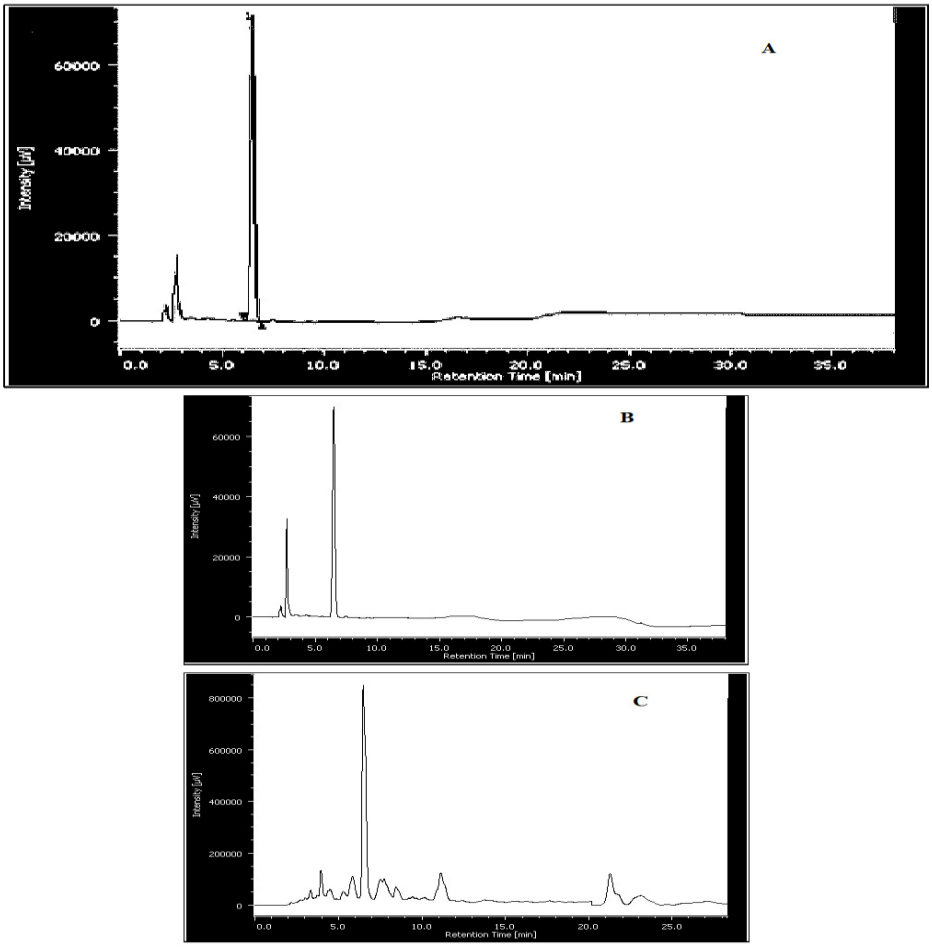
Standardization of extract

The HPLC analysis validated the enrichment of 6-shogaol in GOF as compared to ethanolic extract and content was found to be (6.62% w/w and 23.06 % w/w, respectively) (Figure 1A, 1B and 1C).

3.1. Acute toxicity studies

According to OECD guideline No. 423, administration of 2000 mg/kg of GOF. The test animals did not exhibited any changes in autonomic, behavioral profile and survived beyond acclaimed duration of observation with 2000 mg/kg of GOF (OECD Guideline No. 423). Hence it was found to be safe up to 2000 mg/kg.

Figure 1 Quantitative analysis of ethanolic and enriched extracts of ZO by high performance liquid chromatography



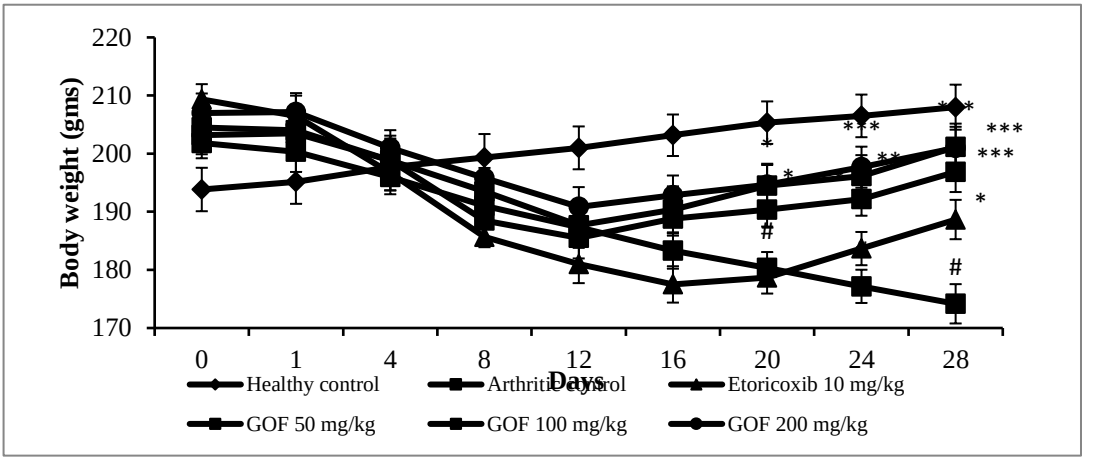

Chromatograph 1A: 6-Shagaol Standard 100 ppm retention time 6.4 minutes, 1B: SZO 100 ppm retention time 6.4 min, 1C: GOF 100 ppm retention time 6.4 minutes

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

Induction of arthritis with FCA produced gradual decrease in body weight of rats. On treatment with etoricoxib there was significant ($P<0.05$) prevention of body weight loss only on day 28 when compared with arthritic control animals. Moreover, treatment with GOF (200 mg/kg) significantly prevented body weight loss on the day 20, 24 and 28 onwards ($P<0.05$, $P<0.001$, $P<0.001$ respectively) when compared to arthritic control group. GOF 100 mg/kg treatment group caused significant ($P<0.05$, $P<0.001$) prevention in body weight loss on day 24 and 28 respectively while GOF 50 mg/kg treatment caused significant ($P<0.05$, $P<0.001$) prevention in body weight loss on day 24 and 28 respectively (

Figure *2*).

Figure 2 Effect of GOF on body weight (gms) in FCA induced arthritis in rats.

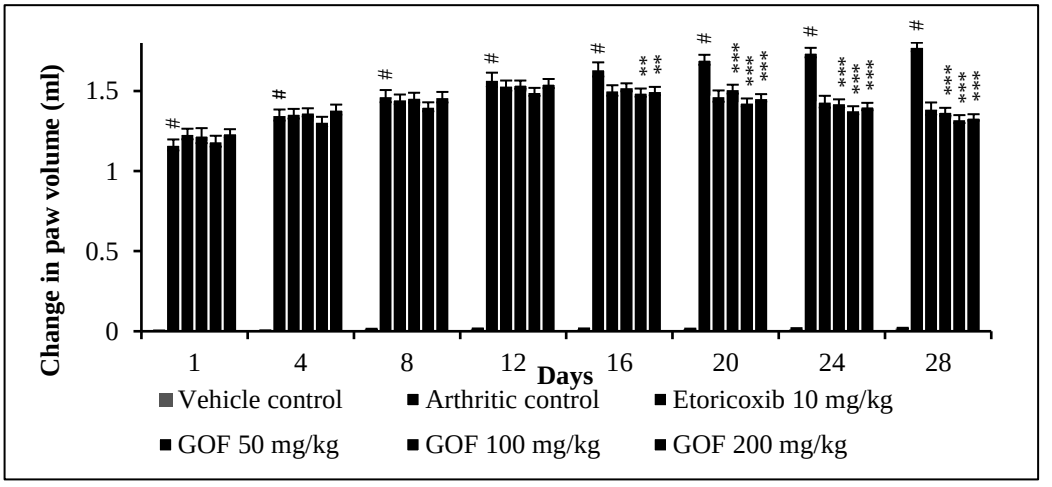



Results are represented as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test Data are compared with arthritic control group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and when compared with vehicle control $^{\#}P<0.001$.

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

A significant (P<0.001) rise in paw volume of right hind paw was observed in all arthritic group animals from day 1 and progressed till day 28. Treatment of etoricoxib (10 mg/kg) caused significant decrease in the paw volume of right hind paw (P<0.05) on day 16 and (P<0.001) on day 20, 24 and 28 respectively. Administration of 100 and 200 mg/kg of GOF caused significant and decrease in the paw volume of right hind paw on day 16 (P<0.01) and (P<0.001) on days 20, 24 and 28, respectively, while administration of 50 mg/kg of GOF caused significant (P<0.05, P<0.001 and P<0.001) decrease in the paw volume of right hind paw on day 20, 24 and 28 respectively. In the case of the left hind paw, no variations in paw volume were observed throughout the study period. (Figure 3)

Figure 3: Effect of GOF on change in paw volume (ml) in FCA induced arthritis in rats.



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

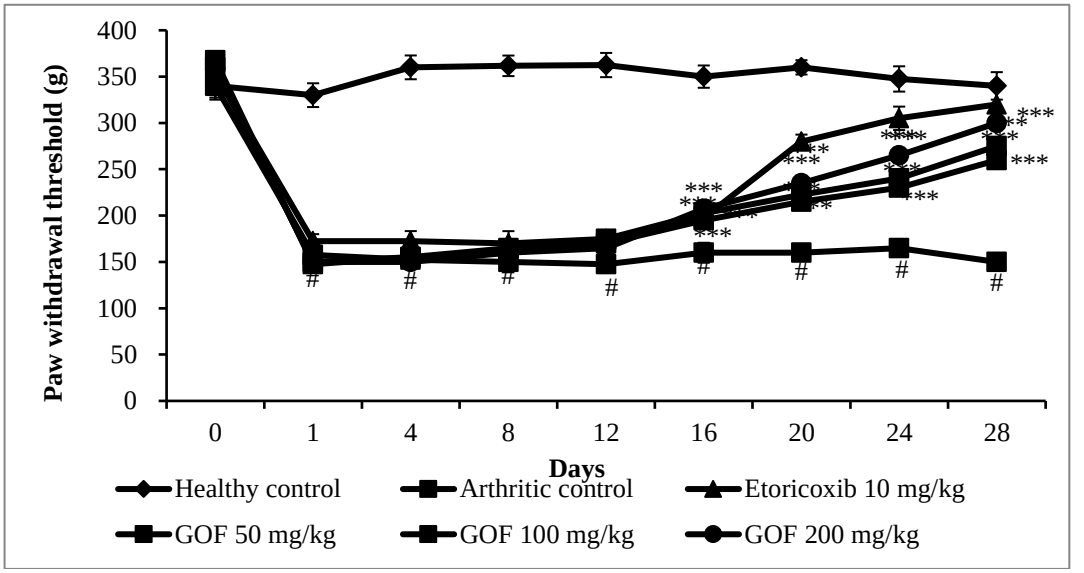
3.4. Effect of GOF on Mechanical hyperalgesia in FCA induced arthritis in rats.

FCA induction resulted in a significant (P<0.001) reduction in paw withdrawal threshold. Maximum decrease in paw withdrawal threshold was noted on day 28 which was recorded as 150.00 ± 5.48gm. Administration of etoricoxib (10 mg/kg) significantly (P<0.001) enhanced the paw withdrawal threshold on days 20, 24 and 28. Administration of GOF at dose 200 mg/kg caused significant (P<0.001) improvement in paw withdrawal threshold on days 16, 20, 24 and 28 respectively when compared to arthritic control, while administration of GOF at dose 50mg/kg caused significant (P<0.05,) on day 16 and(P<0.001)on days 16, 20, 24 and 28 respectively, while GOF 100 mg/kg caused significant (P<0.01, P<0.001, P<0.001, P<0.001)on days 16, 20, 24 and 28 respectively improvement in paw withdrawal threshold when compared to arthritic control .(Figure 4).

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

FCA induction resulted in a significant (P<0.001) decrease in paw withdrawal latency in all the rats. Maximum decrease in paw withdrawal latency was noted on day 28 which was recorded as 5.10 ± 0.24 sec. Oral administration of 10 mg/kg of etoricoxib caused significant (P<0.001) recovery from decreased right hind paw withdrawal latency from day 20 onwards. Administration of GOF at dose 100 and 200 mg/kg caused significant (P<0.001) improvement in paw withdrawal latency on days 16, 20, 24 and 28 respectively, when compared to arthritic control, while administration of GOF at dose 50 mg/kg caused significant improvement in paw withdrawal latency on days 20, 24 and 28 (P<0.05, P<0.001, P<0.001, P<0.001). (Figure 5).

Figure 4: Effect of GOF mechanical hyperalgesia in FCA induced arthritis in rats.

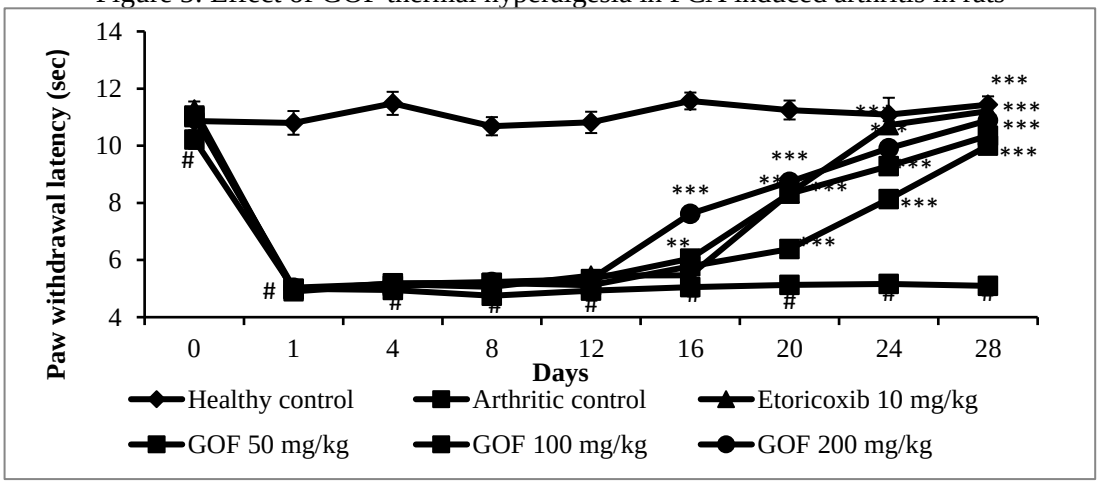


Results are represented as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test Data are compared with arthritic control group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and when compared with vehicle control $^{\#}P<0.001$.

3.6. Effect of GOF on tactile allodynia in FCA induced arthritis in rats

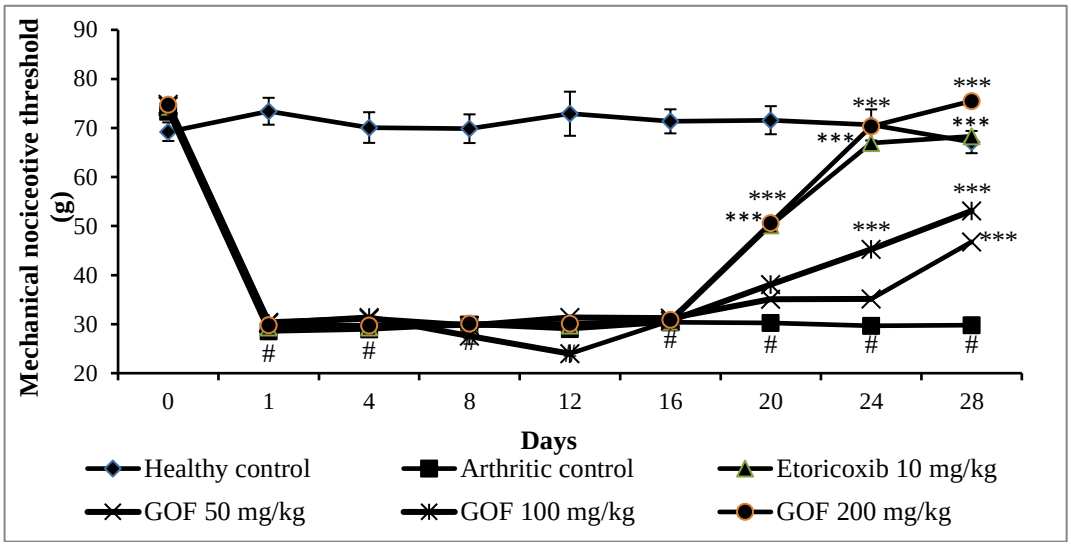
FCA induction resulted in significant decrease in mechanical nociceptive threshold in arthritic animals till day 12. Administration of etoricoxib (10 mg/kg) significantly (P<0.001) enhanced the mechanical withdrawal threshold from day 20 onwards in comparison to arthritic control while treatment with GOF (200 mg/kg) was found to be significantly (P<0.001) effective in improving the mechanical withdrawal threshold from day 20 onwards when compared to arthritic control. Administration of GOF 100 mg/kg significantly (P<0.001) increased mechanical nociceptive threshold on day 24 and 28 while GOF (50 mg/kg) caused significant (P<0.001) improvement in the mechanical withdrawal threshold only on day 28 when compared with arthritic control group (Figure 6).

Figure 5: Effect of GOF thermal hyperalgesia in FCA induced arthritis in rats




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

Figure 6: Effect of GOF on tactile allodynia in FCA induced arthritis in rats




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

3.7. Effect of GOF on haematology and serum parameters in FCA induced arthritis in rats.

The challenge with FCA (0.1ml) caused significant (P<0.001) decrease in the RBC count and increase in the WBC count when compared to arthritic control which was found to be 3.266±0.0738 ($10^3$cells/mm$^3$) and 15.40±0.2556 $10^3$cells/mm$^3$ respectively. Etroicoxib 10 mg/kg caused significant (P<0.001) elevation in the RBC count and decrease in the WBC count while compared to arthritic control. The treatment with GOF 200, 100 and 50 mg/kg caused significant (P<0.001, P<0.01, P <0.01 respectively) elevation in the RBC count as compared to arthritic control. (Table 1)

The challenge with FCA (0.1ml) caused significant (P<0.001) decrease in the haemoglobin count when compared to vehicle control which was found to be 8.90±0.1818 gm/dL. The treatment with etoricoxib 10 mg/kg caused significant (P<0.001) increase in the haemoglobin count as compared to arthritic control, whereas GOF 200, 100 and 50 mg/kg significantly (P<0.001, P<0.001, P<0.01 respectively) increased haemoglobin count when compared to arthritic control (Table 1).

The challenge with FCA (0.1ml) caused significant (P<0.001) elevation in the platelet count when compared to vehicle control which was found to be 18.46 ±0.3516 $10^3$cells/mm$^3$. Etoricoxib 10 mg/kg caused significant (P<0.001) decrease in the platelet count when compared to arthritic control. The treatment with GOF 200, 100 and 50 mg/kg caused significant (P<0.001, P<0.001, P<0.01 respectively) decrease in the platelet count when compared to arthritic control.

The ESR of the vehicle control was 8.350± 0.189 mm/h, which was significantly (P<0.001) increased in arthritic control group and found to be 15.40± 0.339mm/h. Etoricoxib 10 mg/kg

caused significant (P<0.001) decrease in ESR when compared to arthritic control group. Treatment with GOF 200, 100 and 50 mg/kg exhibited a significant (P<0.001, P<0.001, P<0.01) decrease in ESR when compared to arthritic control. (Table 1)

The serum CRP level of the vehicle control was 1.6020±0.034 mg/lit, which was significantly (P<0.001) increased in arthritic control group and found to be 7.1770±0.1666 mg/lit. Etoricoxib 10 mg/kg caused significant (P<0.001) decrease in serum CRP level when compared to arthritic control group. Treatment with GOF 200, 100 and 50 mg/kg exhibited a significant (P<0.001,) dose dependent decrease in serum CRP level when compared to arthritic control. (Table 1)

The serum TP level of the vehicle control was 7.450±0.0646 gm/dL, whereas arthritic group caused significant decrease which was found to be 5.626±0.2288 gm/dL. Etoricoxib 10 mg/kg significantly (P<0.001) increased total protein level when compared with arthritic group. Administration of GOF 200, 100 and 50 mg/kg caused significant (P<0.05, P<0.001, P<0.001 respectively) increase in serum TP level when compared with arthritic group. (Table 2)

The serum AST, ALT and ALP level of the vehicle control was 41.17 ±1.4570 U/L, 52.34±1.5340 U/L and 74.94±2.0760 U/L respectively which was significantly (P<0.001) increased in arthritic control group and found to be 126.90±1.5570 U/L, 188.60±1.7300 U/L and 451.60±2.5140 U/L respectively. Treatment with etoricoxib 10 mg/kg caused significant (P<0.001) decrease in serum AST, ALT and ALP level when compared to arthritic control group. Treatment with GOF 200, 100 and 50 mg/kg exhibited a significant (P<0.001) decrease in serum AST, ALT and ALP level when compared to arthritic control. (Table 2)

The serum TNF- α, IL - 1β and IL - 6 level of vehicle control group was found to be 38.34 ± 0.9888 pg/ml, 120.50± 0.7801 pg/ml and 142.20± 1.1850 pg/ml respectively which was significantly (P<0.001) increased in arthritic control group and found to be 121.2 ± 1.558 pg/ml, 428.90± 1.6570 pg/ml and 407.30 ± 1.8990 pg/ml respectively. Etoricoxib 10 mg/kg significantly (P<0.01, P<0.05 and P<0.01) decreased serum TNF- α, IL - 1β and IL – 6 level when compared to arthritic control group. Administration of GOF 200, 100 and 50 mg/kg significantly (P<0.001) reduced serum TNF- α level when compared to arthritic control group (Table 2).

The liver SOD and GSH level of the vehicle control was 4.508±0.0246 mU/mg protein and 71.02±0.4216 mU/mg protein which was significantly (P<0.001) decreased in arthritic control group and found to be 2.392±0.0313 mU/mg protein and 45.16±0.7465 mU/mg protein. Etoricoxib 10 mg/kg caused significant (P<0.01, P<0.001) increase in liver SOD level when compared to arthritic control group. Treatment with GOF 200, 100 and 50 mg/kg exhibited a significant (P<0.001) increase in liver SOD level when compared to arthritic control. (Table 3)

The liver MDA level of the vehicle control was found to be 1.9980±0.0296 n mole MDA/mg protein which was significantly (P<0.001) increased in arthritic control group and found to be 3.448±0.01564 n mole MDA/mg protein. Etoricoxib 10 mg/kg caused significant (P<0.001) decrease in liver MDA level when compared to arthritic control group. Treatment with GOF 200, 100 and 50 mg/kg exhibited a significant (P<0.001) decrease in liver MDA level when compared to arthritic control. (Table 3)

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

| Groups | RBC($10^6$cells/mm3) | WBC ($10^3$cells/$mm^3$) | Hb (gm/dL) | Platelet ($10^5$cells/$mm^3$) | ESR (mm/h) | CRP (mg/lit) |
|---|---|---|---|---|---|---|
| Healthy Control | 6.846±0.1132 | 7.626±0.2118 | 14.38±0.3222 | 9.238±0.1322 | 8.326±0.2870 | 1.6020±0.034 |
| Arthritic Control | 3.266±0.07378$^{\#}$ | 15.40±0.2556$^{\#}$ | 8.90±0.1818$^{\#}$ | 18.46±0.3516$^{\#}$ | 15.46±0.5332$^{\#}$ | 7.1770±0.1666$^{\#}$ |
| Etoricoxib 10 mg/kg | 6.034±0.1188$^{***}$ | 13.10±0.3058$^{***}$ | 13.22±0.1520$^{***}$ | 13.94±0.3862$^{***}$ | 9.926±0.536$^{***}$ | 3.6820±0.1408$^{***}$ |
| GOF 50 mg/kg | 4.326±0.2080$^{**}$ | 14.24±0.4204$^{*}$ | 10.50±0.1602$^{**}$ | 16.46±0.3978$^{*}$ | 13.36±0.3708$^{**}$ | 5.477±0.04978$^{***}$ |
| GOF 100 mg/kg | 4.574±0.2124$^{***}$ | 14.00±0.3600$^{**}$ | 11.04±0.2780$^{***}$ | 14.91±0.2890$^{***}$ | 12.10±0.2274$^{***}$ | 4.807±0.0281$^{***}$ |
| GOF 200 mg/kg | 5.326±0.0818$^{***}$ | 12.34±0.3266$^{***}$ | 11.60±0.5266$^{***}$ | 13.36±0.3804$^{***}$ | 11.08±0.2496$^{***}$ | 3.7370±0.0346$^{***}$ |

GOF: 6-shogaol rich extract of ZO; FCA: Freund's complete adjuvant, RBC: red blood cell, WBC: white blood cell, Hb: hemoglobin, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein.

Results are represented as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. Data are compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001 and when compared with vehicle control #P<0.001.

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 | 7.450±0.0646 | 41.18±1.4570 | 52.34±1.534 | 74.94±2.0760 | 38.34 ± 0.9888 | 120.50± 0.7801 | 142.20± 1.1850 |
| Arthritic Control | 5.626±0.2288$^{\#}$ | 126.90±1.5570$^{\#}$ | 188.6±1.73$^{\#}$ | 451.60±2.5140$^{\#}$ | 121.20 ± 1.5580$^{\#}$ | 428.90± 1.6570$^{\#}$ | 407.30± 1.8990$^{\#}$ |
| Etoricoxib 10 mg/kg | 6.526±0.1652$^{***}$ | 106.10±2.3490$^{***}$ | 172.2±2.923$^{***}$ | 438.80±1.4380$^{***}$ | 115.80± 0.7032$^{**}$ | 410.50± 0.9717$^{*}$ | 397.20 ± 2.1280$^{**}$ |
| GOF 50 mg/kg | 6.300±0.1080$^{*}$ | 111.50±4.2610$^{***}$ | 170.30±0.8256$^{***}$ | 419.60±1.3410$^{***}$ | 105.00 ± 1.8260$^{***}$ | 347.10± 4.8720$^{***}$ | 374.10 ± 1.8130$^{***}$ |
| GOF 100 mg/kg | 6.750±0.1556$^{***}$ | 98.76±1.7080$^{***}$ | 152.90±1.0740$^{***}$ | 402.10±1.4460$^{***}$ | 91.50 ± 0.5628$^{***}$ | 297.20± 2.9290$^{***}$ | 339.20 ±1.5760$^{***}$ |
| GOF 200 mg/kg | 6.976±0.0854$^{***}$ | 93.41±2.3860$^{***}$ | 140.10±1.1380$^{***}$ | 392.80±0.9480$^{***}$ | 87.50 ± 0.7638$^{***}$ | 244.40± 3.3920$^{***}$ | 315.50± 1.3800$^{***}$ |

GOF: 6-shogaol rich extract of ZO; 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

Results are represented as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. Data are compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001 and when compared with vehicle control #P<0.001.

Table.3.Effect of GOF on antioxidant parameters in FCA induced arthritis in rats.

| Groups | SOD (mU/mg protein) | MDA (nmole MDA/mg protein) | GSH (µmol/mg protein) |
|---|---|---|---|
| Healthy Control | 4.5080±0.02469 | 1.9980±0.0296 | 71.02±0.4216 |
| Arthritic Control | 2.3920±0.03136$^{\#}$ | 3.448±0.01564$^{\#}$ | 45.16±0.7466$^{\#}$ |
| Etoricoxib 10 mg/kg | 2.5320±0.01448$^{**}$ | 2.9220±0.0228$^{***}$ | 50.24±0.9446$^{***}$ |
| GOF 50 mg/kg | 2.5250±0.0138$^{***}$ | 3.3150±0.0329$^{***}$ | 49.16±0.6988$^{***}$ |

| | | | |
|---|---|---|---|
| GOF 100 mg/kg | 3.3500±0.0216*** | 2.7030±0.0266*** | 55.20±0.9852*** |
| GOF 200 mg/kg | 3.5000±0.0139*** | 2.5180±0.0144*** | 62.58±0.3166*** |

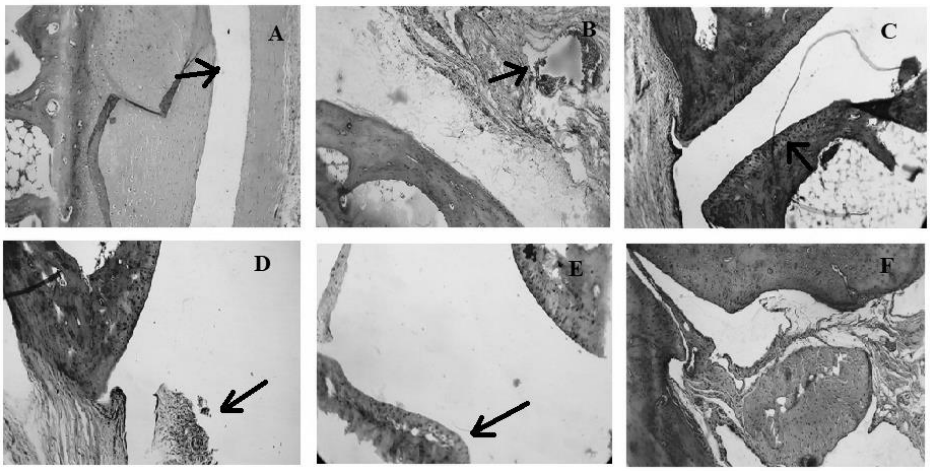
GOF: 6-shogaol rich extract of ZO; FCA: Freund's complete adjuvant, SOD: Superoxide dismutase, MDA: Malondialdehyde, GSH: Glutathione reductase.

Results are represented as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test. Data are compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001 and when compared with vehicle control #P<0.001.

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

Histopathology examination of ankle joint of normal rats revealed intact morphology of synovium and synovial lining. No inflammation and/or influx of inflammatory cells was observed. While, arthritis induction with FCA rats caused cartilage destruction, influx of inflammatory cells, pannus formation, disturbed synovial lining and chronic inflammation. Etoricoxib 10 mg/kg treatment in animals caused protection against cartilage destruction, vascular proliferation and synovial space thickening, low influx of inflammatory cells and no pannus formation. GOF 200 mg/kg and 100 mg/kg treatment to rats caused no cartilage destruction, synovial space thickening, vascular proliferation, no influx of inflammatory cells and no pannus formation. While, GOF 50 mg/kg treatment in rats caused minimal inflammation, influx of few inflammatory cells in synovium with evidence of disturbed synovial lining or pannus formation (Figure 7).

Figure 7: Effect of GOF Histopathology of ankle joint in FCA induced arthritis in rats.




7A: Vehicle control: Joint- Joint Bone with no infiltration of inflammatory cells exudate in joint tissue H&E 10X; Thickness: 5µ; Magnification: 40X 7B: Arthritic Control: Joint- Joint bone with maximum infiltration of inflammatory cells exudate in joint tissue. H&E 10X; Thickness: 5µ; Magnification: 40X 7C: 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 7D: GOF 50mg/kg: Joint- Joint Bone with Moderate infiltration of inflammatory cells and exudate in joint H&E 10X; Thickness: 5µ; Magnification: 40X 7E: GOF 100mg/kg: Joint- Joint Bone with minimal infiltration of inflammatory cells and fibrous

tissue H&E 10X; Thickness: 5µ; Magnification: 40X 7 F: GOF 200mg/kg: Joint- Joint Bone with no infiltration of inflammatory cells only minimal exudate in joint tissue H&E 10X; Thickness: 5µ; Magnification: 40X.

## 4. Discussion

RA is a chronic illness which is characterized by the patient's immune system attacking the joints, invading the synovial membrane of the joints which results in inflammation. Approximately 1% of the world's population is affected, with women being more susceptible which could be related to genetic or hormonal factors. Cytokines such as TNF and IL-6 promote cell migration and sustain the inflammatory response, thus contributing to RA pathology. Common anti-inflammatory agents such as NSAIDs take away pain, yet long-term use causes more issues and still not successful in arresting the disease progression. Even if biological treatment is effective, their high cost prevents a large part of the population from having easy access in developing countries (Luo et al., 2020). All these highlights the need for therapies targeting underlying mechanisms. Herbal medicines offer a cost-effective, holistic approach, leveraging bioactive plant compounds and traditional knowledge. However, rigorous scientific research is essential to confirm their efficacy for chronic conditions like RA (Kola-Mustapha et al., 2021).

Ginger has long been used to reduce pain and inflammation, with 6-shogaol recognized for its anti-inflammatory, analgesic, and immunomodulatory effects. Our study investigated the anti-arthritic potential of 6-shogaol-rich fractions in a rat model of FCA-induced arthritis. We focused on preparing an enriched 6-shogaol extract to enhance its therapeutic efficacy, using a time-efficient, organic solvent-free extraction method that preserves heat-sensitive compounds. The HPLC method validated the enrichment of 6-shogaol in the fraction compared to the ethanolic extract.

FCA-induced arthritis model in rats is a widely used experimental system that mimics key aspects of human arthritis. Its relevance in replicating pathophysiological and immunological changes observed in clinical arthritis makes it a valuable tool in preclinical research for investigating potential therapeutic interventions for chronic polyarthritis, including rheumatoid arthritis (Patel et al., 2021).

The present study proved that treatment with GOF dose-dependently alleviates adjuvant-induced arthritis and facilitated recovery as demonstrated by a reduced inflammation, hyperalgesia, cytokine levels, which are further supported by histopathology of ankle joints.

Rheumatoid cachexia is reported to be a common complication in individuals with RA. The cytokines TNF-α and IL-1 induced inflammation, increased rate of protein breakdown and disease severity result in body weight loss in the animals. However, the GOF treatment had significantly prevented the loss of body weight which might be due to their ability to suppress these proinflammatory cytokines (Patel et al., 2021).

Neutrophils contribute to the pathogenesis of rheumatoid arthritis. They can enhance cell migration, exhibit pathological inflammatory activity, generate oxidative stress, and release neutrophil extracellular traps. Altogether leads to the inflammatory processes in RA. In the

present study GOF significantly inhibited the paw volume and associated inflammatory signs (Luo et al., 2020, Loh et al., 2022).

Inflammation is associated with hyperalgesia, which refers to an increased sensitivity to pain. (Hans-Georg, 2014, Jalal et al., 2013). Due to this hyperalgesia day to day activities of RA patient progressively gets decreased ultimately affecting the quality of life (McNamee et al., 2011). In the present study, the pain threshold in arthritic control group significantly decreased while GOF treatment increased pain threshold in mechanical hyperalgesia, thermal hyperalgesia and tactile allodynia screening. This confirms the anti-inflammatory and analgesic potential of GOF.

The serum concentration of CRP, a common acute-phase protein indicative of inflammation, was significantly reduced in arthritic animals treated with GOF (Spasovski et al., 2023). This suggests that GOF has a hepatoprotective activity against inflammatory reactions in hepatic tissues during the development of arthritis.

Inflammatory cytokines, such as TNF-α, IL-1β and IL-6, are often elevated and are crucial players in the pathogenesis of rheumatoid arthritis (Adhikary et al., 2016). Targeting these cytokines and their downstream signaling pathways is a common therapeutic approach in managing rheumatoid arthritis (Fikrya, et al., 2019). Treatment with GOF has significantly reduced the serum cytokine levels of arthritic animals.

The FCA challenge in investigational animals results in observable changes in biochemical and hematological parameters (Raj et al., 2024). Specifically, a marked reduction in red blood cells (RBCs) and hemoglobin (Hb) statuses is noted, indicating the presence of anemia.

Anemia is attributed to factors such as low erythropoietin, destruction of erythrocytes, decreased bone marrow activity, and diminished plasma iron level. These conditions are often associated with the influence of inflammatory cytokines such as IL-1.

Proinflammatory cytokines play a key role in attracting and recruiting immune cells, particularly leukocytes, to the inflamed joints. This results in altered heamtological parameters Treatment with GOF dose dependently restored heamtological parameters.

According to recent studies, an imbalance between antioxidant and ROS generation, known as redox stress, is responsible for the destruction of synovium in the context of rheumatoid arthritis, in which RA antioxidant enzymes (such as catalase & glutathione and superoxide dismutase) are unable to cope with reactive oxygen species-mediated lipid peroxidation. The role of inflammation is quite complicated; its dysregulation that is oxidative stress may lead to the pathophysiology of RA. In present study treatment GOF exhibited significant antioxidant activity and hence added potential in treating RA (Luo et al., 2020).

One of the detrimental effects of prolonged inflammation in RA is the degradation of cartilage. Proinflammatory cytokines contribute to this process by promoting the production of enzymes and mediators that lead to the breakdown of joint tissues, including cartilage. Treatment with GOF has protected the joints from destruction as indicated in histopathology compared to joint destruction seen in arthritic joints (Fikrya, et al., 2019).

## 5. Conclusion

This study validated the traditional use of ginger in managing chronic inflammatory conditions, particularly rheumatoid arthritis (RA). The 6-shogaol-rich fraction (GOF) demonstrated significant anti-arthritic properties, highlighting its potential as an effective treatment. The enrichment of 6-shogaol in the formulation likely enhanced its bioavailability, contributing to its superior therapeutic efficacy. The findings underscore the value of ginger-derived compounds in developing alternative treatments for RA, offering a natural and potent adjuvant treatment for managing inflammation and joint damage associated with the disease.

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