

Immunomodulatory and antiarthritic activities of *Smilax zeylanica*

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Abstract

Smilax zeylanica Linn has been traditionally used in the treatment of rheumatoid arthritis but, no scientific data has been published supporting the claimed ethnomedical use. This study was designed to investigate the immunomodulatory and antiarthritic activities of *Smilax zeylanica*.

Methanolic extract of *Smilax zeylanica* (MESZ) roots was tested for its immunomodulatory activity by NBT reduction test. Antiarthritic activity of the same was tested by *in vitro* protein denaturation and *in vivo* complete Freund's adjuvant (CFA) induced arthritis.

MESZ showed its significant effect on both cell mediated and humoral immunity to suppress stimulated immune responses in NBT reduction test. It also markedly inhibited protein denaturation in *in vitro* model. Extract at 200 mg/kg and

400 mg/kg showed statistically significant inhibition ($p < 0.05$) of the edema formation in CFA model. Histopathological studies of ankle joints also supported this finding.

The presence of steroids in the extract might be responsible for the prominent immunomodulatory and antiarthritic activities of the plant. Hence the present study concluded that *Smilax zeylanica* holds immunomodulatory and antiarthritic activities.

Keywords: *Smilax zeylanica* Linn, Steroids, Immunomodulatory activity, Antiarthritic activity.

Introduction

Rheumatoid arthritis is a disease that affects the joints. It causes pain, swelling, and stiffness. This disease often occurs in more than one joint and can affect any joint in the body. Nearly 1% of the world population is affected by rheumatoid arthritis. It is characterized by a series of pathological process of the joints, such as leukocyte infiltration, pannus formation and extensive destruction of the cartilage and bone [1]. Macrophages and neutrophils take part in acute inflammation while T-cells and plasma cells are leading candidates in chronic inflammation. There are many anti-inflammatory drugs (NSAIDs) and anti-rheumatic drugs (DMARDs) that have wide applications in clinical conditions. The main drawback of using these drugs is that they cannot arrest progression of the disease even though they reduce its symptoms. In addition they are supplemented with an array of side effects like Gastrointestinal ulcers, Osteoporosis, Serious infections like sepsis, Tuberculosis, Development of various lymphomas etc [2]. Hence, majority of the patients are seen to shift towards the CAMs such as Ayurveda, Unani, Siddha and Homeopathy. Among these ayurveda is the most widely followed system of medicine in which different parts of the herbs are used. Herbal medicine focus on enhancing biological functions to improve overall health and well-being [3].

Smilax zeylanica, a woody climber found in temperature zones, tropics and subtropics worldwide. Their own, *Smilax zeylanica* Linn plants will grow as shrubs, forming dense impenetrable thickets. They will grow over trees and other plants up to 10 m high.

It has been traditionally used for venereal diseases, diabetes, inflammation and ulcers. *Smilax zeylanica* has been traditionally used in the treatment of rheumatoid arthritis but no scientific data has been published supporting the claimed ethnomedical use. As rheumatoid arthritis is an autoimmune disorder, so it is expected that it must have the potential of immunomodulation. Hence this study is designed to investigate the immunomodulatory and antiarthritic potentials of the methanolic root extract of the *Smilax zeylanica* Linn [4].

Materials and methods

Plant material

The roots of *Smilax zeylanica* were collected from Tirupati forest. This material was identified and authenticated by Dr. K. Madhava chetty, Assistant Professor, Department of Botany, Sri Venkateshwara University, Tirupati (517502), India. The herbarium has been deposited in the department of pharmacology, Gokaraju Rangaraju College of Pharmacy, Hyderabad bearing voucher number 1217.

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The root powder of *Smilax zeylanica* was dried. Then the powder was extracted with methanol by simple distillation technique. The solvent was completely removed under reduced pressure and a semisolid mass was obtained and stored for further study. The extractive value was found to be 2.5% (w/w).

Animals

Wistar albino rats were procured from Gentox Bioservices, Hyderabad. All animal experiments were strictly complied with the ethical standards of animal handling and approved by Institutional Animal Ethics Committee (Reg.No.1175/PO/Ere/S/08/CPCSEA). The animals were housed in poly acrylic cages with not more than six animals per cage, with 12h-light/12h-dark cycle. Rats had free access to standard diet and drinking water *ad libitum*. The rats were allowed to acclimatize to the laboratory environment for a week before the start of the experiment.

Phytochemical screening

The extract was subjected to preliminary phytochemical investigation to identify various phytoconstituents like alkaloids, carbohydrates, flavonoids, glycosides, saponins, sterols, terpenes, tannins.

Acute toxicity testing

Studies were carried out in order to check the toxic effects of extract. The study was performed as per Organization for Economic Cooperation and Development (OECD) guideline no. 425.

In vitro antioxidant assays

Reducing power assay

To 1 mL of test and standard compounds added 2.5 mL of potassium ferricyanide (1 % w/v), 2.5 mL of phosphate buffer pH 6.6 and incubated at 50^o C for 30 min. To 2.5 mL of above supernatant liquid added 2.5 mL of distilled water and 0.5 mL of FeCl₃ solution (0.1% w/v). The absorbance of ferric ferrous complex was measured using phosphate buffer pH 6.6 as control at 700 nm using UV-Visible spectrophotometer and estimated the increase in absorbance [5].

The percent increase in reducing power was calculated using the following equation,

$$\% \text{ increase in reducing power} = \frac{\text{Abs test} - \text{Abs blank}}{\text{Abs test}} \times 100$$

H₂O₂ radical scavenging assay

A solution of hydrogen peroxide (2 mmol/l) was prepared in phosphate buffer (pH 7.4). Test compounds (10–100 µg/mL) were

added to hydrogen peroxide solution (0.6 mL). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide and compared with ascorbic acid, the reference compound [6].

$$\text{Hydrogen peroxide activity (\%)} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

Immunomodulatory activity

Animals were immunized by intraperitoneal injection of 1 mL of 20% SRBCs suspension. The day of immunization was considered as day '0'.

Nitroblue tetrazolium (NBT) reduction test

Experimental design

Animals were divided into different groups each containing 6 animals.

Group I: Rats received normal saline.

Group II: Rats received 1 mL of SRBC *i.p.*

Group III: Rats received 1 mL of SRBC *i.p.* (0th day) + 200 mg/kg bd.wt of MESZ *p.o.* (1st to 5th day).

Group IV: Rats received 1 mL of SRBC *i.p.* (0th day) + 400 mg/kg bd.wt of MESZ *p.o.* (1st to 5th day)

Group V: Rats received 1 mL of SRBC *i.p.* (0th day) + 2 mg/kg bd.wt. of Azathioprine, *i.p.* (1st to 5th day)

Method

On 5th day after challenge, blood was withdrawn from retro-orbital plexus of each animal. Smears of collected blood samples were made on glass slides. They were treated with 0.4 mL of NBT medium and incubated at 37 C for 30 min. Then washed with cold saline water after, the slides are stained with safranin solution. The slides were examined under microscope. The percentage of cells with reduced NBT dye was determined [7].

In vitro antiarthritic models

Inhibition of albumin denaturation method

0.2 mL of fresh hen's egg albumin, 2.8 mL of phosphate buffered saline (pH 6.4) and test solution of different concentrations (2, 5, 10, 20, 30 and 60 µg/mL) or standard drug diclofenac sodium (0.05 ml) of different concentrations (2, 5, 10, 20, 30 and 60 µg/mL) were mixed to form a reaction mixture of 5 mL. Double distilled water of same volume served as control. The samples were incubated at 37±2 °C in a BOD incubator for 15 min followed by heating at 70 °C for 5 min. UV-Visible spectrophotometer was used to measure the absorbance at 660 nm. The percentage inhibition of protein denaturation was calculated by the following formula [8]:

Percentage inhibition = $100 \left[\frac{\text{absorbance of test sample}}{\text{absorbance of control}} - 1 \right]$

In vivo antiarthritic models

Complete Freund's Adjuvant (CFA) induced arthritis

Experimental animal groups: The rats were selected randomly for the study and were subdivided into following groups.

Group I: Rats received saline (*p.o*)

Group II: Rats received 0.1 mL of Complete Freund's adjuvant (*sub plantar* route)

Group III: Rats received 0.1 mL of Complete Freund's adjuvant (*sub plantar* route) + MESZ (200 mg/kg bd. wt (*p.o*), 8-21 days)

Group IV: Rats received 0.1 mL of Complete Freund's adjuvant (*sub plantar* route) + MESZ (400 mg/kg bd. wt (*p.o*), 8-21 days)

Group V: Rats received 0.1 mL of Complete Freund's adjuvant (*sub plantar* route) + diclofenac sodium (15 mg/kg bd. wt (*p.o*), 8-21 days)

Procedure

Rats were injected, subcutaneously, 0.1 mL of complete Freund's adjuvant into the plantar region of the left hind paw. The changes in the paw volume (left and right) were measured on various days up to 21 days. The herbal product at (200, 400 mg/kg/day) and diclofenac sodium at (15 mg/kg/day) doses were administered orally for 14 days after 7 days of Freund's adjuvant administration. On the 21st day rats were anaesthetized with diethyl ether and ankle joints were isolated from the injected hind paw [9].

Histopathological study

The ankle joints were amputated from the rest of the body from each animal. Isolated joints were cleared of extraneous tissue and rinsed in cold physiological saline. Then fixed in 10% neutral

buffered formalin solution. The slides were examined under light microscope to study microscope architecture of the ankle joint [10].

Statistical analysis

All the values were expressed as mean \pm SEM. The significance of the differences of the mean values with respect to control group was analyzed using one-way analysis of variance (ANOVA) followed by Dunnet's 't' test.

Results

Preliminary phytochemical analysis

Phytochemical screening of the methanolic root extract revealed the presence of Glycosides, Saponins, Flavanoids, Phytosterols, Alkaloids, Carbohydrates, Fats, Phenols compounds, amino acids and tannins.

Acute toxicity studies

Administration of MESZ at the dose of 2000 mg/kg resulted in no mortality or evidence of adverse effects implying that *Smilax zeylanica* is nontoxic. This shows that *Smilax zeylanica* was safe up to a dose of 2000 mg/kg.

Antioxidant activity

Reducing power assay

The reducing power of the extract increased with increase in concentration. Similarly the reducing power of reference compound ascorbic acid was also concentration dependent and the potency of test extract was comparable to that of reference compound (Figure 1).



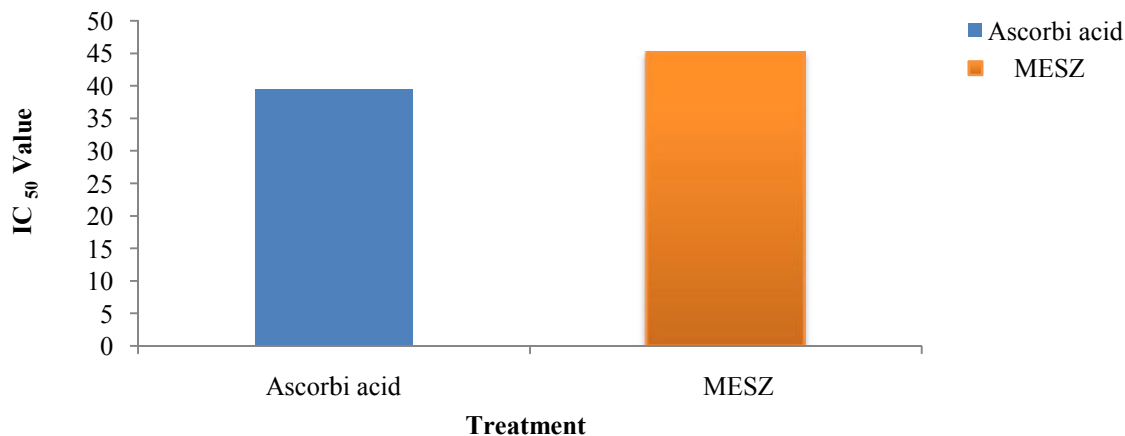


Figure 1: Reducing power assay of methanolic root extract of *Smilax zeylanica*.

The inhibitory concentration-50 (IC₅₀) of ascorbic acid is 39 µg/mL and that of methanolic extract of *Smilax zeylanica* is 45 µg/mL.

Hydrogen peroxide radical scavenging assay

The hydrogen peroxide scavenging activity of MESZ was evaluated and compared with ascorbic acid. The percentage inhibition at

various concentrations of MESZ and ascorbic acid was found to be dose dependent. The IC₅₀ values were calculated from graph and were found to be 22 µg/mL (MESZ) and 12 µg/mL (ascorbic acid) (Figure 2).

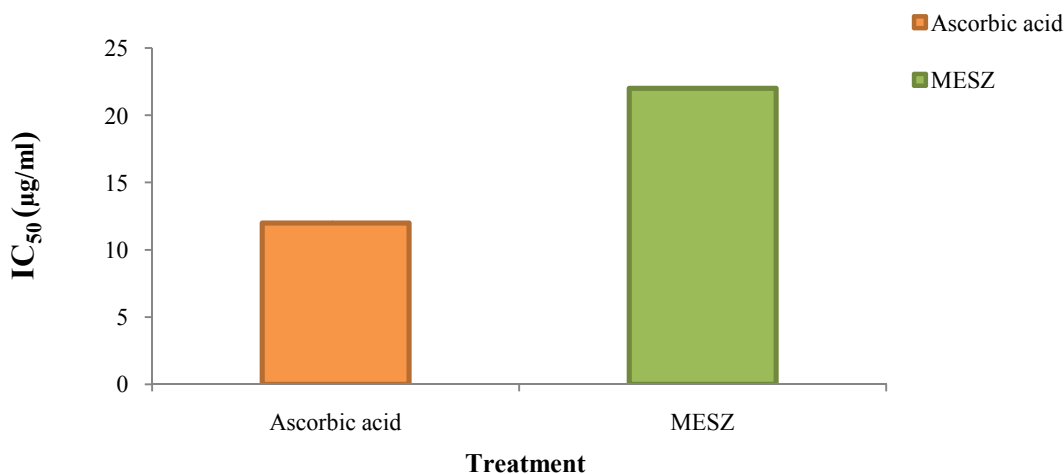


Figure 2: H₂O₂ radical scavenging assay of methanolic root extract of *Smilax zeylanica*.

The inhibitory concentration-50 (IC₅₀) of ascorbic acid is 12 µg/mL and that of methanolic extract of *Smilax zeylanica* is 22 µg/mL.

In vitro immunomodulatory activity

Nitro blue tetrazolium (NBT) reduction test

Methanolic extract of *Smilax zeylanica* has stimulated the neutrophils to phagocytic activity to the extent of 38% and 43% at concentration of 200 mg/kg and 400 mg/kg respectively, when compared to normal control (81%) and to standard i.e., azathioprine (56%) at 2 mg/kg concentration (Table 1).



Table 1: Immunomodulatory activity of methanolic extract of *Smilax zeylanica* by using Nitro blue tetrazolium (NBT) reduction test.

Groups	Treatment	Dose	Total number of positive cells	% of NBT positive cells
I	Normal control	Saline	81±0.9	81%
II	SRBC	1 mL	18±0.6 ^a	18%
III	SRBC+MESZ	1 mL + 200 mg/kg	38±0.7 ^{*,B}	38%
IV	SRBC+MESZ	1 mL + 400 mg/kg	43±0.8 ^{**A}	43%
V	SRBC+Azathioprine	1 mL + 2 mg/kg	56±0.4 ^{**a}	56%

Values were expressed as mean ± SEM (n=6). Statistical analysis was performed by using ANOVA followed by Dunnet's t test by comparing with normal control, disease control and standard. Significant values were expressed as control group (a=p<0.01, b=p<0.05), disease control (**p<0.01, *p<0.05) and standard (A=p<0.01, B=p<0.05).

In vitro antiarthritic activity

Albumin denaturation method

In *in vitro* antiarthritic activity by albumin denaturation method at concentration of 100, 200, 300 and 400 µg/mL showed 65.43, 79.01, 88.88 and 91.35% stabilization of albumin whereas, standard diclofenac sodium at 400 µg/mL showed 93.53% stabilization of albumin (Table 2).

Table 2: Antiarthritic activity of methanolic extract of *Smilax zeylanica* by using albumin denaturation method

S. No	Treatment	Concentration (mg/mL)	Absorbance (660)	% Stabilization
1	Normal control	—	8.1	—
2	MESZ	100	2.8	65.43
3	MESZ	200	1.7	79.01
4	MESZ	300	0.9	88.88
5	MESZ	400	0.7	91.35
6	Diclofenac sodium	400	0.2	97.53

In vivo antiarthritic activity

Complete Freund's adjuvant induced arthritis (CFA)

There was a significant increase in paw volume in CFA injected rats (Group II) when compared to the normal control (Group I). Methanolic extract treatment at the dose of 200 mg/kg and 400 mg/kg showed significant reduction in rat paw edema volume when

compared with the CFA treated and standard groups. After 21 days it was found that methanolic extract significantly showed dose dependent inhibition in paw thickness i.e., the chronic inflammation induced by adjuvant showed decrease in paw thickness. Standard diclofenac sodium significantly decreased the paw thickness after induction of Freund's adjuvant; whereas the extract dose dependently and significantly decreased the paw thickness (Table 3).

Table 3: Antiarthritic activity of methanolic extract of *Smilax zeylanica* by using CFA method.

Groups	Treatment	Dose (mg/kg)	Mean paw volume (mL)			
			0 Day	7 Day	14 Day	21 Day
I	Normal control	Saline	0.09±0.1	0.09±0.02	0.09±0.01	0.09±0.02
II	CFA	0.1mL	0.07±0.04	0.22±0.02 ^a	0.19±0.04 ^a	0.12±0.04 ^a
III	CFA +MESZ	0.1mL+200 mg/kg	0.10±0.02	0.14±0.04 [*]	0.12±0.02 ^{*,B}	0.09±0.01 ^{**B}
IV	CFA +MESZ	0.1mL+400 mg/kg	0.08±0.06	0.15±0.02 ^{*,A}	0.11±0.03 ^{**A}	0.09±0.03 ^{**A}
V	CFA +Diclofenac sodium	0.1mL+15 mg/kg	0.07±0.03	0.13±0.03 ^{**a}	0.11±0.01 ^{**a}	0.08±0.02 ^{**a}

Values were expressed as mean ± SEM (n=6). Statistical analysis was performed by using ANOVA followed by Dunnet's t test by comparing with normal control, disease control and standard. Significant values were expressed as control group (a=p<0.01, b=p<0.05), disease control (**p<0.01, *p<0.05) and standard (A=p<0.01, B=p<0.05).

In the present study, it is clear from the data obtained that there is a close relationship between the extent of joint inflammation and the degree of weight loss. In the first week after adjuvant injection, the

arthritic rats showed marked weight loss, followed by little weight gain in the subsequent weeks, whereas the MESZ at 200 mg/kg

and 400 mg/kg and standard drug treated groups did not show any weight loss instead showed normal weight gain (Table 4).

Table 4: Body weight of rats in CFA induced arthritic model

Groups	Treatment	Dose (mg/kg)	Body weight of rats (in grams)			
			0 th Day	7 th Day	14 th Day	21 st Day
I	Normal control	Saline	171.66±1.12	168.33±0.7	175.39±1.99	177.15±2.13
II	CFA	0.1mL	183.63±2.3	178.73±2.09 ^a	180.13±1.20 ^a	181.23±1.99 ^a
III	CFA +MESZ	0.1mL+200 mg/kg	182.83±3.11	175.42±1.60 [*]	191.39±8.33 ^{*,B}	212.32±9.33 ^{*,B}
IV	CFA +MESZ	0.1mL+400 mg/kg	189.63±4.96	181.34±2.20 ^{**,B}	200.46±8.99 ^{**,A}	214.32±9.33 ^{**,A}
V	CFA +Diclofenac sodium	0.1mL+15 mg/kg	185.92±3.98	178.39±3.33 ^{**,a}	189.23±8.13 ^{**,a}	218.63±13.87 ^{**,a}

Values were expressed as mean ± SEM (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett's t test by comparing with normal control, disease control and standard. Significant values were expressed as control group (a=p<0.01, b=p<0.05), disease control (**p<0.01, *p<0.05) and standard (A=p<0.01, B=p<0.05).

Histopathology of ankle joints

Histopathological studies of the ankle joints showed prominent damage to periosteum and erosion of underlying bone in CFA treated animals (Figure 4). Diclofenac treatment showed normal periosteum with the absence of erosion in bone (Figure 7). MESZ

treated rats produced ankle joint protection compared to arthritic rats by reducing the inflammation and necrosis. Rats treated with MESZ (400 mg/kg) showed mild damage of periosteum and initiation of new bone formation (Figure 6). Dose 200 mg/kg of MESZ showed moderate damage of periosteum and initiation of new bone formation (Figure 5).

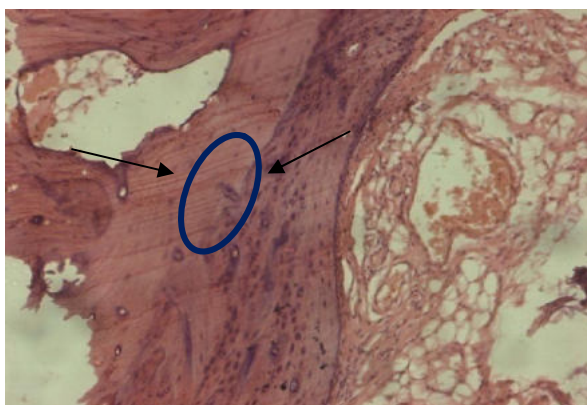


Figure 3: Normal Control

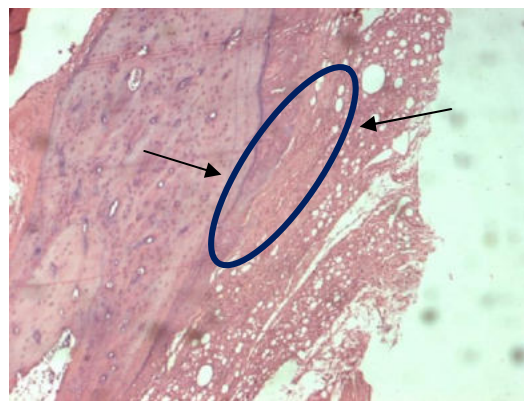


Figure 4: Disease control

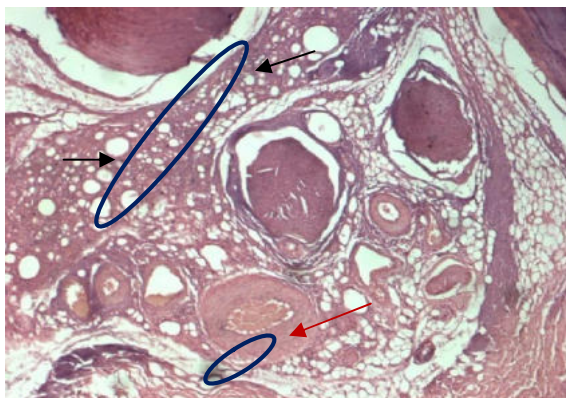


Figure 5: MESZ (200 mg/kg b.w.)

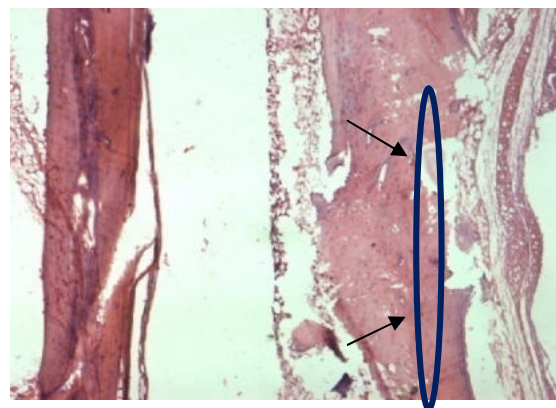


Figure 6: MESZ (400 mg/kg b.w.)



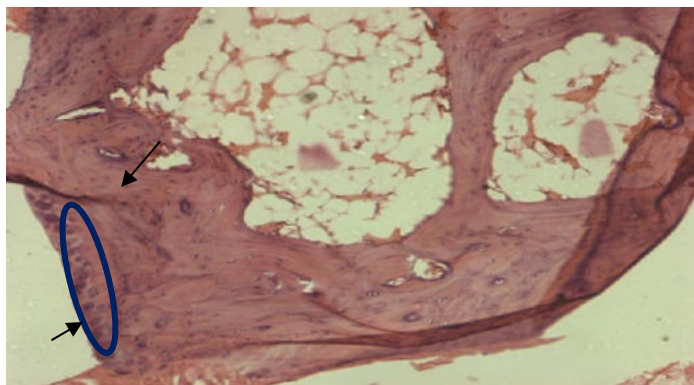


Figure 7: Diclofenac sodium (15 mg/kg b.w.)

Discussion

The immune system is involved in the etiology as well as pathophysiological mechanisms of many diseases. Modulation of the immune functions either by stimulation or suppression may help to maintain a disease-free state. Medicinal plants used in traditional medicines are demonstrated to modulate either cellular or humoral or both arms of the immune responses of the body [11]. Plants contain several chemical constituents, of which some constituents may have immunosuppressive activity, where as others possess immunostimulatory activity [12].

The immunomodulatory activity of the extract was evaluated by NBT reduction test. The NBT reduction test gives the information about the phagocytic and intracellular killing potential of phagocytes [13].

In this test, the NBT dye is readily ingested by phagocytes and consequently reduced by intracellular superoxide anion radicals (O_2^-) to form formazone crystals. The suppression of the intracellular killing potential of phagocytes by MESZ was evidenced by decrease in the intracellular reduction of the NBT dye [14]. The resultant effect of MESZ in intracellular killing activity of phagocytes may be predominantly attributed to the presence of steroidal compound i.e; β -sitosterol.

Rheumatoid arthritis, an autoimmune disease, involves denaturation of proteins and hence production of auto-antigens. *In vitro* antiarthritic activity of the methanolic extract was carried out by the protein denaturation.

Some literature reported that denaturation of protein is one of the cause of rheumatoid arthritis [15]. Production of autoantigens in certain rheumatic diseases may be due to *in vivo* denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding [16]. In our present study, MESZ inhibited heat induced protein denaturation and may be one of the reason of possessing antiarthritic activity.

CFA – induced arthritis is a chronic model used to study the pathogenesis of rheumatoid arthritis for testing therapeutics [17].

One of the reasons for the wide utilization of this model is a strong correlation between the efficacy of therapeutic agents in this model and in rheumatoid arthritis in humans, and this model is characterized by swelling in joints with the influence of inflammatory cells, erosion of joint cartilage and bone destruction and remodeling [18].

Determination of paw edema is according to the grapevine simple, susceptible and rapid procedure to evaluate the degree of inflammation and assess the therapeutic effects of drugs.

CFA induced significant inflammation of the ankle joint of the rats. MESZ at doses 200 and 400 mg/kg b.w. significantly and dose dependently inhibited paw swelling in arthritic rats. The inhibition of inflammation by the extract might be due to inhibition of bacterial peptidoglycan and muramyl dipeptide which are responsible for the swelling, bone and cartilage erosion [19].

Changes in body weight have also been used to assess the course of the disease and the response to therapy using anti-arthritic drugs. Adjuvant arthritis is characterized by reduced body weight, and the weight loss is associated with an increased production of pro-inflammatory cytokines such as TNF- and interleukin-1B [20]. There was significant and dose dependent inhibition of weight loss in the extract treated group and it was comparable to standard diclofenac. This inhibition might be due to prevention of production of cytokine such as TNF- and interleukin-1B by the phytoconstituents present in the extract.

The histopathological studies of the ankle joint in CFA induced arthritic model corroborated antiarthritic potential of the extract.

Standard diclofenac sodium (15 mg/kg b.d.wt.) exerted prominent protection from the damaging effects of CFA evident by mild disintegration of fibrous tissue. MESZ showed significant and dose dependent antiarthritic activity indicated by moderate proliferation of osteoclast/osteoblast and new bone formation.

Although it is difficult to attribute the activity of MESZ to a particular phytochemical present in it, we believe that steroidal compounds (primarily β -sitosterol) present in high concentration in the extract was responsible for the observed immunomodulatory and antiarthritic effects. We base this hypothesis on previously published studies in which β -sitosterol has been evaluated in

inflammation and arthritis [21]. β -sitosterol has been shown to be effective in reducing phlogistic agent induced paw oedema in experimental animals. Additionally, these sterols have also been shown to inhibit the secretion of pro-inflammatory cytokines including TNF- by macrophages which might play an important role in the pathogenesis of RA.

Conclusion

The results obtained in this present study indicates that *Smilax zeylanica* directs towards the control of arthritic progression and/or the inflammation associated with joint synovitis. Hence, orally applicable *Smilax zeylanica* may have great potential as an

alternative to the therapeutic agents currently available for treatment of rheumatoid arthritis.

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Disclosure statement

No competing financial interests exist.

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