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Ethyl-acetate extract of tara mira (*Eruca sativa)* alleviates the inflammation and rheumatoid arthritis in rats

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Abstract: *Eruca sativa*, member of family Brassicaceae, was evaluated for its anti-arthritic potential. Both *in vitro* and *in vivo* models were used to bring out a safe, effective and economical remedy. *In vitro* tests included egg albumin denaturation suppression, bovine serum albumin assay and human red blood cells maintenance assay. While *in vivo* formaldehyde-induced arthritic model was initiated to check effect on paw volume. Similarly, carrageenan produced inflammation was applied to check anti-inflammatory ability of the plant. Acute toxicity studies showed safety margin at 2000mg/kg. The plant showed concentration dependent denaturation protection and membrane stability *in vitro* assays. Likewise, the carrageenan and formaldehyde investigations revealed visible paw volume reduction in dose attributed manner, with maximum outcome at dose of 500mg/kg. Hence, it may be established on the ground of presented results that ethyl-acetate extract of *Eruca sativa* has significant anti-inflammatory and anti-arthritic effects and may be considered for further research to reveal the core mechanism.

Keywords: Arthritis, anti-inflammatory, formaldehyde, carrageenan.

INTRODUCTION

Rheumatoid arthritis, a complex chronic progressive disease whose malady is deeply rooted in the inflammatory response the body makes against itself. Not only affecting joints, it more like a syndrome, involves other body organs (Karami *et al.,* 2019). The pathogenesis is even now ambiguously defined. It is known that ignition of immunogenic insult arise into influx of inflammatory cells, release of cytokines, altered expression of surface adhesion molecules, increased angiogenesis, synovial hyperplasia leading to infiltration of T-cells, B-cells, macrophages and plasma cells (Alapati *et al.,* 2018). The treatment available like DMARDs, glucocorticoids and NSAIDs, no doubt, delays the progression of the disease. However these drugs have devastating side effects like immunosuppression, poor tolerability, gastric ulcers, cardiovascular risks and disease remission (Burmester and Pope, 2017). These drawbacks create room for the search of safer medicines. In the process of discovering new drugs and lead compounds, scientific validation of plants with folkloric medicinal claim has been an inevitable source. Several synthetic drugs available so far have a long history of being used as herbal medicine including aspirin, vincristine, atropine and quinine etc. (Akinyemi *et al*., 2018). Furthermore, in western countries herbal remedies are being considered as alternative means of treatment for uncontrollable diseases. In eastern countries, China, India, Pakistan, Sri Lanka using herbal treatment is a common healthcare practice (Patil *et al*., 2019). In Pakistan, 371 plants are being used as herbal treatment of inflammatory ailments. Many plants still needed to be explored, due to lack of systematic scientific investigation (Alamgeer *et al.,* 2018). The present study was carried out to provide the scientific data that will assist the ethno-medicinal claim of a plant from Pakistan. *Eruca sativa,* family Brassicaceae, locally called Taara Mira in Urdu and rocket in English. It is edible annual herbaceous plant with peppery flavor. Tradionally, it is used as diuretic, astringent, emollient, tonic, laxative, cure bleeding piles and relieve ear pain. Previous scientific work shows that it is antidiabetic, anti-ulcer, anti-cancer, antioxidant, anti-psoriasis and renal as well as hepatoprotective effect (Abbasi et al., 2016; Ahmed *et al.,* 2013; Michael *et al.,* 2011; Yehuda *et al*., 2009). However, there is no work done on its anti-arthritic potential. In this study, we evaluated the anti-inflammatory, anti-arthritic effect of *Eruca sativa* in order to search for a safer anti-arthritic remedy.

MATERIALS AND METHODS

Experimental animals

Healthy Sprague Dawley rats of either sex (130-200g) were placed in the animal house of College of Pharmacy, University of Sargodha. Rats were kept at controlled temperature of 25±2°C under 12hour light/ 12 hour dark

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cycle, given standard pellet diet and water *ad libitium*. They were treated according the standard protocol and the procedures were approved from animal ethical committee of College of Pharmacy, University of Sargodha (Approval No.32A51 CEC UOS).

Plant collection and extraction

Whole plant of *Eruca sativa* (ES) was collected from Khushab district and was further identified by the taxonomist of college of pharmacy, university of Sargodha (voucher No. ES-20-10). The plant was washed, dried in shade and pulverized into fine powder. The extract of powdered *Eruca sativa* was made by sequential cold maceration. The 500g powder was dissolved in 3.5L ethyl-acetate, frequently shaken and kept as such for 3 days. The marc was separated from menstruum and again soaked in ethyl-acetate. The procedure was repeated thrice. The percentage yield of greenish black paste *Eruca sativa* ethyl-acetate (ESE) extract was 6.544%.

Pharmacological investigations

Acute toxicity studies

The acute toxicity study of ESE was conducted in Sprague-Dawley female rats (180- 200g) according to the OEDC guidelines 423. The animals, divided in separate groups of three in each, were given doses of 5mg/kg, 50mg/kg, 300mg/kg and 2000mg/kg. The animals were keenly observed for 30min for signs of toxicity. Additional observations were made within 24 hours. The rats were observed for 7 days to record any mortality. The monitoring was continued till day 14.

Anti-inflammatory activity

The animals (130-180g) of either sex were kept fasted overnight. Animals were divided into groups of five with n=6. Group 1, served negative control in which injection of 0.1ml of 1% w/v carrageenan suspended in normal saline was injected into the sub-planter region of right hind paw, but no treatment was given other than vehicle tween 80 orally. Group 2, standard treatment was given by administering naproxen 20mg/kg orally. Group 3, 4 & 5 were given three different doses of ESE orally i.e. 125, 250 and 500mg/kg respectively, suspended in tween 80; followed by injection of carrageenan. The paw volume was checked to measure inflammation using plethysmometer at 0, 1, 3 and 5 hours. Percentage of inhibition was resolved using formula (Alamgeer *et al*., 2017).

$$\text{Percent inhibition} = \text{Vc} - \frac{\text{Vt}}{\text{Vc}} \times 100$$

Here, Vc = average paw volume (control) and Vt = average paw volume (treated)

In vitro anti-arthritic investigations

Suppression of protein denaturation using bovine serum albumin assay

Bovine serum albumin (BSA) solution 0.5% and phosphate buffer (pH 6.5) were prepared. Control contained BSA solution and distilled water. Standard consisted of naproxen and test solutions had various concentrations of ESE (50, 100, 200, 400, 800, 1600, 3200, 6400µg/mL). The solutions were incubated at 37°C for 20 min then heated at 57°C for 30 min. On cooling; phosphate buffer was poured. Using UV spectrophotometer, absorbance was recorded at 660nm. The percentage inhibition of protein denaturation was calculated using formula (Alamgeer *et al.,* 2017)

$$\% \text{ age inhibition} = 100 \frac{\text{Ts Absorbance} - \text{PC Absorbance}}{\text{Test control Absorbance}} \times 100$$

Suppression of protein denaturation using egg albumin assay

Test sample solutions were prepared by adding 0.2ml egg albumin, 2.8ml phosphate buffer and 2ml of several concentrations of ESE. Similarly, standard naproxen solutions of same concentrations were prepared. Test control solution had double distilled water along with buffer and egg albumin. The solutions were incubated at 37°C for 15 minutes then placed in oven at 70°C for 5minutes. On cooling, absorbance was recorded at 660nm using UV-spectrophotometer (Uttra and Alamgeer, 2017). The percentage inhibition of protein denaturation will be noted using formula as under (Alamgeer *et al*., 2017).

$$\%\text{inhibition} = 100 \times \left(\frac{\text{Control absorbance} - \text{Test sample absorbance}}{\text{Control absorbance}} \right)$$

Human red blood cell (HRBC) membrane stabilization assay

The study was performed as according to our previously published article. The test and standard solutions contained 1mL phosphate buffer, 2mL hypotonic solution, 0.5mL 10% w/v RBCs suspension and 0.5mL ESE and naproxen in different concentrations (50, 100, 200, 400, 800, 1600, 3200, 6400µg/mL) respectively. Test control solution only had 0.5mL HRBC suspension and 2mL distilled water. All solutions were incubated for 30minutes at 37°C, centrifuged at 3000rpm. Hemoglobin content was measured by UV- spectrophotometer at 560nm. Percentage protection in opposition to hemolysis was calculated as following (Alamgeer *et al.,* 2017)

$$\text{percent protection} = 100 - \left\{ \left(\text{Sample absorbance} \Big/ \text{Control absorbance} \right) \times 100 \right\}$$

In vivo formaldehyde-induced arthritis

Animals were divided into five groups n=5. Group 1, diseased group received the vehicle, tween 80 only. Group 2, standard group was given naproxen orally. Group 3, 4 and 5 were orally administered with 125, 250 and 500mg/kg ESE extract suspended in tween 80 respectively.

On day first, 0.1mL 2% formaldehyde injection was administered into sub-planter area of right rat hind paw, 30 minute later to oral drug administration. The injection was repeated on day 3 of ten day treatment plan. Arthritis was assessed by measuring the paw volume through digital plethysmometer (Alamgeer *et al.,* 2017). Percentage inhibition of edema was equated as (Uttra and Alamgeer, 2017).

$$\text{percent inhibition} = \frac{(Vc - Vt)}{Vc} \times 100$$

Here, Vc = Paw volume of control and Vt = Paw volume of treated.

STATISTICAL ANALYSIS

The results obtained were presented as mean ± standard error mean (S.E.M.). Statistical analysis was accomplished by using two-way analysis of variance (ANOVA) test, followed by Bonferroni posttest, using software GraphPad Prism 5. The values were considered significant at $p<0.05$.

RESULTS

Acute toxicity study

Single oral administration of ESE did not produce any signs of toxicity even at day 14. Therefore it was assumed that the LD50 for extract was more than 2000g/kg.

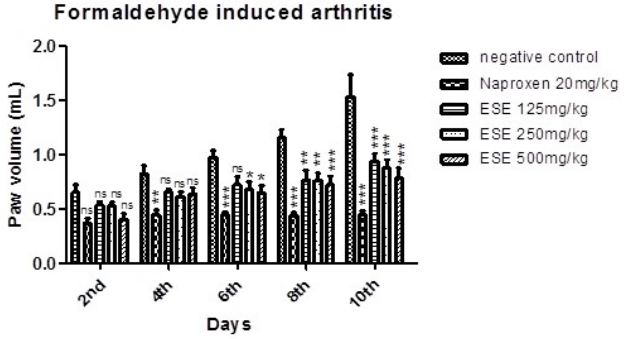

Fig. 1: effect of different doses of ESE on the paw volume of formaldehyde treated rat hind paw. Values are expressed as mean ±SEM n=5. ***= p<0.001, **= p <0.01,*= p<0.05, ns= non-significant compared with arthritic control using two-way ANOVA accompanied by Bonferroni multiple comparison test.

Anti-inflammatory effect

The results in the table 1 revealed anti-inflammatory effect of ethyl acetate extract of *Eruca sativa* (ESE) by countering the rise in paw volume induced by carrageenan. The effect was a dose-dependent one, in which dose of 500mg/kg produced inhibition in rise of paw volume, by 38.851% at 5th hour, when compared with negative control. Standard NSAID naproxen produced better effect on increase of paw volume, 66.488% yet the response by ESE 500mg/kg is significant ($p< 0.001$) enough to consider for further investigation.

Repressive effect of ESE on heat stimulated bovine serum albumin denaturation

Thermally produced protein denaturation in bovine serum was greatly restricted by various concentrations of ESE as shown in fig. 2(a). Maximum percentage protection of 54.032% was evident at concentration 6400µg/mL, which was statistically significant ($p<0.001$) when compared to naproxen. Hence, ESE effectively displayed its protein denaturation repression effect, an *in-vitro* clue of anti-arthritic potential of *Eruca sativa*.

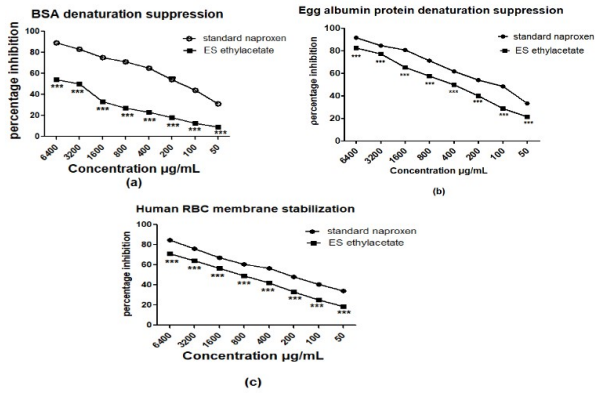

Fig. 2: effect of ES ethyl-acetate on *in vitro* anti-arthritic investigational models. (a) Bovine serum albumin denaturation assay (b) egg albumin denaturation assay (c) HRBC membrane maintenance assay. Values are expressed as mean ± SEM, n=3, * p<0.05, ** p<0.01, *** p<0.001, compared with standard drug naproxen by two-way ANOVA, followed by Bonferroni multiple comparison test.

Repressive effect of ESE on heat stimulated egg albumin denaturation

Protein denaturation tempted in egg albumin on heat provision was suppressed by ESE as shown in fig. 2(b). Both ESE and naproxen presented concentration-dependent protein denaturation inhibition. At 6400µ/mL, ESE extract showed percentage inhibition of 82.456%, which is highly significantly ($p<0.001$). Naproxen percent inhibition was 91.695% at the same concentration.

RBCs membrane protective influence of ESE extract

The membrane bursting phenomenon incited by hypotonicity was greatly prohibited by ESE in a concentration gradient fashion from 50µg/mL to 6400 µg/mL (fig. 2(c)). The statistically significant ($p<0.001$) peak effect observed was 70.836% at 6400 µg/mL. Therefore, it again exhibited the in-vitro anti-arthritic potential through erythrocyte membrane stabilization effect, up to a reasonable extent.

Influence of ESE on formaldehyde provoked arthritis

In vivo arthritic model using formaldehyde revealed the significant potential of *Eruca sativa*. The paw volume increased after formaldehyde injection in arthritic control group. The treatment with ESE extract demonstrated a

Table 1: Anti-inflammatory effect of ESE on carrageenan induced inflammation in right hind-paw of rat

Treatment	Paw volume (mL) at indicated time			
	0 hour	1 hour	3 hour	5 hour
Negative control	0.606 ±0.062	1.054±0.067	1.756±0.151	2.104±0.123
Naproxen (20mg/kg)	0.538±0.041	0.920±0.062^{ns} (28.125%)	1.060±0.083*** (50.434%)	1.156±0.103*** (66.488%)
ESE (125mg/kg)	0.558±0.063	0.976±0.048^{ns} (6.696%)	1.558±0.121^{ns} (13.043%)	1.674±0.081** (23.230%)
ESE (250mg/kg)	0.554±0.039	0.936±0.077^{ns} (9.821%)	1.478±0.125^{ns} (19.652%)	1.656±0.086** (26.435%)
ESE (500mg/kg)	0.534±0.049	0.930±0.044^{ns} (11.607%)	1.478±0.089* (24.521%)	1.450±0.121*** (38.851%)

Data express as mean ±SEM, n=6. Parenthesis shows percentage inhibition from edema. ***= p<0.001, compared with negative control.

Table 2: Effect of ESE on percentage inhibition of paw edema on formaldehyde treated rat hind paw

Treatment groups	Percentage inhibition of paw edema				
	2nd Day	4th Day	6th Day	8th Day	10th Day
Naproxen (20mg/kg)	43.425%	45.893%	54.303%	62.110%	70.741%
ESE (125mg/kg)	17.431%	20.772%	25.614%	33.391%	38.621%
ESE (250mg/kg)	19.571%	22.463%	29.918%	33.910%	43.042%
ESE (500mg/kg)	20.489%	25.603%	33.196%	37.370%	49.024%

significantly reduced paw volume in a dose graded manner, when compared to the arthritic group. Percentage protection imparted at dose 125mg/kg was 38.621% (p<0.001) and 43.042% (p<0.001) at dose 250mg/kg. The maximum percentage inhibition in paw edema observed was 49.024% (p<0.001) at dose of 500mg/kg on 10th day.

DISCUSSION

The consequences obtained from this study evidently display the anti-inflammatory and anti-arthritic potential of *Eruca sativa* in a dose dependent manner, by decreasing the paw edema in *in-vivo* models and repressing the protein denaturation and membrane stabilization in *in-vitro* models. Carrageenan produced paw edema model for acute inflammation, producing response in two phases. Early phase is nearly 1 hour post-injection, results in liberation of bradykinin, histamine and cyclooxygenases induced prostaglandins release. After first hour, delayed phase is accompanying to free radical production, neutrophil infiltration and prostaglandins as well as pro-inflammatory cytokines (TNF-α, IL-1β) release, ending up to edema formation (Makni *et al.,* 2018). In current investigation, ESE produced noticeable anti-inflammatory action in both phases possibly by repressing the synthesis and release of inflammatory mediators, in a dose dependent way. The elicitation of inflammatory response and arthritic ailments is believed to be the outcome of generation of auto-antigens due to protein denaturation. Therefore, remedies that inhibit protein alteration are assumed to be potential candidates for arthritis treatment (Qasim *et al.*, 2020). The present examination revealed that ESE significantly inhibited *in-vitro* protein denaturation in both bovine serum albumin and egg albumin heat-induced denaturation assays, marking its capability to deal with arthritis. Likewise, the extract showed *in-vivo* anti-arthritic effect in formaldehyde produced arthritis by suggestively reducing paw edema during a period of 10 days. It is noteworthy that the family of Brassicaceae has been designated as rich source of antioxidant phytochemicals (Rizwana *et al.,* 2016). Previous research on *Eruca sativa*, member of same family, describes the occurrence of flavonoids (quercetin, erucin, kaempferol and isorhamnetin) in ethyl-acetate extract. Free sinapic acid can be found in ethyl-acetate extract of aerial portion of plant. Additionally, among sterols β-sitosterol, campesterol and brassicasterol are the most abundant (Salma *et al.,* 2018; Khoobchandani *et al.*, 2011).

Quercetin, previously, has been demonstrated to inhibit NF-κB pathway, decrease nitric oxide synthase expression, reduce expression of cyclooxygenase and maintain the mast cell stability thus producing anti-inflammatory response. It can bring down levels of pro-inflammatory cytokines like TNFα, IL-6, IL-1β, IL-17 and interferon-γ (Shen *et al.,* 2021). Kaempferol has been proposed to be anti-inflammatory by directly declining neutrophil infiltration together with IL-1β, IL-18, IL-6, TNF-α and nitric oxide while indirectly NF-κB, COX-2 and reactive oxidative species. Besides, it regulates the MAPK (JNK and p38) pathways, along with inducing apoptosis of synovial fibroblasts and downregulation of matrix metalloproteinases (MMPs) and PGE-2. Moreover,

it down-regulate toll-like receptor4 (TLR4); stamps down NF-κB p65 DNA-binding process (Imran *et al.,* 2018). Furthermore, Pan *et al.,* has found that MAPK pathway is suppressed by kaempferol along with inhibition of reorganization of actin and cytoskeleton, suggesting its powerful therapeutic role in alleviating rheumatoid arthritis. Sinapic acid has been explored as anti-arthritic in rat chondrocytes, inhibiting IL-1β stimulated nitric oxide (NO), prostaglandin E2, COX-2 and MAPK. Also it downregulates the expression of MMP1, MMP3, MMP13 and ADAMTS5 (Huang *et al.,* 2017). Additionally, an antioxidant, β-sitosterol has been demonstrated as anti-inflammatory and anti-arthritic potential, probably by affecting STAT1 and NF-KB pathways (Rashed, 2020; Valerio & Awad, 2011).

Hence, the scientific evidence of the existing phytoconstituents of *Eruca sativa* ethyl-acetate extract strengthens the role of plant in exterminating arthritis.

CONCLUSION

Conclusively, it has been determined that the ethyl-acetate extract of *Eruca sativa* possesses anti-arthritic activity. Advance studies on the possible underlying mechanisms, responsible for this outcome are undergoing.

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