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ANTI-ARTHRITIC ACTIVITY OF HYDRO-ALCOHOLIC EXTRACT OF LEAVES OF *ZIZIPHUS MAURITIANA*

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ABSTRACT

To investigate anti-arthritic activity of hydroalcoholic extract of *Ziziphus mauritiana* (HAZM) in Freund's complete adjuvant (FCA)-induced arthritis in rats. The leaves the plant was collected, dried and extracted (maceration) with 70% methanol. Qualitative analysis of various phytochemical constituents and quantitative analysis of total phenolics and flavonoids were determined by the well-known test protocol available in the literature. Quantitative analysis of phenolic and flavonoids was carried out by Folins Ciocalteau reagent method and aluminium chloride method respectively. The HAZM was prepared and subjected to acute oral toxicity in mice and tested against FCA induced arthritis in rats. Arthritis assessment was done by measuring – paw volume with standard reference drug diclofenac sodium was administered at the dose of 200 and 400 mg/kg body weight. HAZM dose dependently showed anti-arthritic activity which was evident with decrease in paw volume when compared to arthritic control group. Phytochemical analysis revealed the presence of phenols, flavonoids, tannins, saponins, alkaloids, fixed oil and fats. The total phenolics content of leaves hydroalcoholic extract was (0.658 mg/100mg), followed by flavonoids (0.514mg/100mg). The results reveal promising anti arthritic potential of the plant. However further pharmacological investigation using isolated active ingredients can be carried out to confirm its efficacy and mechanism of action.

Key words: Anti-arthritic activity, *Ziziphus mauritiana*, Qualitative and quantitative analysis, Diclofenac sodium.

INTRODUCTION:

Arthritis and related disorders, including rheumatoid arthritis (RA), are common diseases affecting millions of people (Zhang et al., 2009). RA is characterized by articular injuries having an inflammatory propagation of synovial cells, attaining a nearly complete functional defect. It affects about 1% of the general population (Tastekin et al., 2007). RA is a kind of chronic inflammatory autoimmune disease. Although a number of drugs used in the treatment of RA have been developed over the past few decades, there is still a need for more effective drugs with lower side effects (Liu et al., 2005). Conventional medicine, including treatment with steroids, nonsteroidal anti-inflammatory drugs(NSAIDs) and such biological agents as tumour necrosis factor alpha(TNF-α) and interleukin-1 beta (IL-1β) antagonists, has shown only limited success against RA. Such therapies are helpful in controlling the symptoms of acute RA, but their effects on chronic, prolonged RA are unsatisfactory. Moreover, the adverse effects

of drug therapy are significant and include gastrointestinal disturbances, infections and cardiovascular risks (Fan et al., 2005). Freund's complete adjuvant (FCA)-induced arthritis in rats has been employed widely as a model for chronic systemic inflammation and possesses many features in common with human rheumatoid arthritis (Geetha et al., 1999). FCA-induced arthritis is of considerable relevance for the study of pathophysiological and pharmacological control of inflammatory processes, as well as the evaluation of anti-arthritic effects of drugs (Andersen et al., 2004). The FCA-induced arthritis follows a biphasic time course, consisting of an acute local inflammatory reaction that subsides after 3–4 days and a chronic systemic reaction that shows a relapsing-remitting course after the initial two weeks and can persist for several months (Neugebauer et al., 2007). It is not known why this biphasic pattern of activity is seen but it may be due to an initial stimulus caused by the injection of FCA followed by the delayed hypersensitivity response known to be induced by FCA (Chillingworth et al., 2003). *Ziziphus mauritiana* Lam. is a low branched deciduous tree with spreading crown, dark greenish black bark having irregular crack and strong reddish hardwood with oblong and elliptic leaves. It is known as Ber (Hindi), Indian jujube (English) and Badarh (Sanskrit). It is found throughout India, in dry deciduous forests up to 1500 m. In traditional medicine of Ayurveda, unripe fruits are used to pacify "*Vata*", the leaves, fruits, bark & even roots are used to treat a variety of ailments including cold, flu and malnutrition related diseases in children, convulsions and indigestion (Mazumder et al., 2004). The leaves are applied as poultices and are helpful in liver troubles, asthma, fever and to treat sores (Michel et al., 2002) and the roots are used to cure and prevent skin diseases (Adzu et al., 2001). All the parts of this plant are very effective against different types of diseases. Its leaves are useful in the treatment of diarrhoea, wounds, abscesses, swelling and gonorrhoea. The leaves of *Z. mauritiana* are also used in the treatment of liver diseases, asthma and fever (Morton et al., 1987). The fruit has been used as anodyne, sedative, tonic anticancer and potent wound healer (Calabura and Verheij, 1991). The fruit (Ndhala et al., 2006), leaves (Dahiru and Obidoa, 2007) and seed (Bhatia and Mishra, 2009) extracts have been shown to exhibit antioxidant activity, whereas bark (Pisha et al., 1995) is reported to have cytotoxicity against different cancer cell lines. Therefore, the objective of the present study was to determine the efficacy of *Z. mauritiana* (leaves) in Freund's complete adjuvant (FCA)-induced arthritis in rats.

Materials and Methods

Collection of plant material

Fresh plant leaves of *Ziziphus mauritiana* were collected from collected from local area of Bhopal (M.P.). The leaves were washed thoroughly with normal tap water followed by sterile distill water. Then leaves were dried under shaded condition at room temperature. Leaves were crushed to powder using grinding machine. Powder was stored at 4^{o}C in tight air container bottle.

Drugs and chemicals

Complete Freund's adjuvant (CFA) was procured from Sigma Aldrich chemicals Pvt. Ltd, Hyderabad, India. Diclofenac Na was obtained from Akums Drugs and Pharmaceuticals, India. Petroleum ether, Chloroform obtained from SD fine chemicals Pvt. Ltd, Mumbai, India and all other chemicals used in this study were obtained commercially and were of analytical grade.

Preparation of extracts

50 gm powdered sample was weighed and taken separately. The powder was moistening with ammonia and evaporated to dryness (Abdallah et al., 2016; Abdallah et al., 2017). The maceration method was followed for the extraction. 50 gm powder of dried leaves was added into 100 ml of 70 % methanol in an Erlenmeyer flask (250 ml capacity) and resulting mixture was vortexed well. The maceration process was carried out in shaker

incubator at with 50 rpm for 48-72 hrs. After this process, the extracts were filtered Dried extract was stored in refrigerator for their future use in phytochemical analysis.

Phytochemical screening of the extract

The leaves extract of *Z. mauritiana* was subjected to qualitative analysis for the various phytoconstituents like alkaloids, carbohydrates, glycosides, phytosterols, saponins, tannins, proteins, amino acids and flavonoids (Khandelwal, 2005; Kokate, 1994).

Total phenol determination

The total phenolic content was determined using the method of Olufunmiso *et al* [29]. A volume of 2ml of each extracts or standard was mixed with 1 ml of Folin Ciocalteau reagent (previously diluted with distilled water 1:10 v/v) and 1 ml (7.5g/l) of sodium carbonate. The mixture was allowed to stand for 15 min under room temperature. The colour developed was read at 765 nm using UV/visible spectrophotometer. The total phenolic content was calculated from the standard graph of gallic acid and the results were expressed as gallic acid equivalent (mg/100mg).

Total flavonoids determination

The total flavonoid content was determined using the method of Olufunmiso *et al* . 1 ml of 2% $AlCl_3$ methanolic solution was added to 3 ml of extract or standard and allowed to stand for 15 min at room temperature; the absorbance of the reaction mixture was measured at 420 nm using UV/visible spectrophotometer The content of flavonoids was calculated using standard graph of quercetin and the results were expressed as quercetin equivalent (mg/100mg).

Animals

All the experiments were carried out using Swiss albino male rats weighing between 180-250 gm. All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Ethical Committee No. (IAEC No. COPSSUTMS/ANIL/19-10). All animals were housed in polypropylene cages and maintained under standard laboratory conditions. Animals were housed at a temperature of 24±2°C and relative humidity of 65-75% RH. They were fed with a standard diet and water was given *ad libitum* and they were left for a week for acclimatization to animal house conditions. All experiments were conducted after overnight fasting but there was free access to water.

Acute oral toxicity

Acute oral toxicity was performed according to Organization for Economic Co-operation and Development (OECD) guideline No. 420. Swiss albino rat's fasted overnight, accessing water *ad libitum* were used in this study. The extract was administered orally at a dose of 2000 mg/kg body weight and the animals were observed for mortality or any abnormal behavior for first 24 h, then for next 14 days. Further behavioral responses, neurological responses as well as autonomic responses were observed.

The male albino rats were divided into five groups, *i.e.* control, standard, drug treated (two groups of hydro-alcoholic extract of *Z. mauritiana* leaves (low and high dose).

Experimental protocol (Srivastava et al., 2012)

- **Group I (Normal control group):** Rats received 1% CMC (1 ml/kg body weight) only daily for 14 days
- **Group II (Negative control group):** Received 1% CMC (1 ml/kg body weight) +FCA (0.1 ml) daily for 14 days
- **Group III (Standard group):** Diclofenac sodium 15 mg/kg suspended in CMC+FCA (0.1 ml) daily for 14 days
- **Group IV (Treatment control group):** hydro-alcoholic extract of *Z. mauritiana* at dose of 200 mg/kg orally suspended in CMC +FCA (0.1 ml) daily for 14 days
- **Group V (Treatment control group):** hydro-alcoholic extract of *Z. mauritiana* at dose of 400 mg/kg orally suspended in CMC +FCA (0.1 ml) daily for 14 days

Arthritis was induced by injecting 0.1 ml of Freund's complete adjuvant (FCA) into the planter region of the left hind paw. The paw

volumes of both the hind paws were measured using a plethysmometer and body weight was recorded on the day of adjuvant injection. The hydro-alcoholic extract of *Z. mauritiana* (200 and 400 mg/kg) and diclofenac sodium (15 mg/kg) doses were administered orally for 14 days from the day of Freund's adjuvant injection. The changes in the paw volume were measured on various days up to 21 days following FCA injection. The change in the inflammatory reaction was measured by using mercury plethysmometer on 1st, 7th, 14th and 21st day from the day of adjuvant injection. The animals were weighed, using digital weighing balance on 1st, 7th, 14th and 21st day from the day of adjuvant injection.

Percentage inhibition of edema was calculated

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

Where, Vc= Joint edema of negative control group; Vt= Joint edema of treatment group;

Data Analysis

The data is expressed as mean ± Standard Deviation (SD). Results were analyzed using one-way ANOVA followed by Dunnet's test. Differences were considered as statistically significant at $P < 0.05$, when compared with control.

RESULT

The percentage yields of hydroalcoholic leaves extract obtained from *Z. mauritiana* are depicted in the table 1. Preliminary phytochemical studies of the extract were done according to the published standard methods. These tests were broad in scope and used to determine the presence of flavonoids, glycosides, Phenols and saponins. Result was shown in Table 2.

Table 1 % Yield of hydroalcoholic extract

S. No.	Plant material	% Yield (w/w)
1	Leaves of *Ziziphus mauritiana*	8.3%

Table 2 Results of phytochemical screening of leaves of *Ziziphus mauritiana*

S. No.	Phytochemicals	Tests	Observation	Inference
1.	Alkaloids	Iodine Test	No Blue colour	-
		Wagner's Test	No reddish brown precipitate	-
		Dragendorff's Tests	No orange brown precipitate	-
2.	Flavonoids	NaOH Tests	Colourless	+
		Shinoda Tests	Magenta colour	+
3.	Glycosides	Keller-Kiliani Test	Bluish green colour	+
4.	Phenols	Phenol Tests	Blue colour	+
5.	Saponins	Foam Test	Layer of foam	+
6.	Tannins	Gelatin Test	No white precipitate	-
7.	Carbohydrates	Molisch's test	No violet colour	-
		Fehling's test	Reddish orange precipitate	+
8.	Proteins	Millon's test	No white precipitate	-

Among the secondary metabolites that were quantified, the total phenolic content was the highest with (0.658) GE/g of the extract followed by the total flavonoid content with (0.514) QE/g of the extract. The results are tabulated in table 3.

Table 3 Total phenolic and total flavonoid content

S. No.	Solvents→ Bioactive compound↓	Hydroalcoholic extract
		Leaves of *Ziziphus mauritiana*
1.	Total Phenol (Gallic acid equivalent (GAE) mg/100mg)	0.658
2.	Total flavonoid (Quercetin equivalent (QE) mg/100mg)	0.514

No adverse changes and mortality were observed in animals, which orally received hydoralcoholic extract (2000 mg/kg) of *Z. mauritiana*. This indicates that 2000 mg/kg is maximum safe dose. So $1/10^{th}$ and $1/5^{th}$ *i.e.* 200 and 400 mg/kg of body weight, of the maximum safe dose were selected for studying *in vivo* anti-arthritic activity. There is a significant increase in rat paw volume in FCA injected control rats when compared to the standard and drug treated rats. Hydoralcoholic 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 control group. Table 4 shows the effect of extract on Freund′s adjuvant model induced arthritis. After 21 days it was found that hydor-alcoholic extract significantly shows dose dependant inhibition in paw thickness *i.e.* the chronic inflammation induced by adjuvant shows decrease in paw thickness. Standard diclofenac sodium significantly decreased the paw thickness 7.30 ± 0.04 mm i.e. 69.23% inhibition after induction of Freund′s adjuvant; whereas the extract at high dose (400 mg/kg) significantly decreased the paw thickness. It was found that in case of the high dose of the methanolic extract, the percent protection against increase in paw volume was found to be 73.64 % as compared to that of the low dose which was found to be 50.12%.

Table 4 Effect of hydroalcoholic extract *Z. mauritiana* leaves on CFA induced arthritis in rats

Group	Dose of extract (mg/kg, p.o.)	Change in paw thickness (mm)±SD (% inhibition)			
		1 Day	7 Day	14 Day	21 Day
Group I (Normal control)	1% CMC (1 ml/kg body weight)	4.19 ± 0.16***	4.24 ± 0.16***	4.24 ± 0.09***	4.27 ± 0.08***
Group II (Negative control)	1% CMC (1 ml/kg body weight) +FCA (0.1 ml)	5.21 ± 0.04	14.22 ± 0.03	19.50 ± 0.02	22.72 ± 0.04
Group III (Standard control)	Diclofenac sodium (15 mg/kg) +FCA (0.1 ml)	5.90 ± 0.03** (18.16)	9.28 ± 0.03*** (42.78)	7.93 ± 0.03*** (61.34)	7.30 ± 0.04*** (69.23)
Group IV (Treatment Control)	Hydro-alcoholic (200 mg/kg) +FCA (0.1 ml)	6.18 ± 0.09* (14.25)	11.57 ± 0.04*** (28.64)	11.74 ± 0.07*** (42.73)	11.83 ± 0.04*** (50.12)
Group V (Treatment Control)	hydro-alcoholic (200 mg/kg) +FCA (0.1 ml)	5.75 ± 0.04** (20.23)	8.79± 0.03*** (45.76)	7.33 ± 0.09*** (64.27)	6.26 ± 0.05*** (73.64)

Each values represents the mean±SEM; (n=6), *p<0.05, **p<0.01, ***p< 0.001 respectively when compared with toxicant control group (one-way ANOVA followed by Dunnett's test).Values in parentheses indicate percent inhibition activity (H), calculated as 100 x (value of negative control – value of treatment) / value of negative control

Discussion

RA is a chronic inflammatory disease characterized by fibroblastic proliferation, infiltration of the synovial lining by inflammatory cells that leads to expression of proinflammatory cytokines and a paucity of apoptosis resulting in bone and joint destruction. (Ghildiyal et al., 2013) Despite enormous research being carried out for allergic and immune disease, it still remains a disorder which can be ‘’controlled’’ and not ‘’treated’’, since no satisfactory treatment is available in allopathic system. Hence, there is a tremendous interest worldwide for the use of an alternative system of medicine.

The research has been focused on formulations used in traditional medicine for the treatment of RA.(Ram etal., 2012) CFA is used to initiate induction of arthritis. This model is the original model of RA, has been extensively used in preclinical screening of new anti-arthritis compounds and has successfully predicted activity in multiple new therapeutics. After a single injection of the adjuvant, rapid, reliable, robust, and easily measurable poly arthritis develops. The joint pathology seen in this animal model shares the cartilage degradation, bone resorption, and cellular influx seen in human RA. (Andersen et al., 2004; Bendele et al., 1999) Paw volumes and paw thickness are physical indicators of the inflammation in early as well as chronic phase of the disease. The progression of arthritis is characterized by an increase of the paw footpad and tibiotarsal joint diameters after day 14, which can be attributed to the delayed immunological flare in the disease (Andersen et al., 2004). The determination of paw swelling is an apparently simple, sensitive, and quick procedure for evaluating the degree of inflammation and assessing therapeutic effects of drugs. The HAZM -treated group showed significant reduction in paw volume suggesting the anti-arthritic activity of the leaves. T-cell proliferation is an important mechanism of adjuvant diseases, specifically their differentiation into Th-1 helper cells (Weiner, 2001) Therefore, a possible mechanism for reduction in paw edema might be either a suppressive effect on Th-1 helper cells.

Conclusion

Preliminary phytochemical investigations on the hydroalcoholic extract of *Z. mauritiana* were noted the presence of carbohydrates, flavonoids, saponins, alkaloids and glycosides. No mortality or behavioral abnormality recorded in mice during experiments at the highest dose level of 2000 mg/kg tested for LD50 studies. The high dose of hydroalcoholic extract of *Z. mauritiana* exhibited a significant anti-arthritic activity by reducing Paw volumes and paw thickness. Phytochemical constituents like flavonoids, saponins, glycosides and alkaloids were already reported for their anti-arthritic activity and these constituents were present in hydroalcoholic extract of *Z. mauritiana*. Hence these chemical constituents can be accounted for the observed anti-arthritic activities.

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