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Pharmacological Assessment of Morinda CitrifoliaFruit Extract's in Anti-Arthritic Properties in Wistar Rats

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ABSTRACT

Arthritis and related disorders, including rheumatoid arthritis (RA), are common diseases affecting millions of people. 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.*Morinda citrifolia* L(Rubiaceae), commonly called Noni or Indian mulberry, is a small evergreen tree or shrub of Polynesian origin. *M. citrifolia* bears a lumpy, green to yellowish-white fruit, normally 5 to 10 cm in length, with a surface covered in polygonal-shaped sections,The leaves, roots, bark and fruits have all been used medicinally to treat a wide range of ailments. These include, but are not limited to, diabetes, diarrhoea, hypertension, malaria, pain, and topical infections. The fruits are also eaten as a food, but primarily only in times of famine. The present study was designed to investigate anti-arthritic activity of methanolic fruits extract of *M. citrifolia* in Freund's complete adjuvant (FCA)-induced arthritis in rats. Qualitative analysis of various phytochemical constituents was determined by the well-known test protocol available in the literature. The *In vitro* antioxidant activity of methanolic extract of the fruits was assessed against DPPH free radical scavenging assay method using standard protocols. *M. citrifolia* was evaluated for anti-arthritic action by Freund's adjuvant induced arthritis test in adult Albino rats (200±20gm). Rats were injected 0.1 ml of complete Freund's adjuvant into the planter region of the left hind paw. *M. citrifolia* extracts (200 and 400 mg/kg. b.wt.) was given orally to arthritic rats induced with Complete Freund's Adjuvant and changes in rat paw volume and body weight was determined. Phytochemical analysis of methanolic extract of *M. citrifolia* showed the presence of carbohydrate, alkaloids, phenolics, tannin, triterpenoids, steroids, flavonoids and glycoside. In the oral acute toxicity studies, *M. citrifolia* was found to be safe as it did not cause any mortality up to 2000 mg/kg. Hence, 200 and 400 mg/kg doses were selected for the present study.DPPH radical scavenging activity of *M. citrifolia* extract exhibited percent inhibition 60.15% and its IC 50value was found to be 57.10µg/ml. Ascorbic was used as a reference compound which exhibited percent inhibition 86.36% and showed IC 50 value of 19.36µg/ml. *M. citrifolia* administered groups showed marked reduction in paw volume when compared with the negative control group (Group II). It was also found that there was significant weight loss when compared to standard. The results reveal promising anti-arthritic potential of the *M. citrifolia*. However further pharmacological investigation using isolated active ingredients can be carried out to confirm its efficacy and mechanism of action.

Keywords: *Morinda citrifolia, Anti-arthritic activity, Freund's adjuvant induced, Phytochemical analysis, In vitro antioxidant activity*

1. INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease of unknown etiology, affecting 1-1.5% of the population worldwide [1]. The disease is characterized by articular inflammation and by the formation of an inflammatory and invasive tissue, rheumatoid pannus that eventually leads to the destruction of joints. Anti-inflammatory drugs and analgesics including steroids are used to suppress the symptoms, while disease-modifying antirheumatic drugs (DMARDs), newer therapies such as anti-CD20 therapy (rituximab) and abatacept, anti-tumor necrosis factor (TNF)-α therapy (etanercept, infliximab and adalimumab) are often required to inhibit or halt the underlying immune process. However, all of these agents are associated with numerous side effects. In recent days, researchers are directed toward traditional system of medicine for the discovery of drugs that are long-acting anti-inflammatory with minimum side effects. Although there is no ideal animal model for RA at this time, rat adjuvant arthritis shares many features ofhuman RA and the sensitivity of this model to anti- arthritic agents support the view that the adjuvant arthritis is the best available model of RA [2]. Herbal medicines are being accepted and used increasingly by general populations in both eastern and western countries because of the ethnic acceptability and compatibility having fewer side effects [3].*M. citrifolia* (Rubiaceae), popularly known as Indian mulberry or noni, is a plant indigenous to India, Burma, China, and the Polynesian islands [4, 5]. *M. citrifolia* fruits have been used traditionally by native Polynesians to treat diabetes, high blood pressure, cancer, injury, arthritis, digestive distress, arteriosclerosis, pain, and senility [6]. In addition, *M. citrifolia* fruits have also been used as a remedy for halitosis, bacterial and helminthic infection, wound healing, menstrual cramps, arthritis, gastric and oral ulcers, toothache, and indigestion. It improves lactation and also acts as a purgative [6]. Several researchers have confirmed that the immunostimulant properties of *M. citrifolia* significantly contribute to its antitumor potential [6]. Recently, Palu et al. (2008) demonstrated that *M. citrifolia* juice concentrate (NFJC) stimulated the cannabinoid receptors (CB2). In addition, a decrease in interleukin-4 (IL-4) levels with a concomitant increase in interferon-γ (IFN-γ) levels elicited by NFJC indicated the immunostimulant properties of this plant [7].To date, the major chemical constituents of this plant have been found to be anthraquinones, flavonol,glycosides,Iridoid glycosides, lipid glycosidesand triterpenoids [8].Hence, the aim of this study is to prove the therapeutic potential of the plant *M. citrifolia*as an anti-arthritic agent against Freund's complete adjuvant (FCA) induced arthritis.

2. MATERIALS AND METHODS

Collection of plant material

The medicinal plant *M. citrifolia*(300 gm) was collected locally from Bhopal, MP. After cleaning, plant parts were dried under shade at room temperature for 3 days and then in oven dried at 45°C till complete dryness. Dried plant parts were stored in air tight glass containers in dry and cool place to avoid contamination and deterioration.*M. citrifolia* was authenticated by a plant taxonomist in order to confirm its identity and purity.

Chemicals and reagents

Complete friend's adjuvant (CFA)was procured from Sigma Aldrich chemicals Pvt. Ltd, Hyderabad, India. Indomethacin was obtained from Akums Drugs and Pharmaceuticals, India. All other chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), SRL Pvt. Ltd. (Mumbai, India) and Merck Life Sci. Private Ltd. (Mumbai, India).All other chemicals used in this study were obtained commercially and were of analytical grade and triple distilled water was used for whole experiment was generated in house.

Extraction of plant material

Hot soxhlet extraction method

This technique involved gathering, correctly washing, and properly rinsing the blossoms of *M. citrifolia*. They were mechanically pulverised after being shade-dried. The plant material from *M. citrifolia*, either whole or coarsely powdered, was successively extracted using solvents such as petroleum etherand methanol for various lengths of time. The Soxhlet apparatus' chamber was filled with powder using a "thimble" design. The solvent used for extraction was heated in flasks, and its vapours were then condensed in a condenser. The powder is extracted by touch when the condensed extractant is dropped into the thimble holding it. The liquid inside the chamber syphon drops into the flask when the liquid level in the chamber reaches the top of the syphon tube. This procedure was continued until an evaporated drop of solvent from the syphon tube did not leave any residue. The resulting extract was filtered, dried by concentration, weighed, and stored for later use [9]. The following formula is used to determine the extract's yield.

Yield (%) = Weight of the residue obtained×100

Weight of the plant material taken

Phytochemical screening of the extract

A variety of phytoconstituents, including alkaloids, carbohydrates, glycosides, phytosterols, saponins, tannins, proteins, amino acids, and flavonoids were qualitatively analysed in thefruits extracts of *M. citrifolia* [10, 11].

In vitro anti oxidant activity of plant extract

DPPH assay

Free radical scavenging activity of the extracts of *M. citrifolia*fruits, based on the scavenging activity of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method of Ali et al [12]. Different volume of extracts/standard (20-100µg/ml) was taken from stock solution in a set of test tubes and methanol was added to make the volume to 1 ml. To this, 2 ml of 0.1mM DPPH reagent was added and mixed thoroughly. Absorbance at 517 nm was determined after 30 min and the percentage inhibition activity was calculated by using the equation: % scavenging activity=[(A0-A1)/A0] ×100. Where A0 is the absorbance of the control and A1 is the absorbance of the extract. Lower the absorbance, the higher is the free radical scavenging activity. The curves were prepared and the IC50 value was calculated using linear regression analysis.

Animals

Animals will select randomly from animal house of Pinnacle Biomedical Research Institute (PBRI), Bhopal, India and further divided into various treatment groups randomly and kept in propylene cage with sterile husk as bedding. Relative humidity of 30.7 % at 22±2°C and 12:12 light and dark cycle will be maintained in the animal house and fed with standard pellets (Golden Feeds, New Delhi, India) and water will available *ad libitum*. Rats will acclimatize to laboratory conditions for 7 days before carrying out the experiments. Separate group (n=6) of rats will used for each set of experiments. Animal experiments will approve by Institutional Animal Ethics Committee (IAEC) of Pinnacle Biomedical Research Institute (PBRI) Bhopal.

Animals used

- Strain - *Albino Wistar* rats
- Age - 5-6 weeks
- Sex - either sex
- Body weight - 200±20 gm

Acute oral toxicity

Acute toxicity study of the prepared seed extracts of *M. citrifolia* was carried out according to the Organization for Economic Co-Operation and Development (OECD) Guidelines-423 [13] the animals were fasted for 4 h, but allowed free access to water throughout. As per the OECD recommendations, the starting dose level should be that which is most likely to produce mortality in some of the dosed animals; and when there is no information available on a substance to be tested in this regard; for animal welfare reasons, The dose level to be used as the starting dose is selected from one of four fixed levels 5, 50, 300 and 2000 mg/kg body weight. Acute toxicity was determined as per reported method [14].

Induction of arthritis

Arthritis was induced in rats by the intraplantar injection of 0.1 ml of Complete Freund's Adjuvant (CFA) in the left hind paw. The adjuvant contained heat killed Mycobacterium tuberculosis in sterile paraffin oil (10 mg/ml). The paw volume of all the animal groups was measured at 0, 4, 8 and 12 days after the injection of Freund's complete adjuvant [15].

Freund's adjuvant induced arthritic model

Freud's adjuvant induced arthritis model was used to assess the anti-arthritic activity in albino rats. Animals were divided into five groups of six animals each. Group I served as normal control, Group II served as arthritic control, injected with 0.01 ml Freud's adjuvant i.p., Group III served as reference standard, which received 10 mg/kg body weight IP of indomethacin, Group IV received the extract of *M. citrifolia*the dose of 200mg/kg body weight p.o. and Group V received the extract of *M. citrifolia* the dose of 400mg/kg body weight p.o., respectively.

Design of experiment

S.No.	Groups	Number of animals	Dose
1	Normalcontrol	6	0.2 ml
2	Arthritic control (Freud's adjuvant)	6	0.1 ml
3	Reference standard (Indomethacin)	6	10 mg/kg
4	*M. citrifolia* extract	6	200 mg/kg
5	*M. citrifolia* extract	6	400 mg/kg

Evaluation of arthritis

Bodyweight examination

During treatment, the change in body weight was measured with the help of digital weighing balance from the day of CFA immunizationand then subsequently on 0th, 4th, 8th, and 12st days [16].

Paw thickness (Joint diameter)

Joint diameter was measured using a digital vernier caliper (Mitutoyo digimatic caliper, Japan) before adjuvant administration.The joint diameter was measured again on day (0, 4, 8, and 12).

Statistical analysis

The data is expressed as mean ± Standard Deviation (SD). Results were analyzed using one-way ANOVA followed by Bonferroni test. Differences were considered as statistically significant at $P < 0.05$, $P<0.001$ when compared with control.

3. RESULTS

The percentage yields of methanolic extract obtained from *M. citrifolia* 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 carbohydrate, alkaloids, phenolics, tannin, triterpenoids, steroids, flavonoids and glycoside Table 2. In the present investigation, the *in vitro* anti-oxidant activity of extracts of *M. citrifolia was* evaluated by DPPH radical scavenging activity. The results are summarized in Table 3.To determine the safety of *M. citrifolia* for human use, toxicological evaluation is carried out in experimental animals. In the acute toxicity study, no signs of toxicity were found up to the dose of 2000 mg/kg body weight. Hence 1/10th and 1/5th doses i.e. 200 mg/kg and 400 mg/kg have been fixed as ED50 for present study Table 4.Paw swelling is an index of measuring the anti-arthritic activity of *M. citrifolia*at the dose level 200 & 400 mg/kg, p.o.*M. citrifolia* administered groups showed marked reduction in paw volume when compared with the Negative control group (Group II). It was also found that there was significant weight loss when compared to standardTable 5, 6 & Figure 1, 2.

Table 1: Percentage yield of crude extracts of *M. citrifolia*extract

S. No	Plant name	Solvent	Theoretical weight	Yield(gm)	% yield
1	*M. citrifolia*	Pet. ether	300	1.21	0.40%
2		Methanol	283.02	5.99	2.11%

Preliminary phytochemical study

Table 2: Phytochemical testing of extract

S. No.	Experiment	Presence or absence of phytochemical test	
		Pet. ether extract	Methanolic extract
1.	**Alkaloids**		
1.1	Dragendroff's test	Absent	Present
1.2	Mayer's reagent test	Absent	Present
1.3	Wagner's reagent test	Absent	Present
1.3	Hager's reagent test	Absent	Present
2.	**Glycoside**		
2.1	Borntrager test	Present	Present
2.2	Legal's test	Present	Present
2.3	Killer-Killiani test	Present	Present
3.	**Carbohydrates**		

3.1	Molish's test	Absent	Present
3.2	Fehling's test	Absent	Present
3.3	Benedict's test	Absent	Present
3.4	Barfoed's test	Absent	Present
4.	**Proteins and Amino Acids**		
4.1	Biuret test	Present	Absent
5.	**Flavonoids**		
5.1	Alkaline reagent test	Absent	Present
5.2	Lead Acetate test	Absent	Present
6.	**Tannin and Phenolic compounds**		
6.1	Ferric Chloride test	Absent	Present
7.	**Saponins**		
7.1	Foam test	Present	Absent
8.	**Test for Triterpenoids and Steroids**		
8.1	Salkowski's test	Present	Present
8.2	Libbermann-Burchard's test	Present	Present

***In vitro* anti oxidant activity**

Table 3: DPPH assay of ascorbic acidand methanolic extract

S. No.	Conc. (µg/ml)	Ascorbic acid (% Inhibition)	methanolic Extract (% Inhibition)
1.	**20**	52.757	42.919
2.	**40**	56.919	46.871
3.	**60**	65.660	49.835
4.	**80**	71.279	53.238
5.	**100**	86.368	60.153
IC 50 Value		**19.36**	**57.10**

Table 4: Acute oral toxicity of*M. citrifolia*extract

S. No.	Groups	Observations/ Mortality
1.	5 mg/kg Bodyweight	0/3
2.	50 mg/kg Bodyweight	0/3
3.	300 mg/kg Bodyweight	0/3
4.	2000 mg/kg Bodyweight	0/3

Freund's adjuvant induced arthritic model

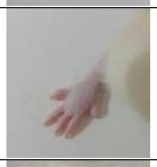
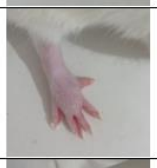
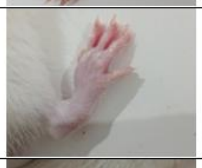
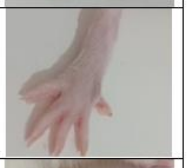
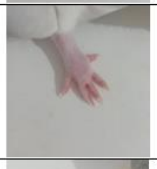
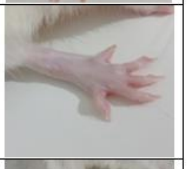
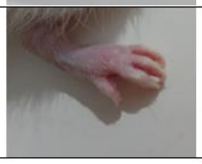
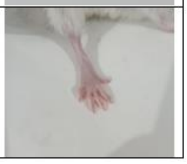
Table 5: Body weight

Body weight		
	Initial weight	**Final weight**
Normal control	210±1.62	216±2.89
Arthritic control (Freud's adjuvant)	211±1.66	221±2.49
standard (Indomethacin) 10 mg/kg	201±0.18	213±1.30
M. citrifolia extract(200 mg/kg)	206±1.05	215±0.35
M. citrifolia extract(400 mg/kg)	205±1.52	218±7.39

Table 6: Freund's adjuvant induced arthritic Model

Rat paw volume				
Groups	**Day 0**	**Day 4**	**Day 8**	**Day 12**
Normal control	0.23±0.05	0.25±0.10	0.26±0.09	0.25.99±0.08
Arthritic control (Freud's adjuvant)	0.28±0.07	0.71±0.08	0.77±0.12	0.80±0.09
Standard (Indomethacin) 10 mg/kg	0.25±0.01	0.53±0.03	0.42±0.07	0.40±0.21
*M. citrifolia*extract(200 mg/kg)	0.27±0.15	0.59±0.21	0.48±0.13	0.46±0.24
*M. citrifolia*extract(400 mg/kg)	0.26±0.04	0.54±0.11	0.45±0.19	0.44±0.17

Table 7: Histology of anti arthritic activity

Group	Day 0	Day 4	Day 8	Day 12
I. Normal control				
II. Arthritic control (freud's adjuvant)				
III. standard (Indomethacin) 10 mg/kg				
IV. *Morinda citrifolia*extract(200 mg/kg)				
V. *Morinda citrifolia*extract(400 mg/kg)				

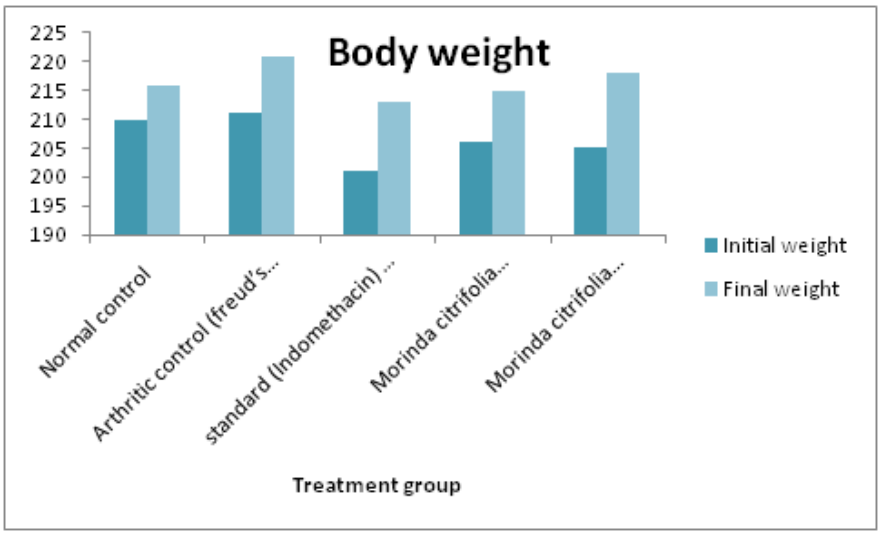


Figure1: Represent the body weight

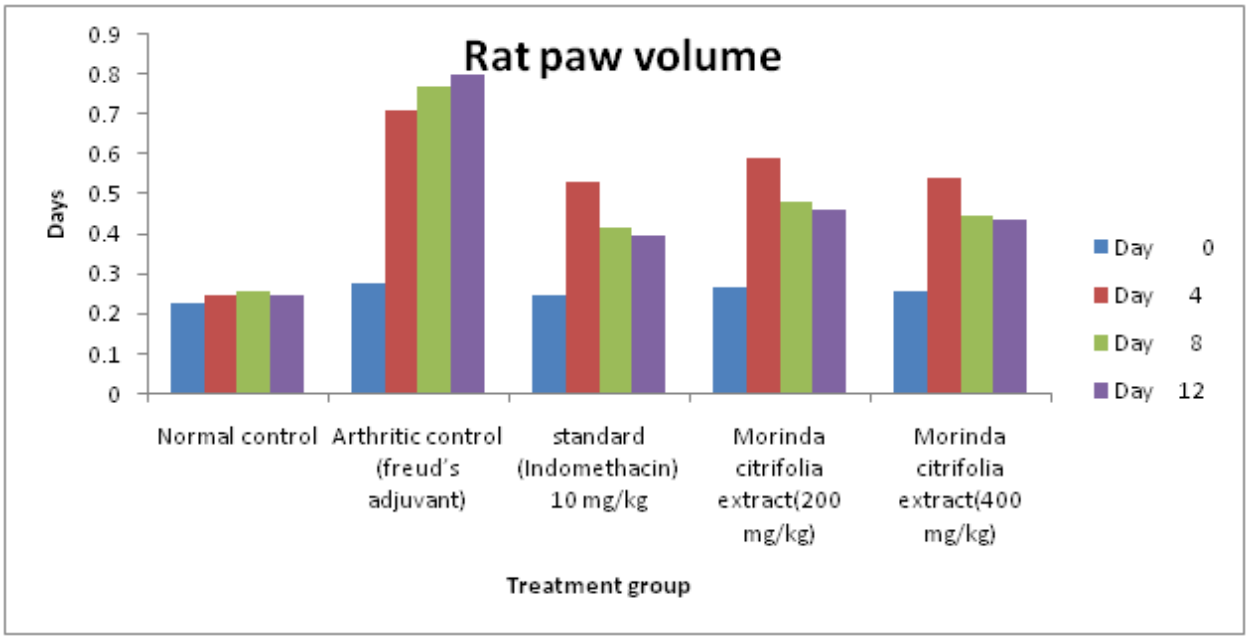


Figure2: Rat paw volume

4. DISCUSSION

Rheumatoid arthritis is an autoimmune disorder, the immunologically mediated complete Freund's adjuvant induced arthritic model of chronic inflammation is considered as the best available experimental model of rheumatoid arthritis. Complete Freund's adjuvant-induced arthritis is a model of chronic polyarthritis with features that resemble rheumatoid arthritis [17]. The determination of paw swelling is apparently simple, sensitive and quick procedure for evaluating the degree of inflammation and assessing of therapeutic effects of drugs [18]. In adjuvant-induced arthritis model rats developed a chronic swelling in multiple joints with influence of inflammatory cells, erosion of joint cartilage and bone destruction and remodeling which have close similarities to human rheumatoid disease. These inflammatory changes ultimately result in the complete destruction of joint integrity and functions in the affected animal. Also, the CFA administered rats showed soft tissue swelling around the ankle joints during the development of arthritis, which was considered as edema of the particular tissues [19].

In our study the methanolic extract of *M. citrifolia* exhibited a significant anti-arthritic activity in a dose dependent manner. In the present study, we showed that methanolic extract of *M. citrifolia*could significantly inhibit the progression of the rheumatoid arthritis in treated animals. However, standard drug andmethanolic extract significantly suppressed the swelling of the paws in both acute and chronic phase which may be due to the suppression of inflammatory mediator released due to

induction of Freund's adjuvant. Though the actual mechanism of suppressing inflammation is not known but it can be correlated with the presence of flavonoids in suppressing the inflammation and anti-oxidant activity. Numerous studies have suggested a role of oxidative stress in the pathogenesis of rheumatoid arthritis [20]. Therefore; it was assumed that the reported and well-established antioxidant properties of *M. citrifolia* and its ability to block the COX-2 pathway during the progression of inflammation justify the usage of the plant extract in the treatment of rheumatoid arthritis. There was a significant reduction in the paw volume in Freund's complete adjuvant induced arthritic rats. The cardinal signs of the chronic inflammatory reactions like redness, swelling, arthalgia and immobility of affected joints were significantly less in the drug treated animal than those of the control. The pathogenesis or reasons for development of arthritis following injection of FCA are not fully understood.The result of the present study also indicates that there is a close relationship between the extent of inflammation, loss of body weight and arthritic index. The arthritic scoring was done on the basis of visual observation where it can be seen that there is a marked reduction in the swelling and joint damage of the drug treated groups [21]. It was also noted that the high dose *Morinda citrifolia*. Extract proved its efficacy to reduce the inflammation of the paws.

5. CONCLUSION

Preliminary phytochemical investigations on the methanolic extract of *M. citrifolia* 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 methanolic extract of *M. citrifolia* exhibited a significant anti-arthritic activity by reducingPaw volumes and increase in body weight. Phytochemical constituents like flavonoids, saponins, glycosides and alkaloids were already reported for their anti-arthritic activity and these constituents were present in methanolic extract of *M. citrifolia*. Hence these chemical constituents can be accounted for the observed anti-arthritic activities.

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