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Antiarthritic Activity of *Murraya exotica* Linn Against Formaldehyde Induced Arthritis In Wistar Rats.

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ABSTRACT

The study was aimed to evaluate anti-arthritic activity of hydro-alcoholic extract of leaves of *Murraya exotica* Linn against formaldehyde induced arthritis in wistar rats. Arthritis was induced using 0.1ml (2% v/v) of formaldehyde administered into plantar region of the left hind paw on day 1 & 3. Concurrent administrations of Aceclofenac (50 mg/kg, i.p.)and hydro-alcoholic leaf extract of *M.exotica* (200 & 400 mg/kg, b.wt.,p.o)for 10 days served as standard & test control. Arthritic activity was evaluated by measuring the changes in paw diameter during study and also at the end of the study, alterations in hematological and biochemical parameters were estimated supported by radiographical examination of joints & histopathology of liver were compared to arthritic control. The results demonstrated significant anti-arthritic activity of *M.exotica* at both doses (200 & 400 mg/kg, b.wt., p.o) by ameliorating the changes in physical, hematological & biochemical parameters as compared to arthritic control evidenced by the radiographical examinations and histopathology of liver.

Keywords: Anti-arthritic activity, *Murraya exotica* Linn, Aceclofenac, Radiographical examination.

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INTRODUCTION

Traditional medicinal plants were used for the treatment of various diseases from many centuries where gained their importance in traditional knowledge periodically[1]. India is known as the Emporium of Medicinal plants due to availability of several thousands of medicinal plants in the different bioclimatic zones. Anti-inflammatory diseases including rheumatoid arthritis are still one of the main health problems of the world's population. Plant drugs are known to play a vital role in management of inflammatory diseases [2]. The phytoconstituents from plant origin are potential against arthritis. The use of natural remedies for the treatment of inflammatory and painful conditions has a long history, starting with Ayurvedic treatment and extending to the European and other systems of traditional medicines.

Rheumatoid arthritis (RA) is a chronic, systematic, autoimmune, progressive, inflammatory disorder which affecting the synovial joints and produced the inflammation in the synovial membrane; that leads to joint destruction, disability and restricted joint movements. The most common effect of the RA is pain, inflammation and tissue damage [3]. Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 are important mediators of the disease perpetuation. The arthritis usually begins in the small joints of the hands and the feet, spreading later to the larger joints, the inflamed joint lining or synovial extends and then erodes the articular cartilage and bone, causing joint deformity and progressive physical disability. Extra-articular features include nodules, pericarditis, pulmonary fibrosis, peripheral neuropathy and amyloidosis [4].

The prevalence of rheumatoid arthritis varies between 0.3% and 1% worldwide and is more in developed countries mainly affecting women than men (3:1). Generally, it strikes between 30 and 55 years affecting 0.5-1.0% of adults with 5-50 per 1,00,000 new cases annually [5].There is no known cure for RA but several drugs such as anti-inflammatory and disease modifying anti-rheumatoid drugs are used in mono or combination therapies to inhibit the disease process. However, prolonged use of these drugs is associated with deleterious side effects such as gastric ulceration, hemorrhage, anemia, and kidney dysfunction [6]. Thus, in recent times, researchers have been directed towards the use of biologics and plant-derived drugs providing an effective cure for the treatment of RA and to overcome the serious drawbacks.

Murraya exotica Linn.Mantiss., synonym *Murraya paniculata* Linn Jack locally known as Kamini; *Murraya* is one of the 150 genera from the family Rutaceae. Among the 14 global species belonging to this genus, *Murraya koenigii* and *Murraya exotica* have been found in India [7].Traditionally the plant possesses stimulant, astringent, abortive properties and is used to treat diarrhea, dysentery, cuts, body aches and venereal diseases. Infusion of the leaves and flowers is tonic and stomachic. It is said to be aromatic, refrigerant, digestive and beneficial in coughs, giddiness, hysteria, thirst and burning of the skin [8]. Phytochemical screening of leaf extract reveals the presence of alkaloids (tamynine) [9], Coumarins (paniculatine, coumurrayin) [9,10]. A complex mixture of 70 Polymethoxylated Flavonoids (PMFs) have been reported from leaves of the plant, which included 45 flavones, 17 flavanones and 8 PMFs glycosides [11].Various pharmacological uses reported for *Murraya exotica* Linn such as Anti-diabetic, Anti-oxidant, Anti trypanocidal, Antimalarial, Antibacterial, Antifungal [12,13], Antinociceptive, Stimulant & Astringent[14], Anti-inflammatory, Antiviral [15,16], Anti-diarrhoeal, Oxytocic, Anti-Fertility [17] the essential oils was reported to possess anti-amebic activity [18], Anticoagulant [19], Antimycobacterial, Antitumor [14,20,21,], Antibiotic activity against *Micrococcus pyogenes*, Var. aureus and E.coli [22].

Figure 1: *Murraya exotica* plant

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Figure 2: *M.exotica leaves* after collection

Taking these facts into considerations, the present study was aimed at evaluating the antiarthritic activity of the hydro-alcoholic extract of leaves of *Murraya exotica* Linn against formaldehyde induced arthritic rats.

MATERIALS AND METHODS

Collection, identification& authentication of plant material

The leaves of *Murraya exotica* were collected from seshachalam hills, Tirupati, Andhra Pradesh. The samples of the plant were identified and authenticated (voucher No.: NN253) by Dr. N. Nagaraju, Asst. Professor in Botany & Head, Dept. of Microbiology, Sri Venkateswara Arts College (UG & PG), Tirupati, Andhra Pradesh.

Preparation of plant extract

The collected leaves were separated from undesirable materials. They were dried in open air for 4 weeks as shown in figure 3. The shade dried leaves were ground into a coarse powder with the help of a pulverizer. The powdered drug was subjected to percolation with 70% ethanol using soxhlet apparatus in 1:4 ratio. The extraction was carried out until the solvent becomes colorless. The extract was evaporated on a hot plate at 40^{o}C. The marc of crude drug obtained was set for complete drying in a dessicator using calcium chloride until dried powder of the crude drug was obtained. The powdered form of crude drug was suspended in 1% CMC (Micro Crystalline Cellulose), to produce a concentration of 200mg and 400 mg/kg, b.wt.as low dose & high dose.

Figure 3: *M.exotica* leaves set for shade drying

Figure 4: Marc of crude drug set for drying in Desicator

Animal husbandry

Healthy, Adult, Male, Wistar rats weighing 130-150gms were acclimatized under standardized conditions with 12-hour light/dark cycle, 24°C and 35 to 60% humidity, provided free access to pellet diet and purified drinking water *adlibtium* [23]. The experimental protocol was approved by Institutional Animal Ethical Committee (Approval no. 1995/PO/Re/S/17/CPCSEA).

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Induction of arthritis

Arthritis was induced using 0.1ml (2%v/v) of formaldehyde solution administered at planter surface of the left hind paw on day 1 & day 3 of experimental period [2, 24]. The animals were divided into five groups each consisting of six animals.

Experimental Protocol:

Group 1 Animals received 0.5 mL normal saline per oral (p.o)
Group 2 Animals received 0.1 ml 2% (v/v) of formaldehyde solution administered at planter surface of the left hind paw [2, 24] on day 1 & 3.
Group 3 Animals received Aceclofenac sodium 50 mg/kg, b.wt., i.p for 10 days in animals pretreated with formaldehyde.
Group 4 Animals received HAME 200 mg/kg, b.wt., p.o for 10 days in animals pretreated with formaldehyde.
Group 5 Animals received HAME 400 mg/kg, b.wt., p.o for 10 days in animals pretreated with formaldehyde.

Assessment of Anti-arthritic activity

The rat paw diameter was measured by using digital vernier calipers [2, 24] on day 1, 2, 4, 6, 8, 10 of experimental period. On 10th day, animals were sacrificed& blood was collected by cardiac puncture for estimation of hematological such as RBC, WBC & Hb [25] and serum biochemical parameter such as SGOT, SGPT, ALP, TP, ALB [26] by using various diagnostic kits. Radiological examination was done for the knee joints on day 10 after the last dose administration of test extract and standard drug. Histopathology of liver tissue was done on day 10 of experimental period.

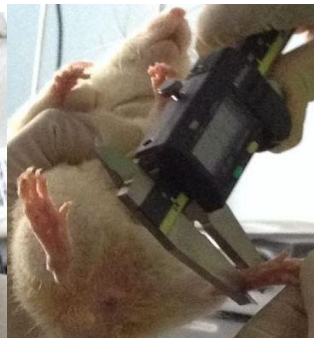

Figure 5: Digital Vernier Caliper **Figure 6 & 7: Recording of Paw diameter using digital vernier calipers**

RESULTS

Table 1: Results of phytochemical screening of HAME

Test for phytochemical group	Reagent	Results of HAME
Carbohydrates	Fehling's Test	+
Alkaloids	Dragendroff's Test	+
Tannin	Ferric chloride test	+
Saponin	Frothing Test	+
Amino Acids	Ninhydrin Test	-
Glycosides	Legal's Test	+
Cardiac Glycosides	Raymonds Test	-
Flavonoids	Shinoda test	+

+: Positive result; –: Negative result

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Table 2 showed a significant increase in paw diameter in formaldehyde induced arthritic group until day 6 with further constant paw diameter.The treatment with HAME (200 & 400 mg/kg, b.wt.) and standard (Aceclofenac 50mg/kg, b.wt.) showed reductionin paw diameter compares to arthritic control which was depicted in graph 1.

Table 2: Effect of HAME on paw diameter in formaldehyde induced arthritic rat

Groups	PAW DIAMETER (mm)					
	Day 1	**Day 2**	**Day 4**	**Day 6**	**Day 8**	**Day 10**
Arthritic [0.1ml of 2% v/v formaldehyde in normal saline]	4.252±0.1487	4.700±0.111	5.263±0.119	5.798±0.117	6.335±0.113	6.853±0.103
Standard [Aceclofenac -50 mg/kg, b.wt.]	4.140±0.054***	4.522±0.048***	5.353±0.0449***	6.077±0.0243***	5.560±0.0273***	5.025±0.027***
HAME – I [200 mg/kg, b.wt]	4.388±0.059***	4.802±0.034***	5.887±0.0149***	6.588±0.0175***	5.885±0.0227***	5.012±0.0317***
HAME – II [400 mg/kg, b.wt.]	4.937±0.045***	5.410±0.0375***	5.963±0.248***	6.777±0.0176***	6.220±0.0203***	5.413±0.0168***

Values are expressed as mean ± SEM (n=6). *P<0.05,**P<0.01,***P<0.001. as compared with arthritic control (One-way ANOVA followed by Dunnett's test).

Graph 1: Effect of HAME on Paw diameter in Formaldehyde induced arthritic rats

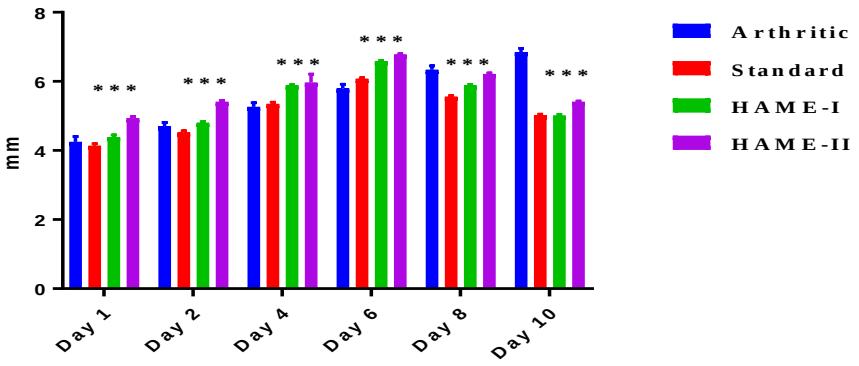

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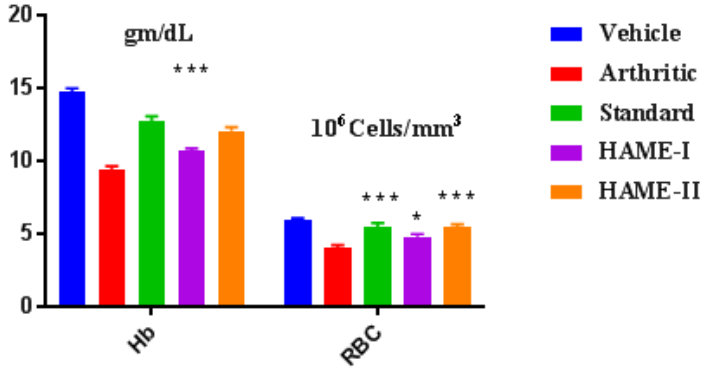
Table 3 showed an increase in WBC and decrease in the RBC &Hb levels in formaldehyde induced arthritic rats compared to vehicle control. The levels of RBC, WBC &Hb were ameliorated upon administration with test extracts at both dose levels (200 & 400 mg/kg, b.wt.) & standard dose (50 mg/kg, b.wt) shown in graphs 2 & 3.

Table 3: Effect of HAME on hematological parameters in formaldehyde induced arthritic rats

Groups	WBC (Cells / mm^3)	RBC (millions of cells / mm^3)	Haemoglobin (gm %)
Vehicle [0.5 mL of Normal saline]	7374±173.1	5.987±0.091	14.76±0.220
Arthritic [0.1ml of 2% v/v formaldehyde in normal saline]	13465±487.4	4.020±0.229	9.35±0.294
Standard [Aceclofenac-50 mg/kg, b.wt.]	8027±151.4***	5.490±0.273***	12.75±0.301***
HAME – I [200 mg/kg, b.wt]	12277±312.5*	4.822±0.167*	10.71±0.153***
HAME – II [400 mg/kg, b.wt.]	9157±294.9***	5.473±0.1926***	12.07±0.251***

Values are expressed as mean ± SEM (n=6). *P<0.05, **P<0.01, ***P<0.001. as compared with arthritic control (One-way ANOVA followed by Dunnett's test).

Graph 2: Effect of HAME on Hb & RBC in Formaldehyde induced arthritic rats



Graph 3: Effect of HAME on WBC in Formaldehyde induced arthritic rats

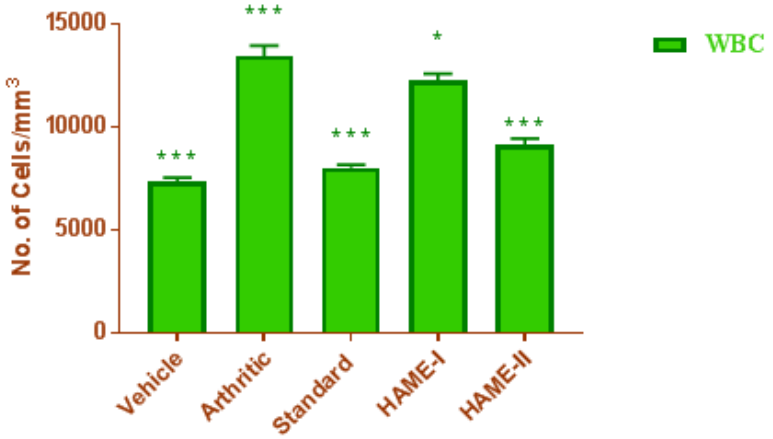


Table 4 showed a significant increase in SGOT, SGPT, ALP and decrease in the TP, ALB levels in formaldehyde induced arthritic rats compared to normal vehicle control. The abnormal serum biomarkers levels were

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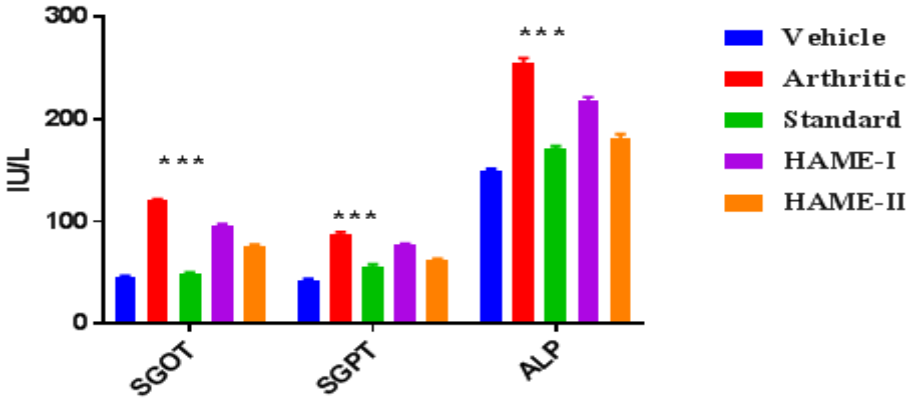
restored upon administration with test extracts at both dose levels (200 & 400 mg/kg, b.wt) & standard dose (50 mg/kg, b.wt) shown in graph 4 & 5.

Table 4: Effect of HAME on biochemical parameters in Formaldehyde induced arthritic rats

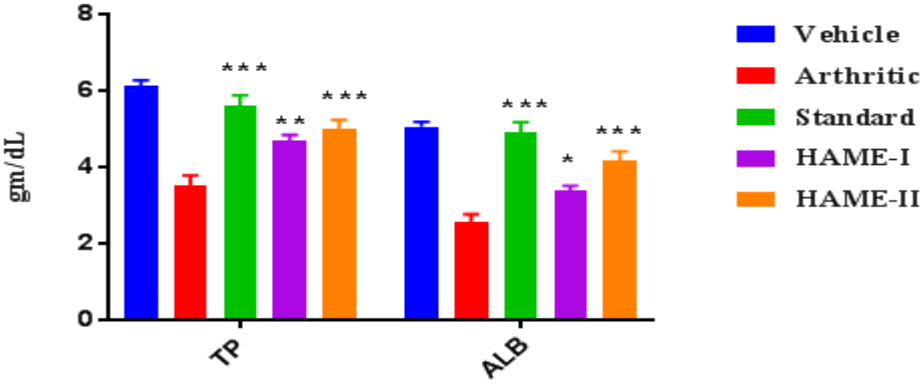
Groups	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	TP (g/dL)	ALB (g/dL)
Vehicle [0.5 mL of Normal saline]	45.62±1.216	42.98±1.162	150.10±1.538	6.128±0.1385	5.047±0.129
Arthritic [0.1ml of 2% v/v formaldehyde in normal saline]	120.5±1.337	87.10±2.453	255.0±5.006	3.532±0.246	2.560±0.205
Standard [Aceclofenac -50 mg/kg, b.wt.]	48.95±1.416***	55.52±2.652***	171.50±2.426***	5.603±0.276***	4.915±0.252***
HAME – I [200 mg/kg, b.wt]	96.43±1.311***	76.86±1.423***	217.90±3.992***	4.688±0.152**	3.392±0.118*
HAME – II [400 mg/kg, b.wt.]	76.22±1.331***	62.10±1.564***	181.90±3.716***	4.995±0.237***	4.153±0.256***

Values are expressed as mean ± SEM (n=6). *P<0.05,**P<0.01,***P<0.001. as compared with arthriticcontrol (One-way ANOVA followed by Dunnett's test).

Graph 4: Effect of HAME on Biochemical parameters in Formaldehyde induced arthritic rats



Graph 5: Effect of HAME on Total protein & Albumin parameters in Formaldehyde induced arthritic rats



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Radiographic Analysis:

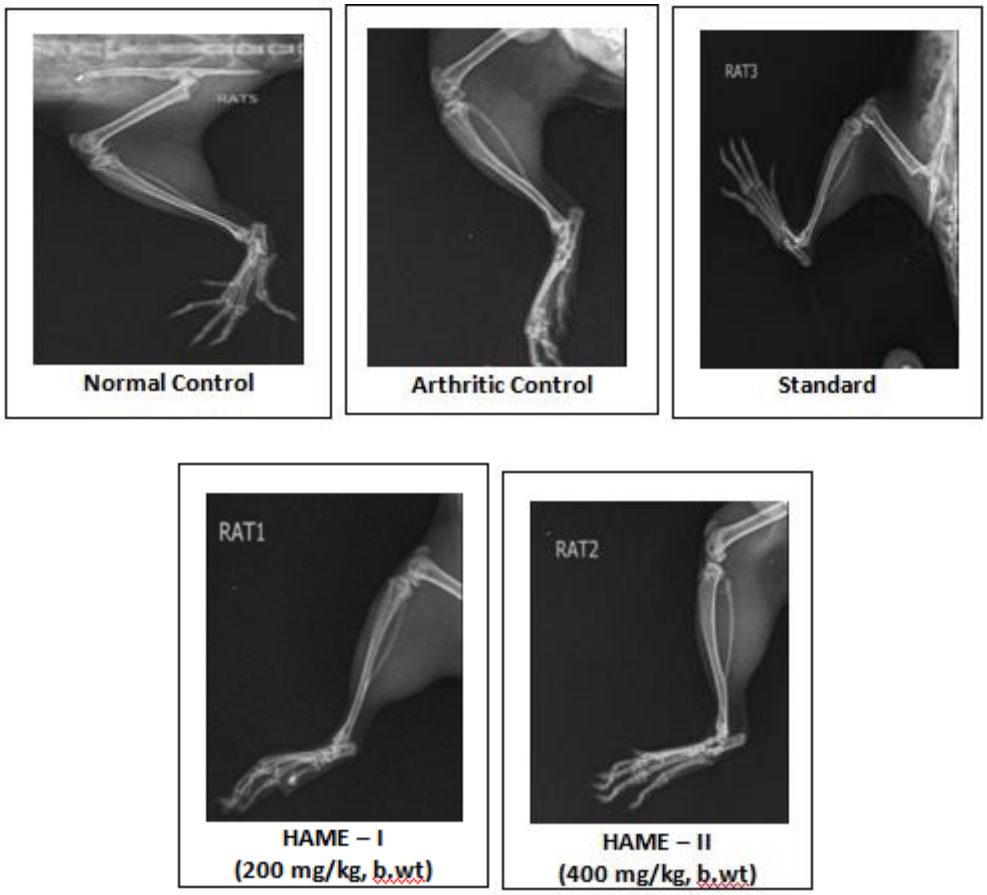
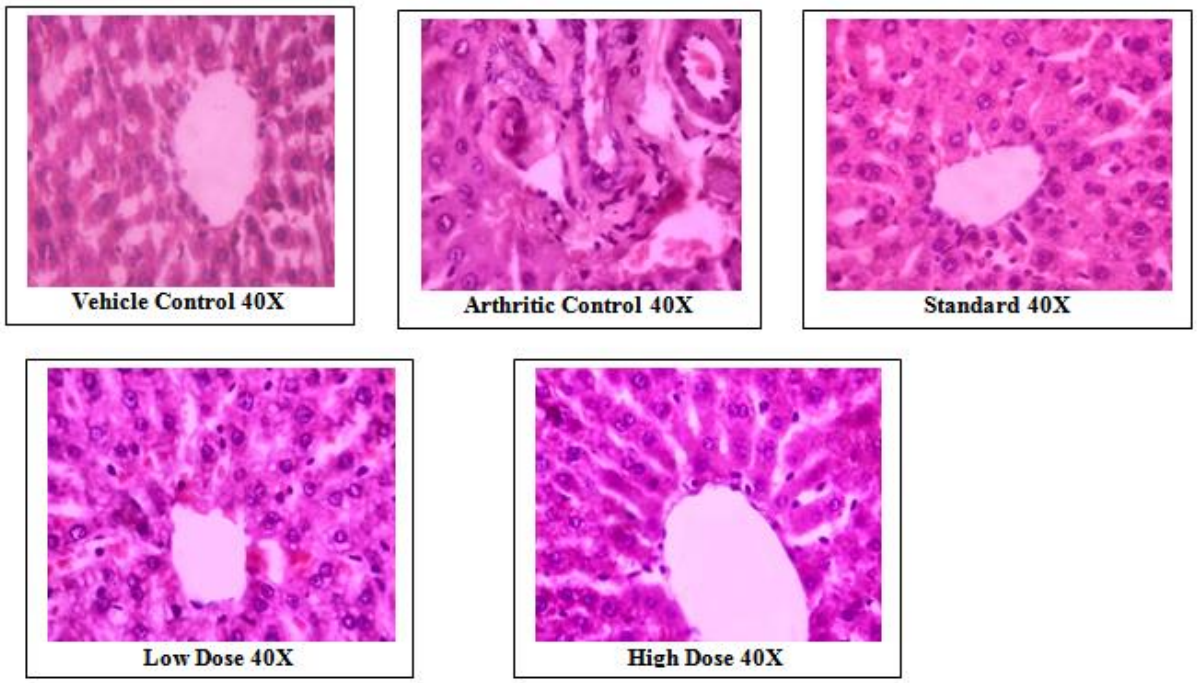


Figure 8: Radiographical examinations

HISTOPATHOLOGY OF LIVER:



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DISCUSSION

Rheumatism was one of the oldest diseases of mankind affecting large population in the world[23]. It is a complicated refractory autoimmune disease characterized by a number of inflammatory and destructive events such as joint pain and swelling, synovial hyperplasia, pannus formation, cartilage and bone erosions, joint malformation etc.,[27]. The RA due to the presence of pro-inflammatory markers, cytokines and leukotrines. The primary inflammatory markers are IL-1, TNF-α, IL-6, IL-15, IL-16, IL-17, IL-18, IFN-γ and granulocyte macrophage colony stimulating factor, chemokines such as IL-8, macrophage inflammatory protein-1 and monocyte chemo attractant protein-1. TNF-α blockade, IL-1 blockade, B cells therapy, IL-6 blockade and Angiogenesis blockade, these are therapeutic target for its treatment[5].

In RA, destructive molecules produced by an abnormal immune system response which is responsible for continuous inflammation of the synovium. Collagen is gradually destroyed, narrowing the joint space and finally damaging bone. In a progressive RA, destruction of the cartilage accelerates. Further pannus (thickened synovial tissue) formation occurs due to the accumulation of fluid and immune system cells in the synovium. The pannus produces more enzymes which destroy nearby cartilage, worsening the area and attracting more inflammatory white cells. There are two most important components of immune system i.e. B cells and T cells lymphocytes that play important role in inflammation associated with RA. If the T cell recognizes an antigen as "non-self," it will produce chemicals (cytokines) which cause B cells to multiply and release antibodies circulate largely in the bloodstream, recognizing the foreign particles and triggering inflammation in order to rid the body of the invasion[28]. There are various steps involved in inflammatory responses in RAdisease[29].

In current study, the anti-arthritic activity of HAME has been carried out using formaldehyde induced arthritic rat model. Here the plant leaves were powdered and subjected to percolation with 70% ethanol using soxhlet apparatus in 1:4 volumes. The extraction was carried out until the colourless solvent appears in the siphon tube. The solvents were removed from the extract by subjecting to the temperature of 40^0 C using heating mantle. The dried extract thus obtained was kept in desiccator until for further use. The extract was suspended using 1% CMC powder in distilled water[2]. The extraction yield was found to be 23%.

The phytochemical analysis of HAME revealed the presence of alkaloids, Flavonoids, carbohydrates, tannins, glycosides and saponins. Flavonoids and Tannins possess different mechanisms in various pharmacological activities[30]. Based on the previous data of acute toxicity studies conducted as per OECD - 423, high dose and low doses were selected as 10% and 20% of administered maximum dose of 2000 mg/kg, b.wt.[31].The choice of the animal strain has been found to be very important for the performance of this test. Wistar albino rats have been proven to be very suitable in contrast to other substrains.This was mainly due to the structural homology of rat TNF and similarity in CYP 450 enzyme system [32].

The formaldehyde-induced model of arthritis is useful to assess the potential anti-arthritic and anti-inflammatory effects of a substance, partially resembling the characteristics of human arthritis [33-36].Screening of anti-arthritic activity using formaldehyde induced arthritis in rats is considered as a modern, scientific, internationally approved standard experimental procedure [37].

In the current study, RA was induced using 0.1ml of 2% v/v formaldehyde in normal saline into the planter surface of the left hind paw, on the first and third day of the experimental period [38]. Formaldehyde injection causes a chronic inflammation of the rat foot, which involves the proliferation phase of inflammation elicited by COX mediators [33-36]. Swelling around the ankle joint and paw of arthritic rat is considered to be due to the oedema of particular tissue such as ligament and capsule[39]. Formaldehyde injection elicits localized inflammation and pain in the early phase followed subsequently by a phase of tissue mediated response [40]. This late phase produces proliferative joint inflammation leading to articular changes similar to those seen in RA [36]. The development of oedema in the paw of the rat after injection of formaldehyde is due to the release of histamine, serotonin and the prostaglandin like substances at the site of injection. Prostaglandins are generated in primary inflammatory phase and auto antibodies are generated in secondary immunological state. Release of various inflammatory mediators including cytokines (IL-1B and TNF-α), interferon and PDGF are responsible for the initiation of pain along with swelling of the limbs and joints, bone deformations and disability of joint function[41].

Aceclofenac (AC) is an effective non-steroidal anti-inflammatory drug, which possess remarkable anti-inflammatory, analgesic and anti-pyretic properties. The analgesic and anti-inflammatory efficacy of AC is generally equivalent to that of comparator non-steroidal anti-inflammatory drugs with similar onset of action [42]. It is also used for the treatment of Rheumatoid arthritis and Osteoarthritis, which reduces levels of prostaglandin E_2 in the synovial fluid and suppresses its production by blood polymorphonuclear leukocytes or mononuclear cells. The oral administration of AC causes GI ulcers and bleeding with chronic use [43]. AC is available as oral, rectal and injectable formulation [44].

The injection of formaldehyde significantly increased the diameters of rat paw and ankle as compared to the vehicle control due to the soft tissue swelling around the ankle joints appeared during the progress of arthritis, which was considered as oedema of the exacting tissues[45]. Treatment with standard (aceclofenac 50 mg/kg, b.wt.) and test extract at both dose levels (200 & 400 mg/kg, b.wt.) reduced the paw edema when compared to the arthritic control group,this may be due to inhibition of the release of inflammatory mediators owing to its anti-inflammatory activity.

In the current study, the rats with formaldehyde induction exhibited a reduction in RBC and Hb level causing anaemia as a common diagnostic feature with chronic arthritis [46].The treatment with HAME-II (400mg/kg, b.wt.) exhibited a significant increase in RBC count and with HAME-I (200mg/kg, b.wt.) exhibited a less significant increase in RBC count when compared to that of arthritic model and causes recovery from the anaemic condition compared to arthritic control.

Increase in WBC count is the indications for the infectious and inflammatory diseases attributed to systemic response of the rats to paw inflammation induced by formaldehyde [47]. The significant increase in Total WBCs count in arthritic control group may be due to the stimulation of immune system against the invading antigens [48]. The migration of leukocytes is more significantly suppressed in Standard control & HAME-II (400 mg/kg, b.wt.) and less significantly suppressed in HAME-I (200 mg/kg, b.wt.).

Elevated level of serum SGPT and SGOT in formaldehyde induced arthritic rats can be due to increase in the liver and bone fraction, implicating a localized bone loss in the form of bone erosion, as the enzyme is released into the circulation in the course of bone formation and resorption [49,50]. Cytoplasmic cellular enzymes, such as alkaline phosphatase (ALP) membrane bound indicator of type 2 cell secretory activity or the lysosomal enzyme β- glucuronidase, an indicator of phagocytic activity, can also be used as sensitive markers of cellular integrity and cellular toxicity induced by pathological conditions [51]. Increase in ALP level in formaldehyde induced arthritic rats was due to disease causing bone remodelling causing its elevation [52, 53]. The treatment with the HAME-I & II (200 mg/kg, b.wt., 400 mg/kg, b.wt.) significantly reduced the elevated levels of SGOT, SGPT & ALP when compared to the arthritic control.

Formaldehyde induces arthritis by denaturing proteins at the site of administration, which produces immunological reaction against the degraded product [54] resulting in decrease of serum total protein**.** The reduced protein levels were reverted back upon treatment with test extracts (200 & 400 mg/kg, b.wt.) when compared to the arthritic control.

Previous studies have reported that protein denaturation is one of the leading causes of inflammatory as well as arthritic diseases, which causes production of auto antigens, progressing to certain rheumatic diseases [55]. The main mechanism involved in protein denaturation is characterized by changes or alterations in hydrophobic, electrostatic, hydrogen and disulphide bonding among the protein molecules[56].The treatment with the HAME-II (400 mg/kg, b.wt.) has shown more and HAME-I (200 mg/kg) has shown less significant increase in albumin levels when compared to the arthritic control.

Radiographic changes in RA condition is useful diagnostic measures which indicate the severity of the disease. Soft tissue swelling is the earlier radiographic sign, whereas prominent radiographic changes like bony erosions and narrowing of joint spaces can be observed only in the developed stages (final stages) of arthritis [57]. In formaldehyde induced rat (group II), soft tissue swelling along with narrowing of the joint spaces were observed which implies the bony destruction in arthritic condition. The Aceclofenac treated groups augments the biosynthesis of extracellular matrix imparting chondroprotective effect in RA[58,59]. Extract treated groups also shows significant recovery form bony erosion and regain of normal architecture of joint occurs.

Induction of formaldehyde appears to be associated with hepatotoxicity depends on the route of exposure could include direct effects on hepatocytes or indirect effects through immune system. There is loss of normal lobular architecture of the liver indicating infiltration of mononuclear inflammatory cells in portal tracts compared to vehicle control [60].Administration with acute over doses of Aceclofenac (50mg/kg b.wt.) can cause potentially liver damage showing mild necrosis and inflammation within the hepatic parenchyma [61,62]. Treatment with test extracts at both dose levels (200 mg/kg b.wt, 400 mg/kg b.wt) restored the architecture damage due to formaldehyde indicating protective effect against reactive metabolites.

CONCLUSION

Medicinal plants are the Heart of nature and are considered as the back bone of Traditional medicine used in various ailments. From the above considerations it can be concluded that the HAME possess antiarthritic activity, it might be due to the presence of phytochemical constituents such as flavonoids and tannins which restored the altered hematological and biochemical parameters evidenced by radiographical examinations. Moreover, in the above study, histopathology of liver has been performed revealed the effect of Aceclofenac at acute overdoses and formaldehyde causing damage to the architecture of hepatocytes, the test drugs at both dose levels (200mg/kg b.wt. and 400 mg/kg b.wt.) restored architecture of hepatocytes due to formaldehyde induced hepatic damage. Further investigation is required to isolate the active principle responsible for the chondroprotective effect without inducing liver damage.

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