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Research Article

THERAPEUTIC EFFECT OF A POLY-HERBAL PREPARATION ON ADJUVANT INDUCED ARTHRITIS IN WISTAR RATS

MANGESH S BANSOD [*,1], VIRENDRA G. KAGATHARA [1, 2], ROHINI R. PUJARI [1, 3], VIVEK B. PATEL [2], HARDIK H. ARDESHNA [2]

[1] Dept. of Pharmacology, AISSMS College of Pharmacy, Pune-411001, Maharashtra, India, [2] Faculty of Pharmacy, Dharmsinh Desai University, Nadiad-387001, Gujarat, India, [3] Dept. of Pharmacology, Modern College of Pharmacy, Borhadewadi, Dehu-Alandi Road, Moshi, Pune, Maharashtra, India Email: viru_maitri24@yahoo.co.in, mee.mangesh@gmail.com.

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ABSTRACT

The present study was aimed to assess the anti-arthritic nature of polyherbal formulation containing *Cissampelos Pereira* Linn, *Pongamia pinnata* (Linn) Pierre and *Vitex negundo* Linn. against Freund's complete adjuvant induced arthritis in rats. The degree of inflammation was evaluated by hind paw sweeling, body weight and haematologicalc study supported by histopathology of ankle joints and radiological study. Polyherbal formulation (200 mg/kg, 400 mg/kg and 600 mg/kg) reduces hind paw swelling and body weight alongwith significant improvement in haematological study, while histopathology reveals the significant reduction in mononuclear infiltration, pannus formtion and bone erosion. The radiological pictures of the joints particularly metatarsal, phalanges and the ankle joint space of polyherbal formulation treated group animals showed protective effect against adjuvant induced arthritis.

Keywords: *Cissampelos Pereira* Linn, Freund's complete adjuvant, Poly-herbal Formulation, *Pongamia pinnata* (Linn) Pierre, *Vitex negundo* Linn.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, progressive, systemic inflammatory disorder affecting the synovial joints and typically producing symmetrical arthritis that leads to joint destruction, which is responsible for the deformity and disability. The consequent morbidity and mortality has a substantial socio-economic impact [1]. The prevalence of Rheumatoid arthritis is consistent worldwide affecting, about 0.5- 1 % of the population. It usually occurs in the people between 25 and 55 year of age. Women are affected more often than men at ratio of 3 to 1 [2]. Adjuvant induced arthritis (AIA) in rats is a chronic inflammatory disease characterized by infiltration of synovial membrane in association with destruction of joints resembles Rheumatoid arthritis in humans [3]. Rheumatoid arthritis progress in three stages. The first stage is the swelling of the synovial lining, causing pain, warmth, stiffness, redness and swelling around the joints. Second is the rapid division and growth of cell, or pannus, which causes the synovium to thicken. In the third stage, the inflamed cell releases enzyme that may digest the bone and cartilage, often causing the joints to loses its shape and alignments, more pain and loss of movements [4]. The most commonly prescribed medication for Rheumatoid arthritis treatment is steroidal, non-steroidal anti-inflammatory, disease modifying anti rheumatic and immunosuppressant drugs. Though the goal of these drugs have been to relieve pain and to decrease joint inflammation, to prevent joint destruction and to restore function of disabled joints, these drugs are known to produce various side effects including gastrointestinal disorders, immunodeficiency and humoral disturbances [5]. Accordingly, reducing side effects should be considered while designing improved therapeutics for Rheumatoid arthritis, besides enhancing medicinal effectiveness. The Siddha and Ayurvedic systems of treatment are being increasingly recognized as an alternate approach to arthritic treatment.

Cissampelos Pereira Linn. var. hirsuta (C.P.) is a very variable, lofty, slender, dioecious, perennial, climber commonly distributed throughout topical and sub topical India, ascending up to an altitude of 2,000 m, traditionally known as Laghupatha in Ayurveda, an Indian traditional system of medicine [6,7]. The presence of two crystalline alkaloids, hayatin and hayatinin were reported along with the other constituents as quercitol and a sterol. Plant alkaloid has shown inhibitory activity against human carcinoma cells of the naso-pharynx in cell culture [8]. Plant has been documented for potent diuretic [9], neuromuscular blocking and anti-tumor [10], antibacterial against Gram-positive bacteria [11], anticonvulsants [12], antimalarial [13], antidiarrhoeal [14], antioxidant, antimicrobial and β-glucosidase inhabitation [15], immunomodulatory [16], anti-inflammatory and antiarthritic activities of root [17].

Pongamia pinnata (Linn) Pierre (Leguminosae, Papilionaceae) (P.P.) (synonym: Pongamia glabra Vent), popularly known as 'Karanj' or 'Karanja' in Hindi, is a medium sized glabrous tree, found throughout India and further distributed eastwards, mainly in the littoral regions of South Eastern Asia and Australia [18]. This plant indicated the presence of abundant prenylated flavonoids such as furanoflavones, furanoflavonols, chromenoflavones, furano-chalcones, and pyranochalcones [19-22]. The seed and seed oil of this plant have been recommended for the treatment of various inflammatory and infectious diseases such as leucoderma, leprosy, lumbago, muscular and articular rheumatism [23]. The leaves are hot, digestive, laxative, anthelmintic and cure piles, wounds and other inflammations [7]. A hot infusion of leaves is used as a medicated bath for relieving rheumatic pains and for cleaning ulcers in gonorrhea and scrofulous enlargement [24].The different extracts of roots and seeds (ethanol, benzene, petroleum ether) of Pongamia pinnata have already been reported to possess anti-inflammatory and antinociceptive activities [25-27]. And also, 70% ethanol extract of *Pongamia pinnata* leaves (PLE) was evaluated for antiinflammatory activity in rats [28].

Vitex negundo Linn. Verbenaceae, (V.N.) known as Nirgundi in Hindi, grows gregariously in wastelands and is also planted as a hedge-plant. It is an erect, 2–5 m in height, slender tree with quadrangular branchlets distributed throughout India. The leaves have five leaflets in a palmately arrangement, which are lanceolate, 4–10 cm long, hairy beneath and pointed at both ends. The bluish purple flowers are numerous [29]. Among the chemical constituents, it has several flavonoids such as casticin, orientin, isoorientin, luteolin, lutecin-7-O-glucoside, corymbosin; gardenins A and B. Besides, many glycosidic iridoids, alkaloids, and terpenoids have also been isolated [30]. Plant has been documented for potent antiinflammatory, antipyretic and febrifuge properties [31], are claimed, it has also been investigated for an anti-inflammatory [32, 33], anticonvulsant [34, 35], hepatoprotective and bronchial relaxant actions [36]. They are also used as tonics, vermifuge, lactagogue, emmenagogue, antibacterial, antipyretic and antihistaminic agents [37, 38].

The objective of our study was to evaluate the efficacy of poly-herbal formulation and its individual components by virtue of their anti-arthritic potential in laboratory animals.

MATERIAL AND METHODS

Plants material

The Mature fresh leaves (MFL) of *Vitex negundo* and *Pongamia pinnata* were collected from the local region and root of *Cissampelos pareira* Linn were purchase from local vendor. The plant materials were identified and authenticated taxonomically (V. No.BSI/WC/Tech/2009/660) at Botanical Survey of India, Pune.

Preparation of extracts

The roots of *Cissampelos pareira* were washed, cut into small pieces, and dried under shade. Coarse powder of the roots was made and extracted by maceration with 50% aqueous alcohol for 72 h at room temperature. Mature fresh leaves (MFL) of *Vitex negundo* and *Pongamia pinnata* were crushed into powder and extracted by maceration with 50% aqueous alcohol for 72 h at room temperature. The whole extract of individual plants was collected in conical flasks, filtered and the solvents were evaporated to dryness under reduced pressure.

Animals

Wistar rats were used for the study. The animals were housed in solid- bottomed polypropelene cages and acclimatized to animal house conditions. The rats were fed with commercial rat's diet and water ad libitium. The experiments were designed and conducted in accordance with ethical norms approved by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPSCEA) and Institutional Animal Ethical Committee (IAEC).

Preparation of poly-herbal formulation

Poly-herbal formulation was prepared according to ED_{50} of individual herbs. ED_{50} of individual plants was found to be as, *Cissampelos pareira* L. (400 mg/kg), *Vitex negundo* L. (500 mg/kg), *Pongamia pinnata* L (300 mg/kg). The % contents of poly-herbal formulation were calculated from individual ED_{50} of the plants exstracts as, *Cissampelos pareira* L. (33.33 %), *Vitex negundo* L. (41.66%), *Pongamia pinnata* L (25%).

Drugs and dosage

The formulation was administered orally at doses of 200 mg/kg,400mg/kg and 600 mg/kg in the form of suspension prepared in double distilled water containing carboxy methyl cellulose (1%, w/v, CMC). Freund's adjuvant (Complete) was purchased from Sigma-Aldrich USA. Methotrexate tablets (Neotrexate, Mfg by Emil Pharmaceutical's, Tarapur, Thane) was purchased from local market.

Acute toxicity studies

The acute oral toxicity study [39] was carried out as per the guideline set by the Organization for Economic Co-operation and Development (OECD guidelines 425) received from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Drug treatment

Animals were randomly divided into eight groups of five animals each (n=5). Group I served as Control (1% (w/v) CMC in double distilled water p.o). Group II was given reference standard, Methotrexate (0.75 mg/kg p.o). Group III-V served as Test Drug groups as poly-herbal formulation (200, 400 and 600 mg/kg p.o in double distilled water containing carboxy methyl cellulose (1%, w/v, CMC) respectively). Group VI-VIII was given Test Drugs as an individual herb extracts (C.P., 400mg/kg p.o, P.P. 300mg/kg p.o, V.N. 500 mg/kg p.o). The prepared extract was administered once daily for 21 consecutive days.

Freund's adjuvant induced arthritis

Arthritis was induced by injecting a 0.1 ml (0.1% w/v) suspension of killed Mycobacterium tuberculosis bacteria homogenized in liquid paraffin into the left hind paw. Drug treatment was started from the initial day i.e. from the day of adjuvant injection (0 day), 30 minutes before adjuvant injection and continued till 21st day. Paw volume was measured on 4th, 8th, 14th and 21st day by using plethismometer (Panlabs, India). The mean changes in injected paw edema with respect to initial paw volume, were calculated on respective days and % inhibition of paw edema with respect to untreated group was calculated using following formula.

$$i = \left(1 - \left(\frac{\Delta V_{Treated}}{\Delta V_{Untreated}}\right)\right) \times 100$$

Where,

i = % inhibition of paw edema

$\Delta V_{Treated}$ = *Mean change in paw volume of treated rat*

$\Delta V_{Untreated}$ = *Mean change in paw volume of untreated rat*

The changes in body weight were recorded daily. At 22nd day blood was withdrawn through retroorbital vein puncture of all groups by anaesthetizing the animals with diethyl ether and the biochemical parameters like haemoglobin content, total WBC count, ESR and RBC were analysed.

Histopathological analysis

The ankle joint of the hind paw of the rats were removed and separated from the surrounding tissues and weighed. The joints were fixed in 10 % formalin and were decalcified, sectioned and finally stained with haematoxylin and eosin to examine the histopathological changes during the experimental period in all the above groups under light microscope.

Radiological analysis

Before sacrificing the animals, X-rays were taken at the joints of the hind paw of the animals for evaluating the bone damage. Radiographs were taken using X-ray apparatus (Siemans- 60MA, Germany) and industrial X-ray film (Fuji photo film, Japan). The X-ray apparatus was operated at 220 V with a 40 V peak, 0.2 second exposure time, and a 60 cm tube-to film distance for anterior-posterior projection.

Statistical analysis

The experimental results are represented as Mean ± SEM (Standard Error of Mean). Statistical analysis was performed by one-way ANOVA followed by Dunnet's't' test. $P < 0.05$ was considered significant.

RESULTS

From the acute toxicity study, the LD_{50} cut-off dose for poly-herbal formulation extract was found to be 4000 mg/kg body weight. Hence, the therapeutic doses were taken as 600 mg/kg, 400 mg/kg and 200 mg/kg body weight. 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. These inflammatory changes ultimately result in the complete destruction of joint integrity and functions in the affected animal [40].

Methotrexate produced a significant inhibition in the rat paw edema by 72.93 % ($p<0.01$), Individual herb like CP 400 mg/kg, PP 300 mg/kg and VN 500 mg/kg produced 50.41 %, 47.37 % and 44.36% inhibition respectively of rat paw oedema after 21 days . The treatment with poly-herbal formulation (400 mg/kg and 600 mg/kg) produced dose-dependent decreased in the rat paw oedema (70.65 %, 62.35 % and 55.27 % respectively) as compared with the control. However, the poly-herbal formulation (200, 400 and 600 mg/kg) showed synergistic effect in inhibited the rat paw oedema as compared with individual herb like CP 400 mg/kg, PP 300 mg/kg and VN 500 mg/kg (Table 1).

Table 1: It shows effect of poly-herbal formulation on % Inhibition of hind paws edema in rats induced by Freund's adjuvant

Treatments	Paw edema volume					
	Day 1	Day 4	Day 8	Day 14	Day 21	% inhibition on 21st day
Control	1.222 ± 0.07	1.18 ± 0.081	1.116 ± 0.073	1.052 ± 0.089	0.988 ± 0.083	0
Metho.	1.182 ± 0.06	0.798±0.031 **	0.636 ± 0.031**	0.428± 0.069**	0.276± 0.042**	72.93
PF 200	1.078 ± 0.06	0.936 ± 0.026 *	0.81 ± 0.047**	0.6 ± 0.021**	0.442± 0.045**	55.27
PF 400	1.03 ± 0.07	0.856±0.050 **	0.712 ± 0.064**	0.506± 0.046**	0.372± 0.030**	62.35
PF 600	1.026 ± 0.03	0.806±0.047 **	0.692 ± 0.052**	0.442± 0.045**	0.29 ± 0.028**	70.65
CP 400	1.046 ± 0.06	0.926 ± 0.066 *	0.832 ± 0.037*	0.61 ± 0.062**	0.49 ± 0.063**	50.41
PP 300	1.038 ± 0.06	0.936 ± 0.026 *	0.87 ± 0.045*	0.634± 0.047**	0.52 ± 0.064**	47.37
VN 500	1.018 ± 0.02	0.972 ± 0.082	0.882 ± 0.077*	0.622± 0.065**	0.5 ± 0.065**	46.36

Values are expressed as Mean ± SEM; n =5. * *P < 0.05; ** P < 0.01*. CP- *Cissampelos pareira*,PP- *Pongamia pinnata*,VN- *Vitex negundo*.Metho-Methotrexate.

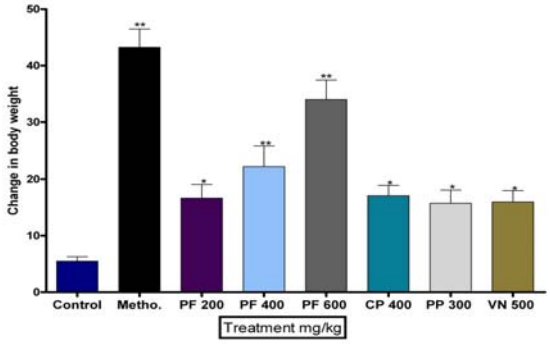


Fig. 1: It shows effects of poly-herbal formulation on change in body weight (n=5).

The mean paw edema counts were expressed as Mean ± SEM, * P<0.05 and ** P < 0.01 (ANOVA followed by Dunnett's test), CP-*Cissampelos pareira*, PP- *Pongamia pinnata*, VN- *Vitex negundo,* PF-Polyherbal formulation.

The loss of body weight was observed during the arthritis condition. The standard drug methotrexate and poly-herbal formulation (200 mg/kg, 400 mg/kg and 600 mg/kg) extract treatment significantly increased the body weight (Figure 1).

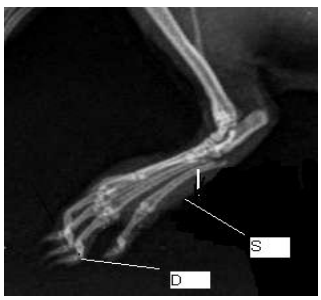
However, Polyherbal formulation (200 mg/kg, 400 mg/kg and 600 mg/kg) extracts and standard drug treated group significantly decreased (P<0.01) the total WBC count. The ESR count, which drastically increased in arthritic control group, has been remarkably counteracted by the standard and poly-herbal formulation, restoring it back to normal, thus justifying its significant roles in arthritic conditions (Table 2).

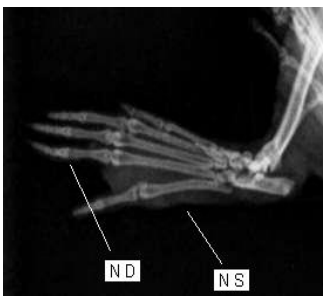
Table 2: It shows effect of poly-herbal formulation on changes in haematological parameters.

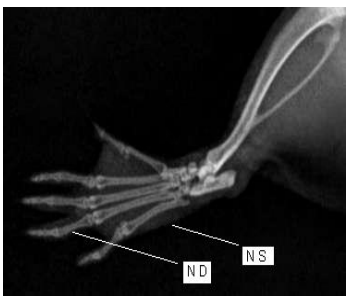
Treatments (mg/kg)	Changes in haematological parameters (Mean ± SEM)			
	Total WBC Count (cells/cu. mm)	RBC Count (million/cu.mm)	Hb (gm%)	ESR (mm/hr)
Control	9300 ± 152.7	5.43± 0.185	7.5 ± 0.152	7 ± 0.577
Metho.	7100 ± 208.1**	7.03± 0.120**	9.466 ± 0.120**	3.6 6 ± 0.333**
PF 200	7930 ± 35.11**	6.16 ± 0.145*	8.8 ± 0.173*	5.66 ±0.333
PF 400	7666.6 ± 88.19**	6.6 ± 0.152**	10.03 ± 0.463**	4.66 ± 0.333**
PF 600	7400 ± 57.73**	6.83 ± 0.202**	10.06 ± 0.388**	4 ± 0.577**
CP 400	8466.6 ± 120.1*	5.3±0.1	7.733 ± 0.166	6.66 ± 0.333
PP 300	8333.3 ± 437.1*	6.03 ± 0.120	7.73 ± 0.284	6.33 ±0.333
VN 500	8466.6 ± 176.3*	6.16 ± 0.260*	7.66± 0.233	7 ± 0.577

Values are expressed as Mean ± SEM; n =5. * *P < 0.05; ** P < 0.01*. CP- *Cissampelos pareira*,PP- *Pongamia pinnata*,VN- *Vitex negundo*.Metho-Methotrexate.

Methotrexate treated group rats show the normal architecture of phalangeal joint space (a), where as individual herb treated group rats show the narrowing of metatarsal and phalangeal joint space and diffused joint in phalangeal region and deformity in shape. Soft tissue swelling and bending of metatarsal and phalangeal joints can be seen. In poly-herbal formulation treated (400 mg/kg and 600 mg/kg) animals, these changes were normalized. The joint space of metatarsal and phalanges were observed to have been regenerated which indicates its protective effect on arthritis (Figure 2).







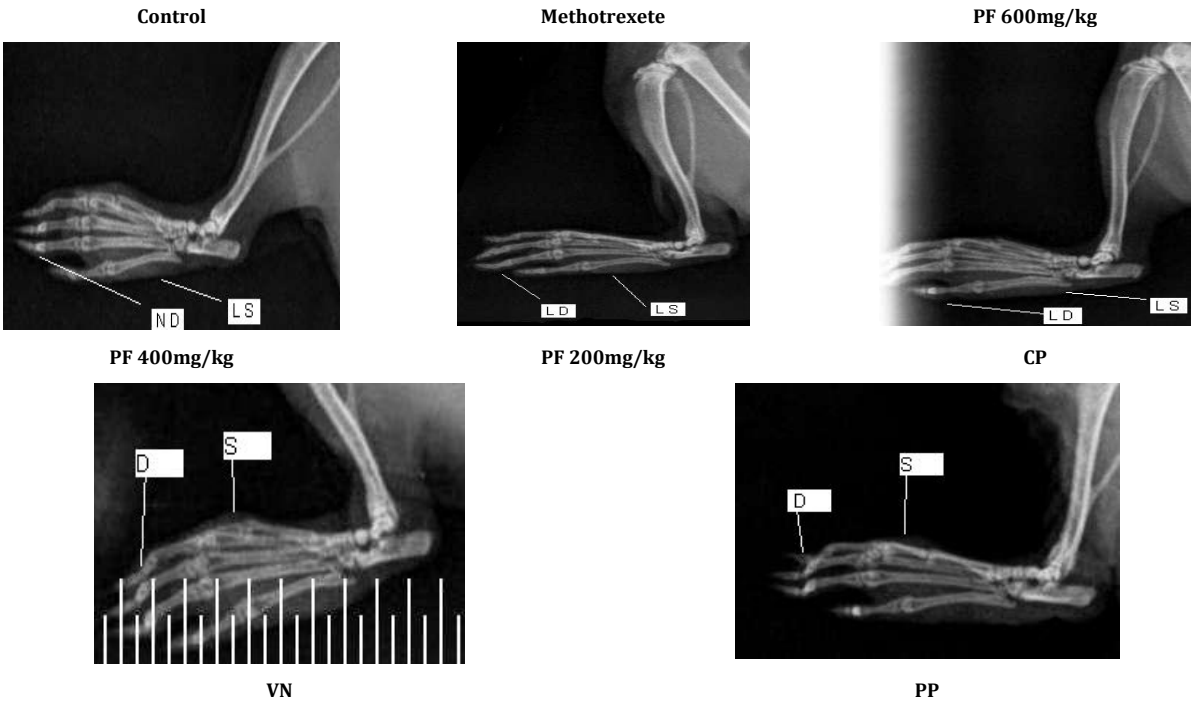
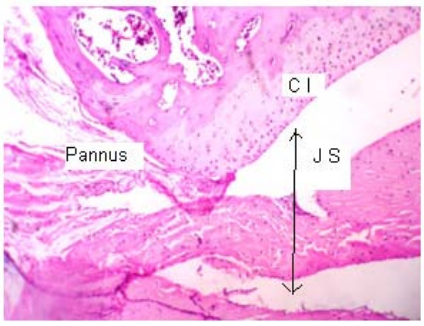


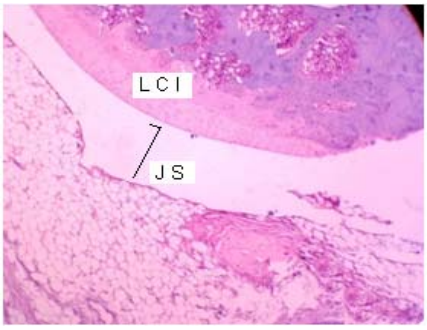
Fig. 2: It shows radiographic analysis, radiograph of the proximal interphalangeal joints of control and experimental animals and effects of poly-herbal formulation, on histopathological study of rats. CP- *Cissampelos pareira*, PP- *Pongamia pinnata*, VN- *Vitex negundo.*

Control animal (Arthritis) showing soft tissue swelling with diffused joint in phalangeal region, bending of phalangeal joints and narrowing of joint space were observed. Polyherbal formulations showing no narrowing of joint space and resembling near normal radiographic pattern of the joints.(D- Deformity, S- Swelling, LD- Low deformity, LS- low swelling, ND- No deformity, NS- No swelling)

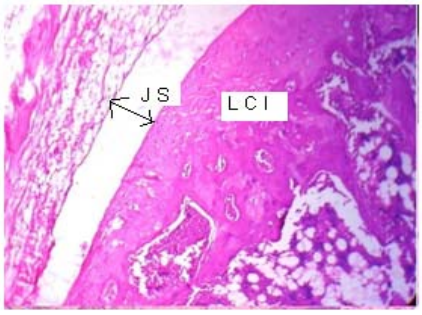
The histological changes in ankle joints of control shows with a massive influx of inflammatory cells, synovial hyperplasia, and accumulation of abundant monomorphonuclear and poly-morphonuclear cells in the joint space and congestion of vessels. Synovial proliferation with granulation tissues adjacent to the damaged articular cartilage was seen. Individual plants extracts showed little massive influx of inflammatory cells, synovial hyperplasia, and accumulation of abundant monomorphonuclear and polymorphonuclear cells in the joint space and congestion of vessels (Figure 3).

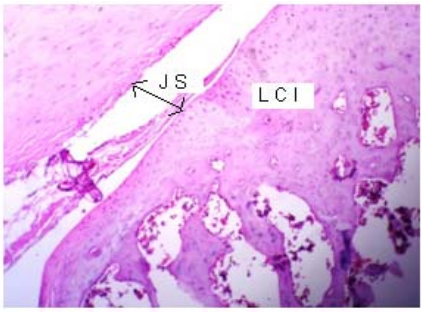


Polyherbal 200 mg/kg



Polyherbal 400 mg/kg





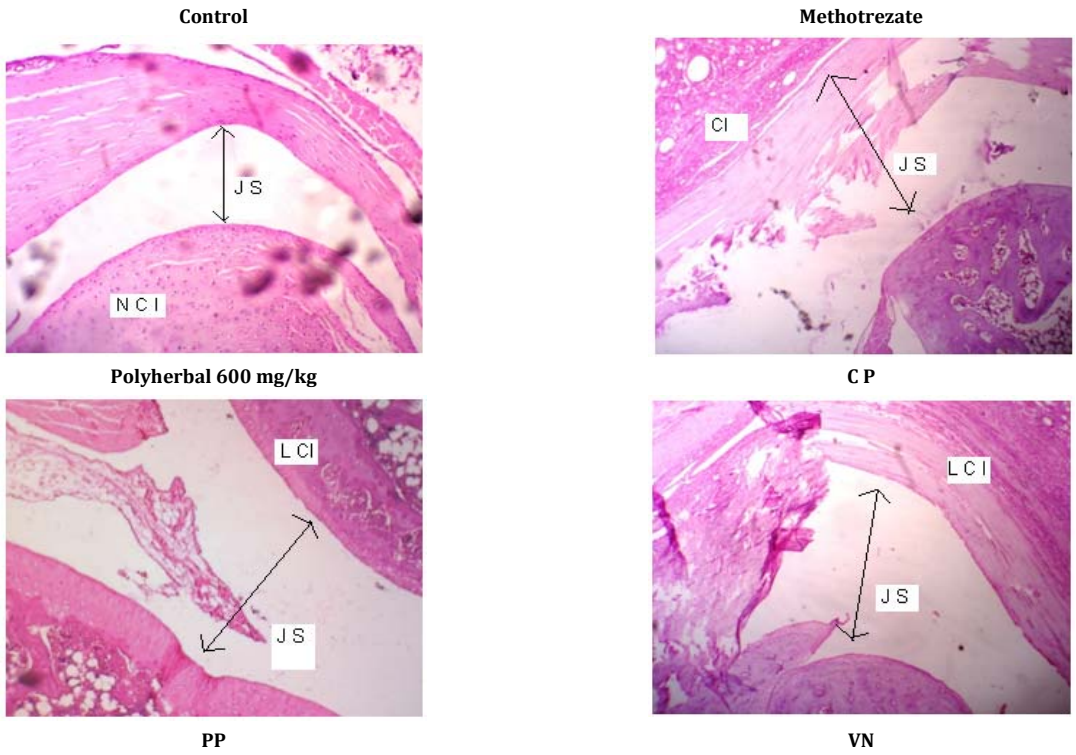


Fig. 3: It shows histological analysis

Figure 3 shows histological changes in joints of control and experimental animals. Standard drug section showing joint cavity with synovial lining and normal joint space in between two articular cartilages . Section of joint cavity of arthritis rats showing proliferation with granulation tissue adjacent to the damaged articular cartilage. Section of joint cavity of arthritis rats showing mononuclear cell infilteration . Section of joint cavity of plyherbal formulations treated rats showing normal architecture of both cartilages with no granulation tissue seen but only small fibrous strands in between the cartilage was observed. CP- *Cissampelos pareira*,PP- *Pongamia pinnata*,VN- *Vitex negundo.*(- joint space, CI- cellular infiltration, LCI – low cellular infiltration)

DISCUSSION

In the present study, rats were selected to induce arthritis because rats develop a chronic swelling in multiple joints with influence of inflammatory cells, erosion of joint cartilage and bone destruction. It has close similarities to human rheumatoid disease [41]. The determination of paw swelling is apparently simple, sensitive and quick procedure for evaluating the degree of inflammation and the therapeutic effects of drugs. Chronic inflammation involves the release of number of mediators like cytokines (IL-IB and TNF-α), GM-CSF, interferon's and PGDF. These mediators are responsible for the pain, destruction of bone and cartilage that can lead to severe disability [42]. However, standard drug methotrexate and poly-herbal formulations extract significantly suppressed the swelling of the paws. In arthritis condition, there is a mild to moderate rise in WBC count due to release of IL-IB inflammatory response. IL-IB increases the production of both granulocyte and macrophages colony stimulating factor [42, 43].

In the present investigation, the migration of leucocytes into the inflamed area is significantly suppressed by the standard drug and poly-herbal formulations extract as seen from the significant decrease in total WBC count. Erythrocyte sedimentation rate (ESR) is an estimate of the suspension stability of RBC's in plasma. It is related to the number and size of the red cells and to the relative concentration of plasma proteins, especially fibrinogen and β globulins. Increase in the rate is an indication of active but obscure disease processes. The acute phase proteins in ESR and C-reactive protein (CRP) share the property of showing elevations in the concentration in response to stress or inflammation like injection, injury, surgery and tissue necrosis. The ESR count, which drastically increased in arthritic control group, has been remarkably counteracted by the standard, poly-herbal formulations extracts and back to normal, thus justifying its significant role in arthritic conditions [43].

Changes in body weight have also been used to assess the course of the disease and the response to therapy of anti-inflammatory drugs [44]. As the incidence and severity of arthritis increased, the changes in the body weights of the rats also occurred during the course of the experimental period. The loss of the body weight during arthritic condition was also supported by earlier observation [45] on alterations in the metabolic activities of diseased rats. Earlier findings suggest that absorption of ^{14}C- glucose and ^{14}C- leucine in rat's intestine was reduced in the case of inflamed rats [46]. But on the treatment with anti-inflammatory drugs, the decrease in absorption was nullified [47] and it shows that the anti-inflammatory drugs correct the decreased/deranged absorption capacity of intestine during inflammation. The increased body weight during treatment of standard drug, poly-herbal formulations extracts may be due to the restoration of absorption capacity of intestine.

It the present investigation the arthritic rats showed a soft tissue swelling that was noticeable around the ankle joints during the acute phase of arthritis and was due to be edema of periarticular tissues such as ligaments and joint capsules. The swelling has been found to be increasing in the initial phase of inflammation and then becomes constant in 2 weeks. These changes in paw volume have been found to be associated with an increase in granulocytes and monocytes [48]. Because, the activation of macrophages results in the production of several cytokines including IL-1, IL-6, interferon-γ (IFN-γ) and TNF-α which have been implicated in immune arthritis [49, 50]. TNF-α is mainly involved in the perpetuation of the

inflammatory cascades in autoimmune diseases, which affect connective tissues where the connective tissues become hypercontracted due to inflammation [51]. Furthermore, macrophage-derived NO may increase vasodilation and vascular permeability at the inflammatory site, which may aggravate the arthritic process [52, 53]. Moreover, prostaglandins greatly potentiate exudates by inducing relaxation of arteriolar smooth muscle cells and increasing the blood supply to the tissue [54].

The potent anti-arthritic effect of poly-herbal formulations as further confirmed by radiological studies. The diagnosis of RA is usually obvious clinically and it allows therapeutic monitoring which remains the standard method in evaluating disease progression. The X-ray appearance, commonly referred to as diminished joint space is the hallmark of arthritis [54]. In control rats, erosion representing bony destruction were evident on bone unprotected by cartilage, since they are exposed directly to cytokines such as TNF- α and IL-1 which stimulate the chondrocytes to produce proteolytic enzymes such as collagenases, glycohydrolases and neutral proteases degrading the cartilage. As a result, the pannus invades the joint and sub-chondral bones and eventually the joint is destroyed and undergoes fibrous fusion or ankylosis. These changes were reverted back to near normal upon poly-herbal treatment.

From the results observed in the current investigation, it may be concluded that the poly-herbal formulation (200 mg/kg, 400 mg/kg and 600 mg/kg) can be employed as potential anti-arthritic formulation since it was active in both the inflammation models and adjuvant.

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