3. Rheumatoid arthritis progress in three stages. The first stage is the...
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.BS1/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 polypropylene cages and acclimatized to animal house conditions. The rats were fed with commercial rat’s diet and water ad libitum. 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 (CPCSEA) and Institutional Animal Ethical Committee (IAEC).

Preparation of poly-herbal formulation

Poly-herbal formulation was prepared according to ED₅₀ of individual herbs. ED₅₀ 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 ED₅₀ of the plants extracts 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 distill 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 plethysmometer (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.

\[ i = \frac{1 - \left( \frac{\Delta V_{\text{Treated}}}{\Delta V_{\text{Untreated}}} \right)}{100} \]

Where,

\( i \) = % inhibition of paw edema

\( \Delta V_{\text{Treated}} = \) Mean change in paw volume of treated rat

\( \Delta V_{\text{Untreated}} = \) 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 test. P< 0.05 was considered significant.

RESULTS

From the acute toxicity study, the LD₅₀ 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).

It shows effect of poly-herbal formulation on % inhibition of hind paws edema in rats induced by Freund’s adjuvant

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day 1</th>
<th>Day 4</th>
<th>Day 8</th>
<th>Day 14</th>
<th>Day 21</th>
<th>% inhibition on 21st day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.222 ± 0.07</td>
<td>1.18 ± 0.081</td>
<td>1.116 ± 0.073</td>
<td>1.052 ± 0.089</td>
<td>0.988 ± 0.083</td>
<td>0</td>
</tr>
<tr>
<td>Metho.</td>
<td>1.182 ± 0.06</td>
<td>0.796±0.031 **</td>
<td>0.636 ± 0.031 **</td>
<td>0.428±0.069 **</td>
<td>0.276±0.042 **</td>
<td>72.93</td>
</tr>
<tr>
<td>PF 200</td>
<td>1.078 ± 0.06</td>
<td>0.936 ± 0.026 *</td>
<td>0.81 ± 0.047 **</td>
<td>0.6 ± 0.021 **</td>
<td>0.442±0.045 **</td>
<td>55.27</td>
</tr>
<tr>
<td>PF 400</td>
<td>1.03 ± 0.07</td>
<td>0.85±0.050 **</td>
<td>0.712 ± 0.064 **</td>
<td>0.506±0.046 **</td>
<td>0.372±0.030 **</td>
<td>62.35</td>
</tr>
<tr>
<td>PF 600</td>
<td>1.026 ± 0.03</td>
<td>0.806±0.047 **</td>
<td>0.692 ± 0.052 **</td>
<td>0.442±0.045 **</td>
<td>0.29±0.028 **</td>
<td>70.65</td>
</tr>
<tr>
<td>CP 200</td>
<td>1.046 ± 0.06</td>
<td>0.926±0.037*</td>
<td>0.832 ± 0.064 **</td>
<td>0.61±0.062 **</td>
<td>0.49±0.063 **</td>
<td>50.41</td>
</tr>
<tr>
<td>CP 400</td>
<td>1.038 ± 0.06</td>
<td>0.936±0.045*</td>
<td>0.87 ± 0.045*</td>
<td>0.63±0.047 **</td>
<td>0.5±0.064**</td>
<td>47.37</td>
</tr>
<tr>
<td>CP 600</td>
<td>1.018 ± 0.02</td>
<td>0.972±0.082</td>
<td>0.882 ± 0.077**</td>
<td>0.62±0.065**</td>
<td>0.5±0.065**</td>
<td>46.36</td>
</tr>
</tbody>
</table>

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.

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 arthritis 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>
<thead>
<tr>
<th>Treatments (mg/kg)</th>
<th>Changes in haematological parameters (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total WBC Count (cells/cu. mm)</td>
</tr>
<tr>
<td>Control</td>
<td>7100 ± 208.1**</td>
</tr>
<tr>
<td>Metho.</td>
<td>7930 ± 35.11**</td>
</tr>
<tr>
<td>PF 200</td>
<td>7666.6 ± 88.19**</td>
</tr>
<tr>
<td>PF 400</td>
<td>7400 ± 57.73**</td>
</tr>
<tr>
<td>PF 600</td>
<td>8466.6 ± 120.1*</td>
</tr>
<tr>
<td>CP 200</td>
<td>8333.3 ± 437.1*</td>
</tr>
<tr>
<td>VN 500</td>
<td>8466.6 ± 176.3*</td>
</tr>
</tbody>
</table>

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).
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 polymorphonuclear 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).
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. 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-1, IL-6, interferon-γ, TNF-α) responsible for proliferation of granulocyte and macrophages colony stimulating factor (IFN-γ) and TNF-α which have been implicated in immune arthritis.

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 (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.

Changes in body weight have also been used to assess the course of the disease and the response to therapy of anti-inflammatory drugs. 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 on alterations in the metabolic activities of diseased rats. Earlier findings suggest that absorption of 14C-glucose and 14C-leucine in rat’s intestine was drastically reduced in the case of inflamed rats. But on the treatment with anti-inflammatory drugs, the decrease in absorption was nullified 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. 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.

TNF-α is mainly involved in the perpetuation of the
inflammatory cascades in autoimmune diseases, which affect connective tissues where the connective tissues become hypervascularised due to inflammation. Moreover, prostaglandins greatly potentiate exudates by inducing relaxation of arteriolar smooth muscle cells and increasing the blood supply to the tissue. The potent anti-arthritic effect of poly-herbal formulations as further confirmed by radiological studies. The diagnosis of RA is based on clinical features and confirmed by radiological studies. The diagnosis of RA is made based on clinical criteria and confirmed by radiological studies.

Furthermore, NO may increase vasodilation and vascular permeability at the inflammatory site, which may aggravate the arthritic process. The X-ray appearance, commonly referred to as diminished joint space is a hallmark of RA.

The potentiating effect of NO on the production of arachidonic acid and the subsequent production of prostaglandins and leukotrienes can contribute to the development of inflammatory diseases.

References

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