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ASSESSMENT OF ANTI-ARTHRITIC ACTIVITY OF A TRADITIONAL POLY HERBAL FORMULATION

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ABSTRACT
Background: The medicinal uses of plants are in many cases based exclusively on traditional knowledge without enough scientific evidences. A polyherbal herbal formulation (Vaatrog Nashak Churna) is traditionally used for treatment of wide variety of inflammatory ailments including arthritis and joints pain.
Objective: The present study aimed to evaluate the anti-arthritic and anti-noceciptive activities of Vaatrog Nashak Churna (VNC) in Complete Freund's Adjuvant (CFA) and Turpentine oil animal models for commercial mass production of botanicals as a medicinal product.
Methods: Complete Freund's adjuvant (CFA)-induced arthritis in rats was used as chronic disease model. CFA-induced inflammatory paw edema, body weight, arthritic index, hematological parameters, serum analysis and histopathological analysis were all evaluated for assessment of disease progression. Turpentine oil model was applied as acute disease model through which joint edema was taken as assessment parameter to check therapeutic potential of VNC.
Results: The analysis of various arthritic assessment parameters used in this study revealed that VNC extracted with 50 % hydro-acetone have a considerable effect in preventing development or ameliorate arthritis disease severity. Moreover, the formulation revealed significant anti-nociceptive activity at in both CFA-induced and Turpentine oil induced arthritis rats.
Conclusion: 50 % hydro-acetone extract of VNC appears to be a really promising as anti-arthritic and analgesic formulation, but larger and more detailed preclinical and clinical studies especially in human is highly recommended.
KEYWORDS: Vaatrog Nashak Churna, Arthritis and joint pain, Complete Freud's Adjuvant, Turpentine oil, 50 % Hydro-acetone extract

INTRODUCTION
Rheumatoid arthritis (RA) is a chronic rheumatic disease, characterized by progressive articular damage and extra-articular manifestations such as inflammation, pain, and stiffness, which can lead to permanent disability and which is associated with a mortality rate higher than that in the general population. RA is the most prevalent systemic autoimmune disease among the rheumatic inflammatory musculoskeletal diseases. It causes significant morbidity and mortality, affecting 1% of the world population. In India, the prevalence of RA is estimated to be 0.7% which is higher than the global prevalence of 0.46%. This inflammatory disorder is more common in females in comparison to males (5:1).
Arthritis is a multifactorial disorder with elusive pathogenesis and the underlying pathogenic mechanisms are in progress to be characterized at the molecular level. This disease prevails in urban areas compared to rural areas due to genetic and socioeconomic risk factors. Any infection or other environmental factor can stimulate these genes which can lead to arthritis.

Normally, the lining of a joint is very thin and has very few blood vessels and white blood cells, but in rheumatoid joints, the lining becomes thick and the number of vessels and white blood cells is increased. Interlukin-1 and TNF-alpha (tumor necrosis factor) are secreted by white blood cells. They cause swelling, pain and joint destruction. Recently, novel cytokines, such as Interleukin-18, Interleukin-17 and Receptor activator of nuclear factor kappa-B ligand were discovered in the arthritis pathogenesis.

Cytokines perpetuate the complement system and immune complex which cause the onset of rheumatoid arthritis. Immune complexes are affected by metalloproteinases. CD4+T-cells are activated by antigens and then stimulate macrophages, monocyte and synovial fibroblasts. As a result, TNFa cytokines interlukin-6 and interlukin-1 are produced. At the start of rheumatoid arthritis, synovial fluid has a large number of neutrophils. Projections are formed by hyperplasia and hypertrophy into joint capsule, IgG/anti-IgG antigen antibody complexes are immune complexes found in synovial fluid. Key pathogenic markers of arthritis are rheumatoid factors (IgM and IgA). The pathophysiology of RA involves a cascade of processes taking place; therefore, arthritis necessitates a multidimensional approach for its management.

Most of RA patients suffer long-lasting illnesses, which significantly reduce their levels of physical activity and negatively impact their quality of life. RA can be difficult to treat, and it generally necessitates lifelong therapy, but advances in management paradigms and the development of more effective treatments have resulted in considerable progress such as biological/targeted disease-modifying anti-rheumatic drugs therapy. But RA treatment is expensive, particularly with biologics/targeted therapies; which has a significant economic impact. In India the healthcare system is characterized by a mix of public and private providers. The majority of Indians seek treatment from the private sector, where over two-thirds of overall health spending is through out-of-pocket. The high cost of care and a lack of health insurance coverage exacerbate the financial strain on households in the lower-socioeconomic strata. On the other hand the effectiveness and safety parameters are still under surveillance as far as newer approaches for therapy is concerned. To overcome the shortcomings of synthetic drugs, continuous efforts have been made to explore the efficacy of medicinal herbs and their phytochemicals in the treatment and prevention of inflammatory diseases.

Since time immemorial, medicinal plants and natural bio-active compounds have been found to be potent against the majority of ailments including inflammatory diseases such as arthritis. Natural products can control arthritic inflammation through multiple pathways, for example, inhibition of effectors molecules, pro-inflammatory cytokines, chemokines etc.

Herbal medicines are in widespread use and although many believe herbal medicines are safe, they are often used by formulating herbs and herbal ingredients into certain formula (known as poly herbal formulation, PHF) have been shown to have potential interaction effects.

A major hypothetical advantage of botanicals over conventional single component drugs is the presence of multiple active compounds that together can provide a potentiating effect that may not be achievable by any single compound. PHF have plant-based pharmacological agents which may exert synergistic, potentiative, agonistic antagonistic actions by virtue of its associated diverse active principles themselves. Various classes of herbal compounds have been clinically evaluated in most recent twenty years with noteworthy anti-inflammatory activity. Flavonoid, phenols, tannins, terpenoids, alkaloids and stilbenoids are the most significant class. Therefore, the search for new anti-arthritic drugs alongwith existing traditional polyherbal formulation for effective therapies is still an important field in drug discovery.

In the present study, we investigated the anti-arthritic potential of Vaatrog Nashak Churna (VNC) using CFA and turpentine oil animal protocol.

MATERIALS

Animals

The study was carried out in Central Laboratory of Chhattisgarh Council of Science and Technology, Raipur, India, after approval of the experimental design protocol (10/IAEC/CCOST/2022) by the Institutional Animal Ethics Committee. Working institute provided adult male Wistar Albino rats (200-250 gm) with basic animal feed. Animals were housed under standard laboratory conditions at 25 ± 20C in groups of three with access to food and water ad libitum. They were acclimatized to the laboratory conditions for a period of 5 days before the study. After completion of the study, all the animals were euthanized by an overdose of anaesthetic di-ethyl-ether and the carcasses were disposed in accordance with institute regulations.

Reagents

The Chhattisgarh Council of Science and Technology's central laboratory in Raipur, India, supplied the Complete Freund's Adjuvant, Turpentine Oil, 1% Gum Acacia, Methotrexate (Mexate 2.5; Zydus, India), Indomethacin (Indocap; Jagsonpal Pharmaceuticals, India) NaCl solution (0.9% w/v).). All other chemicals used in this study were of Analytical grade.

Plant Materials

Herbs were collected from natural habitats around the vicinity of Belgahna, Bilaspur, Chhattisgarh, India; which were collected with the help of a local healer. The plants were taxonomically identified, authenticated (Authentication No. Bot/GGV/2023/81) by Department of Botany, Guru Ghasidas Vishwavidhyalaya, Bilaspur, Chhattisgarh, India. Plant materials were shade dried, cut into small pieces and pulverized using a mechanical grinder. The powder was passed through sieve number 6, 7,8,12 and 18 after that stored in an air-tight container for further use.

Formulation Preparation

Plant material was taken in the proportions as mentioned in Table 1. Plant extract was prepared as per the standard procedure of double phase maceration as mentioned in Ayurvedic Formulary of India. 50 % Hydro-ethanol and 50 % Hydro-acetone solvents were used for extraction. Furthermore UV-Vis spectrophotometric estimation was performed to select the best solvent system for further animal study.

Table 1: Details of individual herbs of VNC with dose

S.No.	Botanicals	Part	Human Dose	Animal Dose*	Quantity for Extraction
1	*Soymida febrifuga (Roxb.) A. Juss.*	Bark	2 gm	0.29 gm	8 gm
2	*Premna herbacea (Roxb.)*	Root	2 gm	0.29 gm	8 gm
3	*Cissus repanda (Wight & Arn) Vahl*	Root	3 gm	0.43 gm	12 gm

* 1 gm/kg dose of VNC was found to be safe according to the analytical data of acute and sub-acute toxicity study.

STUDY DESIGN

Acute Oral Toxicity Study

After receiving a single oral dose of 2000 mg/kg/p.o. of VNC, all five rats survived and exhibited no adverse effects. More than 2000 mg/kg may be the LD50 value. After reviewing

Organization for Economic Cooperation and Development Test Guidelines (OECD TG)-407, the 1000 mg/kg dose was selected for the sub-acute toxicity investigation since it was deemed safe.

UV-Vis Spectrophotometric Estimation

UV-Vis spectrophotometric analysis is one among various quality control parameters which would provide both qualitative and quantitative standards. But both qualitative and quantitative analysis needs markers for confirmation. An attempt is made to study UV-Vis Spectrometric analysis of VNC for understanding qualitative parameters (investigating particular class of herbal chemicals) without markers.

It is well established statement that flavonoid, tannin and phenolic class of herbal chemicals are commonly found in those medicinal herbs which have been scientifically reported for their anti-inflammatory and anti-arthritic activity. Here, VNC was investigated for qualitative analysis of flavonoid, tannin and phenolic herbal constituents.

Sample analysis was conducted at room temperature. System calibration was automatically programmed in the Spectrophotometer. Analysis was done with two cuvettes in which one contained distilled water as a blank solution and another contained 50 % hydro-ethanol and 50 % hydro-acetone extract of VNC separately.

The method of Harborne was adopted for the qualitative preliminary phytochemicals screening:

Estimation of Phenolic Content

The total phenolic content of 50% hydro-ethanol and 50 % hydro-acetone extract of VNC was measured using Folin-Ciocalteu reagent. The extracts were solubilized in distilled water separately. After that 100 µl of sample was mixed with Folin-Ciocalteu reagent (500 µl), sodium carbonates (400 µl) and distilled water (5 ml). This solution was kept at room temperature for 30 min, and the absorbance of solution was measured at 725 nm.

Estimation of Flavonoid Content

The extracts about 500 µl were mixed with methanol (1.5 ml), aluminum chloride (0.1 ml, 10 %), potassium acetate 0.1 ml, 1 M) and water (2.5 ml). The solution was kept at ambient temperature for 40 min, and measured the absorbance of solution at 415nm using spectrophotometer.

Estimation of Tannin Content

0.5 gm of the powdered material was weighed and transferred to a 250 ml conical flask. 75 ml of water was added and boiled for 30 min. The supernatant was collected after centrifuging at 2,000 rpm for 20 min. The volume was made up to 100ml in a volumetric flask. 1 ml of the sample extracts were transferred and added to 75 ml of water. 5ml of Folin-Denis reagent was added to 10ml of sodium carbonate solution and diluted to 100ml with water. All the reagents in each tube were mixed well and kept undisturbed for about 30 min. The absorbance was read at 700nm.

Quantitative estimation was performed by using above mentioned protocols and the findings are exhibited in table 2.

Table 2: Absorbance value of listed extracts at reported wavelength

S.No.	Extract	Wavelength	Absorbance	Inference
1	50 % Hydro-ethanol extract	725	0.133	Phenol
		415	0.227	Flavonoid
		700	0.275	Tannin
2	50 % Hydro-acetone extract	725	0.278	Phenol
		415	0.301	Flavonoid
		700	0.130	Tannin

On the basis of estimated analytical result, it was clear that the absorbance value of 50 % Hydro-acetone extract was comparatively higher than that of 50% Hydro-ethanol extract of VNC. According to Beer- Lambert's law absorbance is directly proportional to concentration; therefore the concentration of phenol, flavonoid and tannin of hydro-acetone extract was expected to be high.
Thus 50% hydro-acetone extract of VNC was selected for further animal study.

CFA (complete Freund's adjuvant) Induced Arthritis Protocol [Chronic model for anti-arthritic activity]
The animals were randomly assigned into four different groups of six animals per group (n=6). Group I (Normal control) no CFA injection, group II (Induced group) no treatment only vehicle, group III given methotrexate 0.25 mg/kg/p.o./day (reference standard group) and group IV (test group) given 1000 mg/kg/p.o./day of VNC.
The bottle of CFA (1 mg/ml *Mycobacterium tuberculosis*, heat killed and dried) was vortexed prior to use to prevent sedimentation of Mycobacteria during storage. 0.5 ml CFA was added to 0.5 ml sterile 0.9% saline to obtain final 0.5 mg/ml mycobacterium emulsion. Then Hind limbs of all groups except group I were shaved, sterilized by 70% (v/v) alcohol subsequently 0.1 mL of CFA was injected subcutaneously in sub-plantar of the left hind paw of each animal (except normal control group) 30 minutes after the administration of vehicle/ drug under mild anesthesia with diethyl ether. The time of adjuvant injection was referred as day 0. The daily oral doses of vehicle/VNC/methotrexate were started on day 0 and continued to day 21 post injection.
The daily oral dose for all treatment groups (group III and IV) and CFA control group were administered at 10 -11 am and the measurements were conducted at 3-4 pm.
On day 21 post CFA injection, all animals were sacrificed and blood as well as hind paw edematous tissue was collected for further investigations.

Turpentine Oil Induced Joint Edema [Acute model for anti-arthritic activity]
Rats were fasted 24 hour before experimentation with free excess of water. Animals were divided into three groups (n=6). Group I served as induced group and received vehicle only, group II received indomethacin (10 mg/kg, p.o) as standard group, and group III (test group) received VNC (1000 mg/kg/p.o.). Acute non- immunological inflammatory joint edema was produced by 0.02 ml of turpentine oil into the synovial cavity of right knee joint. An intra-articular injection was performed in anesthetized rat with the use of a Hamilton syringe, with a 26 G needle inserted through the patellar ligament into the joint space of the knee, 30 minutes after the administration of vehicle/indomethacin/VNC.

Assessment of Anti-Arthritic Activity
Parameters for CFA Model
Effect of VNC on CFA- Induced Paw Edema
The progression of CFA induced inflammation was evaluated by measuring the change in paw

volume on day 5, 9, 13 and 21 after induction of inflammation. At the end of the experiment, change in paw volume with percentage inhibition was determined.
Percent inhibition of edema was calculated as per the following formula:

$$\text{Percent inhibition of paw edema} = \frac{Vc - Vt}{Vc} \times 100$$

Where, Vc = Paw volume of CFA control group animal, Vt = Paw volume of treatment group (reference standard and test) animal.

Assessment of Arthritic Index

The arthritis score was used to evaluate the arthritis damage of both hind paw in different groups at days 5, 9, 13, and 21 post injection of the trial. Development and severity of induced arthritis were evaluated by a visual scoring system (Table 3) of the clinical signs and symptoms. Arthritis scores (for one limb) were defined as follows:

Table 3: Visual scoring system according to manifested signs on affected body part

S.No.	Body Part	Signs and Symptoms	Score
1	One finger	Normal status (no arthritis)	0
		Redness or swelling of one finger	0.1
2	Big joint (ankle, tarsus and wrist)	Normal status (no arthritis)	0
		Mild but definite redness and swelling	0.5
		Severe redness and intense swelling	1

The arthritis score for a given limb ranged from 0 to 1.5 and the global arthritis score (four limbs) ranged from 0 to 6.
The clinical scores were further divided into five grades (Table 4) for arthritic indexing:

Table 4: Clinical scores according to grade for calculating arthritic index

S.No.	Grade	Score
1	0	0
2	1	Between 0.1 and 0.9
3	2	Between 1 and 1.9
4	3	Between 2 and 2.9
5	4	Between 3 and 3.9
6	5	More than 4

Assessment of Body Weight Change

On day 0 and day 21 post CFA injection; the body weights of the rats in the groups were weighed using a precision balance and the results were recorded. The percent body weight change was calculated using the following formula:

$$\%\, Body\ Weight\ Change = \frac{Wt - Wo}{Wt} \times 100$$

Where Wt is the weight of animal at time t and Wo is the weight of animal at day 0.

Assessment of Hematological and Serum Parameters

On day 21 post CFA injection blood was withdrawn through cardiac puncture from all groups under mild anesthesia with diethyl ether and kept in a suitable blood collection tubes (BD Vacutainer®). The hematological parameters including red blood corpuscles (RBC), Hemoglobin (Hb) count, White Blood Corpuscles (WBC), Packed Cell Volume (PCV) and Erythrocyte Sedimentation Rate (ESR) levels; serum parameters including serum Tumor

Necrosis Factor (TNF)-∝ and Interleukins (IL)-1β levels, C-Reactive Protein (CRP) and Rheumatoid Factor (RF) were evaluated immediately after blood sample collection.

Histopathological Analysis of Edematous Paw

For histopathology, the right paw (edematous paw) were removed, washed with saline and stored in 10% formalin. Tissues were dehydrated, processed and embedded in paraffin wax. 4 µm sections were prepared and stained with hematoxylin and eosin (H&E) and observed under light microscope.

Parameter for Turpentine Oil Model

Assessment of Turpentine Oil - Induced Joint Edema

Diameter of the injected joint (right knee) was measured every hour till 6 hours after turpentine oil injection using a calibrated micrometer screw gauge. The percentage inhibition of left paw edema was calculated by following formula:

$$\% \text{ Inhibition of Edema} = \frac{Ec - Et}{Ec} \times 100$$

Where, Ec represents edema at a given time of control group and Et represents edema at a given time of test group.

STATISTICAL ANALYSIS

The values were expressed as mean ± SD. The statistical significance between groups was analyzed by one- way analysis of variance (ANOVA) followed by Tukey's post hoc t-test. $p < 0.05$ was considered to be statistically significant where $*p < 0.05$ for Control vs Induced; Induced vs Standard/Test and $**p < 0.05$ for Standard vs Test.

RESULT

Paw Edema

CFA injected animals exhibited a marked unilateral peripheral edema in paw as compared to standard and test group animals (Figure 1). Standard group, which was treated with the standard drug Methotrexate, showed significant and maximum decrease in paw volume (0.34 ± 0.01 ml, $p < 0.01$) on day 21 of the experiment as compared to CFA induced group. Test group which was treated with VNC, also showed moderate decrease in paw volume (0.55 ± 0.02 ml).

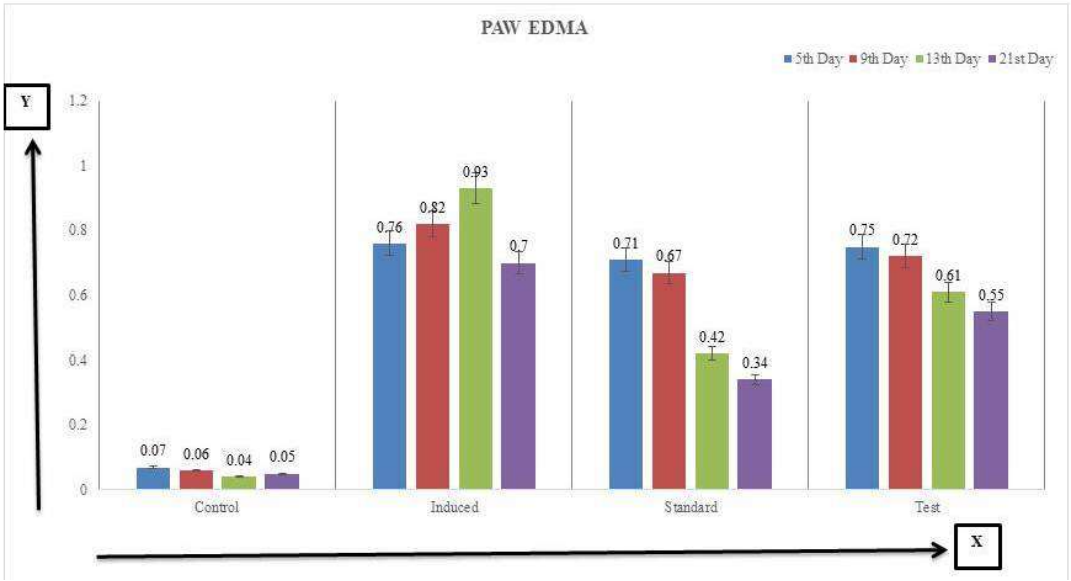

Figure 1: Effect of VNC (test group) on paw edema volume. Y axis represents paw volume (mL) and X axis represents day intervals associated with study groups.

Percent inhibition of paw edema

Figure 2 shows the percentage inhibition of paw edema on 21st day of the experiment. Administration of selected formulations considerably inhibited development of edema induced by CFA, thereby showing the percentage inhibition of edema comparable to that of the standard drugs treated groups.

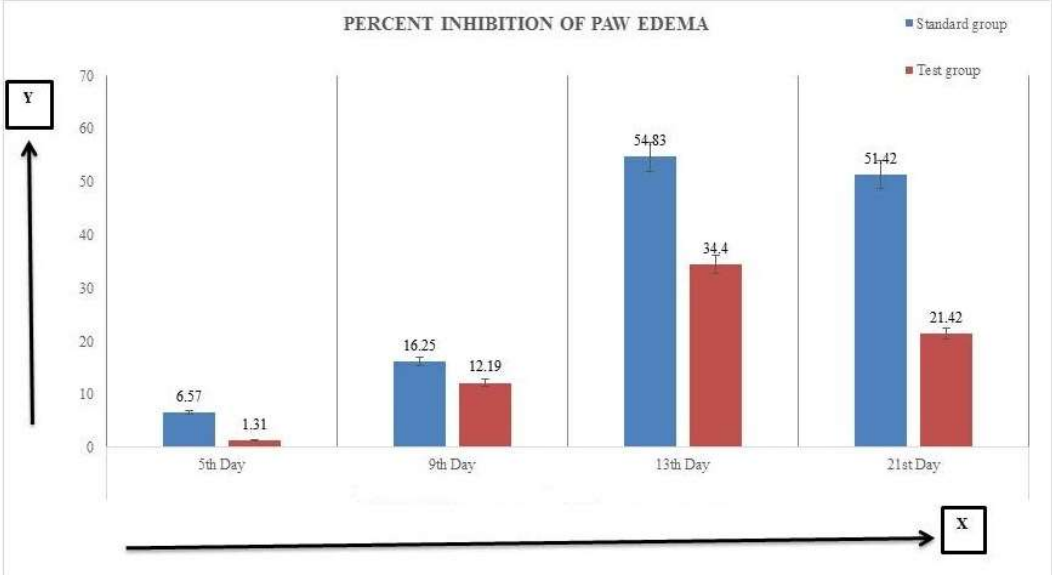

Figure 2: Percent inhibition of paw edema compared with induced group, which is based on statistics of paw edema (Figure 1). Y axis represents percent inhibition value and X axis represents time interval (day-wise) alongwith standard and test group.

Arthritic Index

Recording of arthritic score for both hind paws was recorded at definite time intervals that are 5, 9, 13 and 21 after CFA injection. Development and severity of induced arthritis were evaluated by a visual scoring system of the clinical signs and symptoms such as mild to severe redness and swelling of one finger and big joints. A significant decrease in arthritic index was recorded (Figure 3) for standard group and group given VNC compared to CFA-induced group. On day 21 animal group given VNC showed better arthritic index (2.336), i.e. less clinical signs of inflammation and arthritis, than animal group given standard drug (2.089).

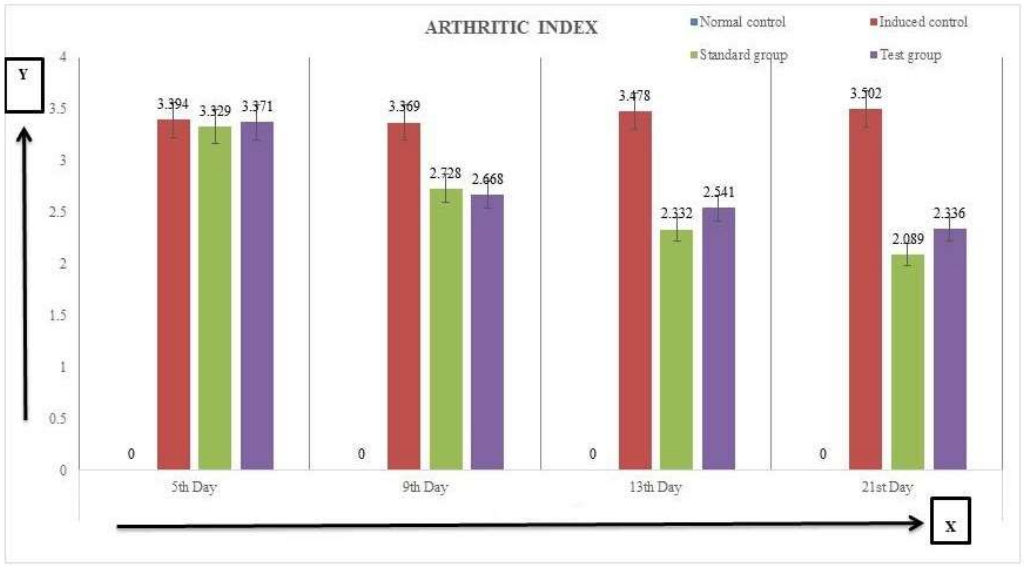

Figure 3: Arthritic index based on grade scoring system. Y axis represents clinical score scale and X axis represents day intervals with study groups.

Body Weight

Figure 4 shows the changes in body weight of rats. It can be observed that in induced group, there was a marked decrease in body weight as compared to other groups. The animals gained weight significantly than control group animals (except CFA induced group). On day 21 it was evaluated that standard group exhibited an increase in body weight (248±2.36); in the test group, VNC caused weight gain (252±2.09) which was comparable to that of all animal groups.

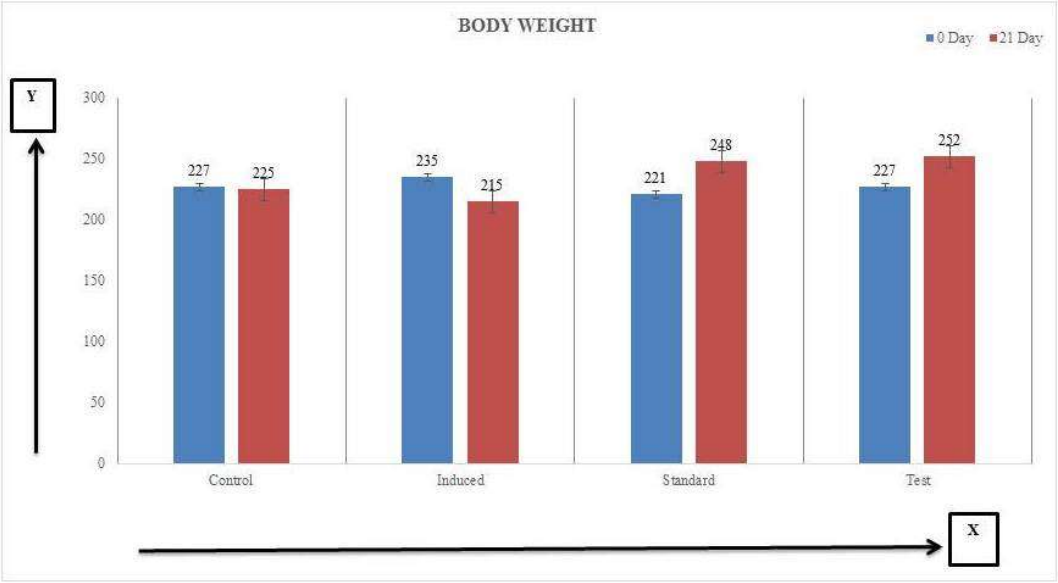

Figure 4: Effect of VNC on body weight. Y axis represents weight (gm) of animals and X axis represents day-wise figures of study groups.

Hematological and Serum Parameters

Hematological alterations in CFA induced rats were normalized after treatment with test

formulation comparable to that of standard drug treatment group (Figure 5). All the evaluated hematological parameters of CFA-induced group showed an out of normal range results, such as decreased hemoglobin count (8.98±0.47), decreased red blood cells count (3.93±0.29), decreased packed cell volume (38.12±0.83), increase in white blood cells count (16.11±0.67) and increased erythrocyte sedimentation rate (1±0.41) were favorably altered by treatment with VNC (13.26±0.64, 5.88±0.22, 43.09±0.56, 12.34±0.56 and 0.5±0.30) respectively.

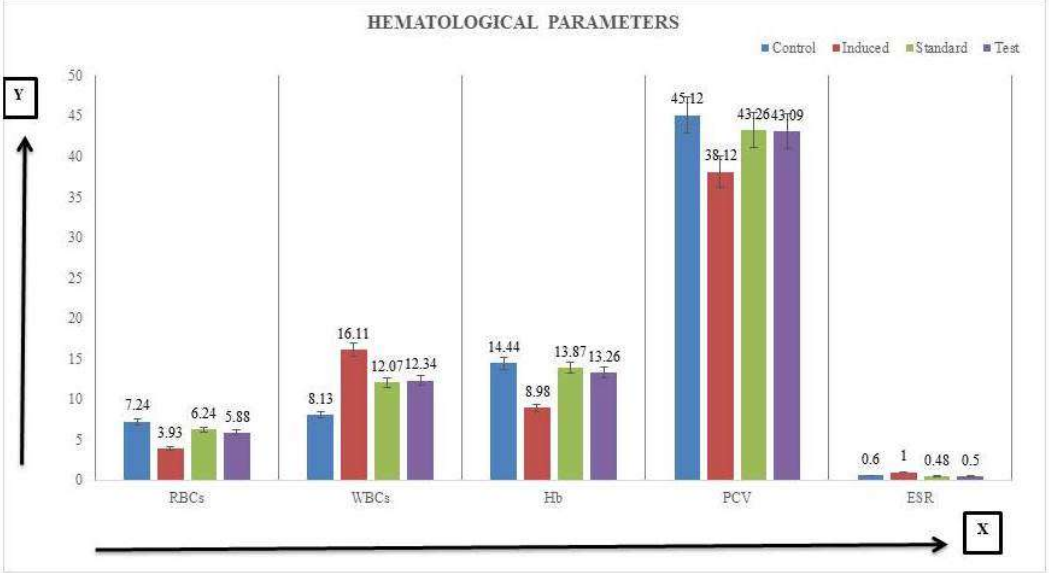

Figure 5: Hematological analysis data of various parameters. Y axis represents unit of hematological parameters and X axis represents selected parameters of study groups.

Figure 6 showed that serum rheumatoid factor (27.57±0.92) and serum CRP (5.20±0.19) were significantly increased in induced group compared with normal control group which were comparatively decreased in VNC treated group (16.46±1.02 and 3.62±0.63) respectively. The expression of key proinflammatory cytokines viz. IL1-β and TNF-α was studied as these cytokines have been reported to be expressed at significant levels during chronic inflammation. IL1-β (364.98±0.75) and TNF-α (128.34±0.61) were increased in induced group rats whereas in VNC treated group both the parameters were significantly lowered down to normal condition (355.16±0.60 and 110.10±0.79) respectively.

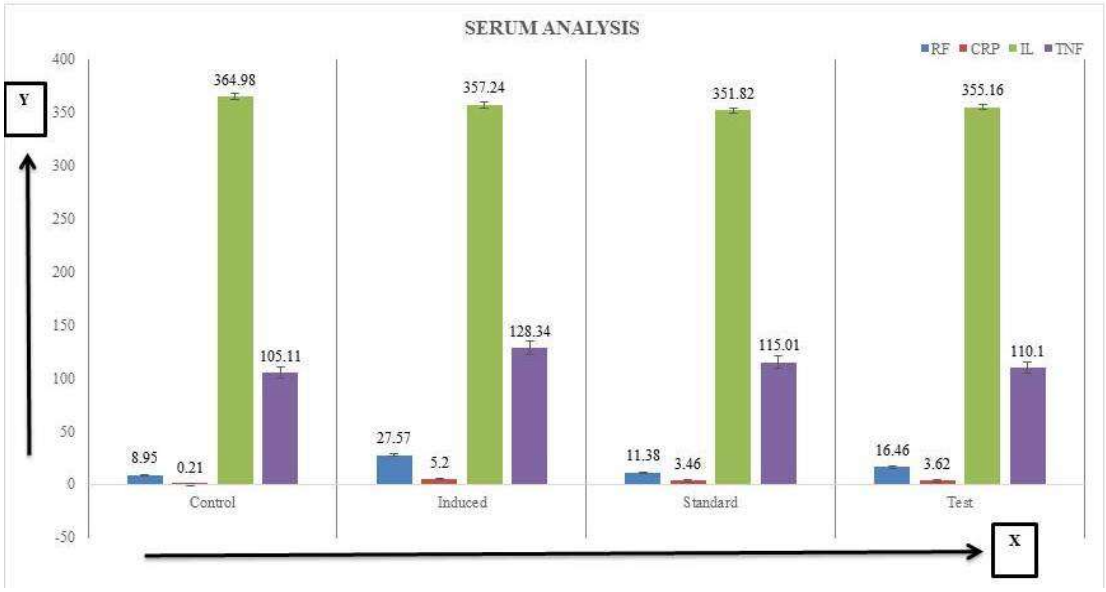

Figure 6: Effect of VNC on serum markers of experimental animals. Y axis represents animal range of value of serum components and X axis represents study groups with various serum parameters.

Histopathology

The histopathological findings observed in the groups were presented in Figure 7. No lesions were observed in the wrist region in the control group. Severe edema under the skin and around the joint, intense mononuclear cell infiltration and a small number of neutrophil leukocyte infiltration were noted in the induced group. In addition, mononuclear cell infiltration (synovitis), an increase in connective tissue, synovial hyperplasia in which lymphocytes are the majority and presence of pannus formation with destruction of joint space were also observed in the synovial membrane. It was also noted that the edema and inflammatory cell infiltrations decreased in standard and VNC treated group.

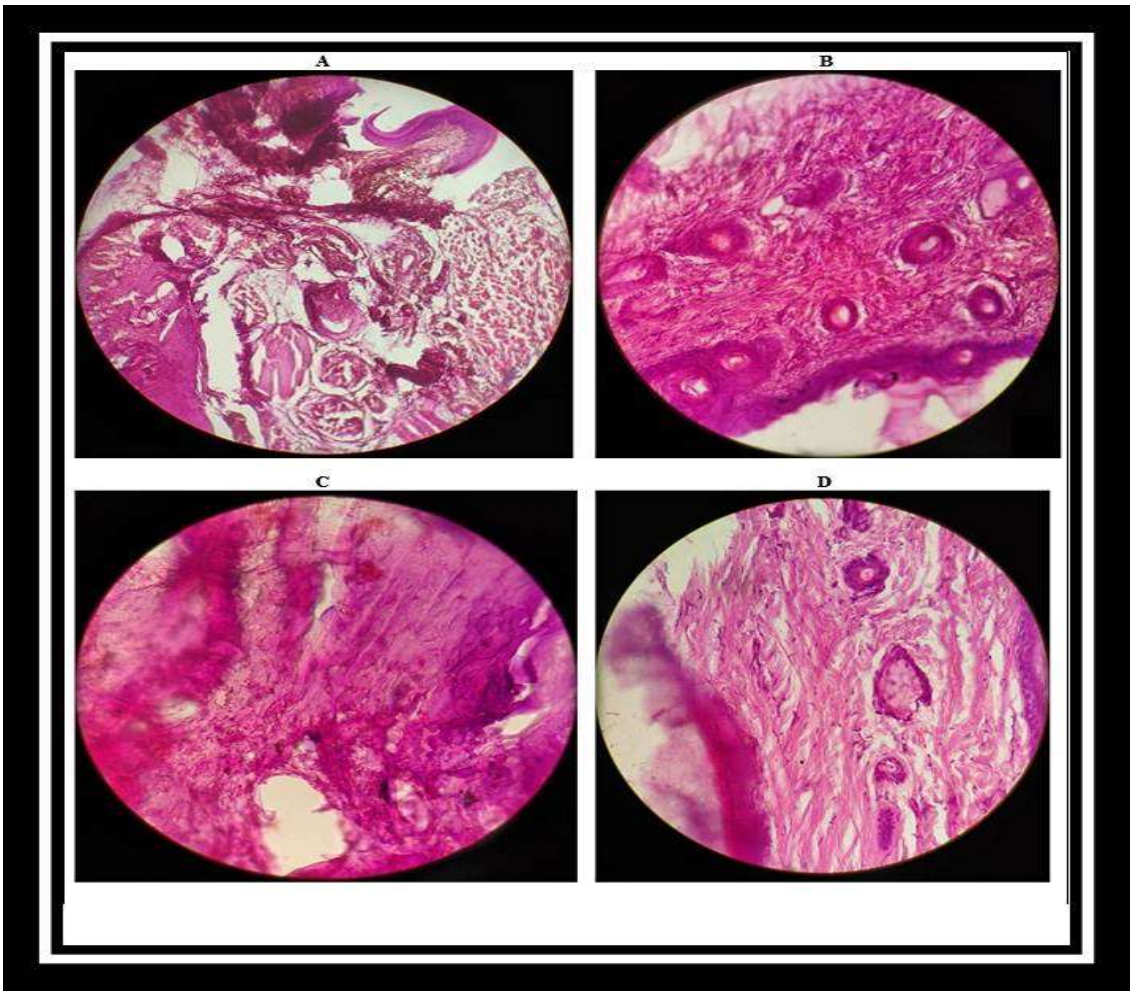

Figure 7: Microscopic view of isolated paw. A = Control group; B = Induced group; C = Standard group and D = Test group.

Joint Edema

In turpentine-induced arthritis, on all evaluatory hours, administration of standard drug resulted in a significant reduction in the diameter of the synovial joint. The test herbal formulation VNC produced a moderately significant reduction in the diameter of the synovial joint when compared with standard group (Figure 8). After 6 h, percentage inhibition of paw edema in test group rats was 74.14%; while standard drug showed 94.67 % of inhibition.

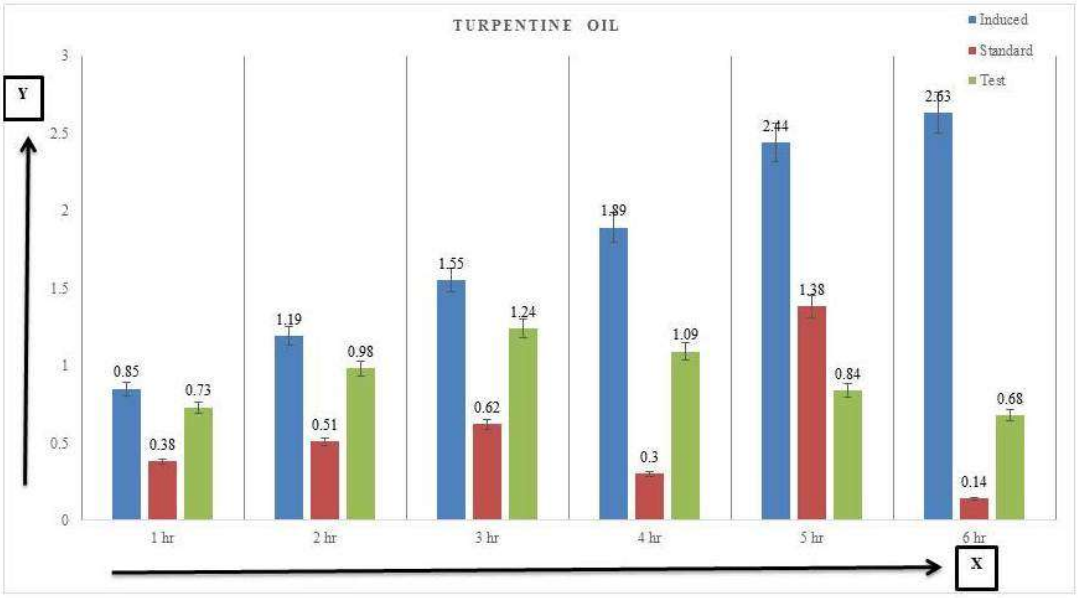

Figure 8: Effect of VNC on turpentine oil induced joint edema. Y axis represents unit (mL) of joint edema and X axis represents duration in hours after administration of inducing agent with study groups.

DISCUSSION

The alarming safety and efficacy issues of many drugs used for the treatment of inflammatory disorders have continued since years. There always has been focus on the biologically active compounds from plants and efficacy of natural products against inflammatory disorders. In the present study we investigated the herbal preparation VNC and compared its anti-arthritic and anti-inflammatory effect with standard drugs by using CFA induced arthritis protocol (Chronic model) and turpentine oil induced joint edema protocol (Acute model). VNC is traditionally used for the treatment of inflammatory disorders especially for arthritis.

The CFA induced experimental model has been extensively used in the study of inflammatory processes and for evaluation of anti-inflammatory agents because this model has many clinical similarities with RA in human patients, including pathological and immunological features. CFA administration into hind paw of rats leads to marked swelling which persists for weeks as a primary reaction. After a few days, the delayed systemic response is also seen in the form of controlateral paw swelling and biochemical alteration.

The determination of paw swelling is apparently simple, sensitive, and quick procedure for evaluating the degree of inflammation and assessing therapeutic effects of drugs. In the present study, the increase in paw volume seen in the induced group is believed to be produced due to elevated immune response during the late phase of the model. There was a decrease in paw volume seen in standard as well as test group suggesting the anti-inflammatory activity of the test formulation. T-cell proliferation is an important mechanism of adjuvant diseases; specifically their differentiation into Th-1 helper cells. Therefore, possible mechanism for reduction in paw edema might be either suppressive effect on Th-1 helper cells.

Arthritic index is considered as an indicator of systemic inflammation. Arthritic index includes the combined index of inflammation, formation of nodules, and extent of spread of the disease to other organs. Inflammation during RA results in redness and swelling in the fingers and joint areas. Animal groups given either standard drug or VNC showed less inflammatory and arthritis symptoms with statistically significant decrease in arthritic index, moreover reduction in the arthritis score also distinguishes the immunosuppressive effects of a drug from its anti-

inflammatory effects as well. Thus the result suggests that VNC is effective in prevention of RA development.

The changes in animal body weight have been used to evaluate the therapeutic effects of treatment in a model of adjuvant-induced arthritis to assess the course of the disease and the response to therapy of arthritic drugs. Finding from the studies implicated that with increase the incidence and severity of arthritis, a decrease in body weight of the rats occurred during the course of the experimental period due to alterations in the metabolic activities of diseased rats. The decrease in body weight gain in the induced group rats compared to the normal control rats in the current study is in concordance with the fact that RA is associated with loss of lean tissues, which contain most of the body's protein. The reduction in body weight gain may also be attributed to muscle wasting in experimental arthritis, occurring due to enhanced protein breakdown by the ubiquitin-proteasome proteolytic pathway. It has been previously reported that decrease in the body weight during inflammation is due to deficient absorption of nutrients through the intestine. Chronic treatment with VNC to arthritic rats significantly improved loss of body weight. These effects may be attributed to either inhibition of loss of lean tissues containing body's protein or inhibition of muscle wasting or improvement in inflammation leads to increase in absorption of nutrients through the intestine. In addition, Weight reduction, expressed as rheumatoid cachexia in RA, is attributed to increased levels of proinflammatory cytokines. Therefore, the anti-arthritis effect of VNC has been demonstrated by its effect on body weight gain. It is possible to say that VNC shows this effect by reducing proinflammatory cytokine levels.

It has been stated that there is a decrease in RBC, Hb, and PCV values and an increase in WBC and ESR levels in rats with arthritis. It is clear that decrease in haemoglobin level and RBC count represents anemic condition in CFA induced model. Iron deficiency anemia is one of the hematological symptoms of RA. Anemia of chronic disease is immune driven; cytokines and cells of the reticulo-endothelial system induce changes in iron homoeostasis, proliferation of erythroid progenitor cells, production of erythropoietin, and life span of red cells, all of which contribute to the pathogenesis of anemia. In a study, it was reported that WBC play an important role in the pathogenesis of RA, and an increase in its count indicates deterioration in inflammation and immune balance. In the study, it was determined that WBC count increased in induced group rats compared to the control group. ESR is an estimate of the suspension stability of RBCs in plasma, related to the number and size of red cells and to the relative concentration of plasma proteins especially fibrinogen and α and β globulins. Increase in ESR is an indication of active but obscure disease processes. Increased ESR is a common diagnostic feature in inflammatory conditions. The ESR level which was markedly elevated in induced group rats. VNC treated groups satisfactorily restored the altered hematological profile levels back to normal by increasing RBC, Hb and PCV counts and decreasing WBC and ESR. The level of WBC of test group was much less than induced group but not significant as compared to normal control group.

In the present study, a significant increase in RF, CRP, TNF-α and IL1-β levels in CFA induced group rats was observed compared to the control group. CRP is one of the blood markers for inflammation and immune response of RA, representing an acute phase plasma protein produced in response to action of IL-6 in inflammatory condition by the liver and adipocytes. High level of CRP was observed in induced group rats. A number of autoantibodies like anti-perinuclear factor, antikeratin antibodies, RF, a-CCP (anti-cyclic citrullinated peptide) and ANA (anti-nuclear antibodies) are known to be associated with RA and the progression of the disease. RF is an autoantibody produced against the Fc (fragment crystallizable) portion of IgG and is most relevant in RA. Rheumatoid factor is the true marker of clinical presentation of RA. Adjuvant disease shows elevated blood levels of rheumatoid factor. Rheumatoid factor generation in arthritis involves B cell activation via toll-like receptors and several genetic

predispositions to arthritic diseases. Induced group showed significantly elevated levels of serum rheumatoid factor compared to rest of the experimental groups. Overproduction of proinflammatory cytokines, particularly TNF-α and IL-1β, play an important role in the pathogenesis and progression of RA. CFA injection caused activation of immune system, resulting in abnormal leukocyte proliferation and differentiation. Dendritic cells react with adjuvants components; it enhanced phagocytosis, proliferation of CD4+ lymphocytes, and secretion of cytokines (TNF-α and IL-1β). Several studies have reported that TNF-α and IL-1β play crucial roles in inflammation and synovial tissue damage, and their levels were increased in experimental group rats. Thus, TNF-α and IL-1β represent crucial targets for treating RA. It is well established that TNF-α binds to two cytokines receptors, namely TNFR (tumor necrosis factor receptor)-1 and TNFR-2. However, major inflammatory reactions are facilitated by TNFR-1. It is possible that VNC prevented joint destruction by suppressing pro-inflammatory cytokine/cytokine receptor levels. In this study, VNC inhibited both TNF-α and IL-1β (not much significant as compared to that of normal control group) when compared with induced group, it has also been reported that these two cytokines caused activation of Signal transducer and activator of transcription 3 (STAT3). Induction of STAT3 further increases the expression of RANKL (receptor activator of nuclear factor-κB-ligand), which promotes osteoclastogenesis and joint destruction. Hence VNC successfully lowered the raised levels of RF, CRP, TNF-α and IL1-β.

Histopathological evaluations of ankle joints in normal control group showed normal joint structure, no cartilage destruction, and no signs of inflammation or other distortion was observed. While in arthritic animals showed mild to moderate hyperplasia of synovium; focal cartilage destruction; presence of pannus formation with destruction of joint space. It also showed marked damage of articular structure indicating joint damage and inflammation. The pannus formation and bone erosion associated with inhibition of neutrophil infiltration. Hyperplastic synovial tissue (pannus) may erode cartilage, subchondral bone, articular capsule, and ligaments. Treatment with standard and VNC groups showed significant improvement in hyperplasia of synovium as compared to disease control group which showed protective effect of formulation on hyperplasia of synovium support its anti-arthritic effect. The inhibition of pannus formation and bone erosion may be associated with inhibition of neutrophil infiltration. Turpentine oil induced paw edema is characterized by a triphasic release of inflammatory mediators. The initial phase is mediated by histamine and serotonin, intermediate phase by kinin like substance and the late phase by cyclooxygenase and lipoxygenase products. In the present study, inhibition of turpentine oil induced paw edema was observed in the test drug treated groups throughout the observation period. This suggests that VNC influenced all the phases of turpentine oil induced inflammation in the rat paw.

CONCLUSION

In summary, this study provides conclusive evidence of the efficacy of a 50% hydro-acetone extract of VNC as an anti-inflammatory, anti-arthritic, and analgesic drug, hence bolstering popular traditional beliefs and applications. The investigated dose had no negative effects however it had a regulating influence on blood parameters, some pathophysiological markers associated with RA, the level of CRP, and a reduction in the proinflammatory mediators and cytokines in the RA model. Furthermore, if harvesting is done properly, using bark and roots is a solid choice for managing biodiversity that is more plentiful and simple to obtain all year round. More preclinical and clinical research, particularly involving human patients, is certainly required and strongly recommended.

REFERENCES

1. Bagepally BS, Kumar SS, Sasidharan A, Haridoss M, Venkataraman K. Household catastrophic health expenditures for rheumatoid arthritis: a single centre study from South India. Sci Rep. 2023;13(1):15385.
2. Bagepally BS, Kumar SS, Sasidharan A, Haridoss M, Venkataraman K. Household catastrophic health expenditures for rheumatoid arthritis: a single centre study from South India. Sci Rep [Internet]. 2023;13(1):15385.
3. Barakat M, Syed NK, Hasen E, Abdulrazzaq SB, Thiab S, Al-Najjar MAA, et al. The effect of natural products on inflammatory cytokines production and secretion. Phytomedicine Plus [Internet]. 2023;3(4):100488.
4. Blainski A, Lopes GC, de Mello JC. Application and analysis of the folin ciocalteu method for the determination of the total phenolic content from Limonium brasiliense L. Molecules. 2013 Jun 10;18(6):6852-65.
5. Dar WR, Mir IA, Siddiq S, Nadeem M, Singh G. The Assessment of Fatigue in Rheumatoid Arthritis Patients and Its Impact on Their Quality of Life. Clin Pract. 2022 Jul 26;12(4):591-598.
6. Dehelean CA, Marcovici I, Soica C, Mioc M, Coricovac D, Iurciuc S, Cretu OM, Pinzaru I. Plant-Derived Anticancer Compounds as New Perspectives in Drug Discovery and Alternative Therapy. Molecules. 2021 Feb 19;26(4):1109.
7. Dubey S, Dixit AK. Preclinical evidence of polyherbal formulations on wound healing: A systematic review on research trends and perspectives. J Ayurveda Integr Med. 2023 Mar-Apr;14(2):100688.
8. Eberl G, Studnicka-Benke A, Hitzelhammer H, Gschnait F, Smolen JS. Development of a disease activity index for the assessment of reactive arthritis (DAREA). Rheumatology [Internet]. 2000 Feb 1;39(2):148–55.
9. Hayat J, Akodad M, Moumen A, Baghour M, Skalli A, Ezrari S, Belmalha S. Phytochemical screening, polyphenols, flavonoids and tannin content, antioxidant activities and FTIR characterization of Marrubium vulgare L. from 2 different localities of Northeast of Morocco. Heliyon. 2020 Nov 28;6(11):e05609.
10. Jain A, Jain P, Bajaj S, Majumdar A, Soni P. Chemoprofiling and antioxidant activity of edible curcuma species. Food Humanit [Internet]. 2023;1:1027–39.
11. Jain A, Jain P, Parihar DK. Comparative study of in-vitro antidiabetic and antibacterial activity of non-conventional Curcuma species. TBAP (Taylor n Francis). 2019;9(6):457 – 464
12. Jain P. Secondary metabolites for antiulcer activity. Nat. Prod. Res. (Taylor n Francis). 2015:1-17
13. Jang S, Kwon EJ, Lee JJ. Rheumatoid Arthritis: Pathogenic Roles of Diverse Immune Cells. Int J Mol Sci. 2022 Jan 14;23(2):905.
14. Jat Diksha, Tyagi CK, Budholiya Prabhakar. Evaluation of anti arthritic potential of hydroalcoholic leaves extract of wrightia tinctoria. International Journal of Pharmaceutics & Drug Research. 2021 March 9;1:27-33
15. Kaithwas G, Majumdar DK. Therapeutic effect of Linum usitatissimum (flaxseed/linseed) fixed oil on acute and chronic arthritic models in albino rats. Inflammopharmacology. 2010 Jun;18(3):127-36
16. Kondo N, Kuroda T, Kobayashi D. Cytokine Networks in the Pathogenesis of Rheumatoid Arthritis. Int J Mol Sci. 2021 Oct 10;22(20):10922.
17. Kondo N, Kuroda T, Kobayashi D. Cytokine Networks in the Pathogenesis of Rheumatoid Arthritis. Int J Mol Sci. 2021 Oct 10;22(20):10922.
18. Kujur Adeep, Tirkey Rakesh, Tripathi Vaibhav, Sahu PK. Promising herbs for the management of inflammation associated with various pathological conditions - A Review.

International Journal of Research in Pharmaceutical Sciences. 2020 Dec 11; (SPL4):3084-3091.
19. Kumar V, Rathore K, Jain P, Ahmed Z. Biological activity of Bauhinia racemosa against diabetes and interlinked disorders like obesity and hyperlipidemia. Clin. Phytoscience (Springer), 2017;3:7
20. Lu R-M, Hwang Y-C, Liu I-J, Lee C-C, Tsai H-Z, Li H-J, et al. Development of therapeutic antibodies for the treatment of diseases. J Biomed Sci [Internet]. 2020;27(1):1.
21. Meena Pooja, Tyagi CK, Patel Narendra, Jhalawa Brajesh, Budholiya Prabhakar. Pharmacological Screening of Aquoues and Methanolic Extract of Moringa Oleifera for Anti-Inflammatory and Analgesic Activity. International Journal of Pharmaceutical Research and Applications. 2020 Sept 5;1:35-41.
22. Miller FW. The increasing prevalence of autoimmunity and autoimmune diseases: an urgent call to action for improved understanding, diagnosis, treatment, and prevention. Curr Opin Immunol. 2023;80:102266.
23. Nasim N, Sandeep IS, Mohanty S. Plant-derived natural products for drug discovery: current approaches and prospects. Nucl. 2022;65(3):399–411.
24. Nisar A, Jagtap S, Vyavahare S, Deshpande M, Harsulkar A, Ranjekar P, Prakash O. Phytochemicals in the treatment of inflammation-associated diseases: the journey from preclinical trials to clinical practice. Front Pharmacol. 2023 May 9;14:1177050.
25. Noh ASM, Chuan TD, Khir NAM, Zin AAM, Ghazali AK, Long I, Ab Aziz CB, Ismail CAN. Effects of different doses of complete Freund's adjuvant on nociceptive behaviour and inflammatory parameters in polyarthritic rat model mimicking rheumatoid arthritis. PLoS One. 2021 Dec 8;16(12):e0260423.
26. OECD (2008), Test No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris,
27. Pal Mudit, Shah SK, Patel Narendra, Tyagi CK. Evaluation of Anti-Arthritis Activity of Butea monosperma Bark Extract on Albino Wistar Rats. International Journal of Pharmacy & Pharmaceutical Research. 2018 July 12;4: 416-428.
28. Pereckova J, Martiniakova S, Payer J, Falk M, Killinger Z, Perecko T. Analysis of hematological parameters in rheumatoid arthritis patients receiving biological therapy: contribution to prevention of avoidable hematological complications. EXCLI J. 2022 Mar 8;21:580-594.
29. Poudel P, Goyal A, Lappin SL. Inflammatory Arthritis. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2024
30. Roshan A, Hathout R, Sherweit, El-Ahmady S, Abdelnasser, Singab B, et al. Breaking the challenge of polyherbal quality control using UV and HPLC fingerprints combined with multivariate analysis. Phytochem Anal. 2021 Oct 28;33.
31. Sagar NA, Pareek S, Gonzalez-Aguilar GA. Quantification of flavonoids, total phenols and antioxidant properties of onion skin: a comparative study of fifteen Indian cultivars. J Food Sci Technol. 2020 Jul;57(7):2423-2432.
32. Saxena V, Mishra G, Saxena A, Kr K. A comparative study on quantitative estimation of tannins in terminalia chebula , terminalia belerica , terminalia arjuna and saraca indica using spectrophotometer. 2013;6:5–6.
33. Scherer HU, Häupl T, Burmester GR. The etiology of rheumatoid arthritis. J Autoimmun. 2020;110:102400.
34. Sharwan G, Jain P, Pandey R, Shukla SS. Toxicity and safety profiles of methanolic extract of Pistacia integerrima J. L. stewart ex brandis (pi) for wistar rats. J. Pharmacopunct. 2016; 19[3]: 253-258

35. Tirkey R, Adeepkujur KY, Tripathi V, Dewangan D, Saraf S. Potential of neoteric phytoactives and herbs for targeting pathophysiological modules of arthritis. Bull. Environ. Pharmacol. Life Sci. 2021 Apr 5;10:273-81.
36. Tiwari V, Jandu JS, Bergman MJ. Rheumatoid Factor. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2024
37. Tripathi Vaibhav, Tyagi CK,Raja Wasim. Safety evaluation of a polyherbal formulation: Acute and sub-acutetoxicity using Wistar Albino rats.International Journal of Experimental Research and Review. 2023 Aug 32:235-245.
38. Tyagi CK, Tripathi V, Kashyap AK. Herbal Resources Having Potential Anti-Inflammatory Activity: An Insight To Ecosystem Diversity Of Chhattisgarh. International Journal of Biology, Pharmacy and Allied Sciences. 2021 Sept 10;9: 3221-3232.
39. Tyagi CK, Tripathi V. Pros and Cons of Animal Models: Special Emphasis on Anti-arthritic Clinical Evaluation Model. Indian Journal of Pharmaceutical Education & Research. 2023 Jan 1;57(1).
40. Vaou N, Stavropoulou E, Voidarou C, Tsakris Z, Rozos G, Tsigalou C, Bezirtzoglou E. Interactions between Medical Plant-Derived Bioactive Compounds: Focus on Antimicrobial Combination Effects. Antibiotics. 2022; 11(8):1014.
41. Wright HL, Lyon M, Chapman EA, Moots RJ, Edwards SW. Rheumatoid Arthritis Synovial Fluid Neutrophils Drive Inflammation Through Production of Chemokines, Reactive Oxygen Species, and Neutrophil Extracellular Traps. Front Immunol [Internet]. 2021;11.
42. Yao Q, Wu X, Tao C, Gong W, Chen M, Qu M, Zhong Y, He T, Chen S, Xiao G. Osteoarthritis: pathogenic signaling pathways and therapeutic targets. Signal Transduct Target Ther. 2023 Feb 3;8(1):56.
43. Zhang QW, Lin LG, Ye WC. Techniques for extraction and isolation of natural products: a comprehensive review. Chin Med. 2018 Apr 17;13:20.