Research Article

Evaluation of Anti-Arthritic Potential of *Vitex Agnus-castus* in Freund's Complete Adjuvant Induced Arthritic Albino Rats

S. Manivannan[1*], S. Bhuvaneswari[2], M. Akshay[3], G. Tharani[1], D. Benasir[1], P. Madhavi[1]

[1*]PG and Research Department of Biotechnology, Bharath College of Science and Management, Thanjavur, Tamil Nadu, India.

[2] Department of Pharmaceutics, Periyar College of Pharmaceutical Sciences, Tiruchirappalli, Tamil Nadu, India.

[3] Department of Pharmaceutical Chemistry, Pushpagiri College of Pharmacy, Thiruvalla, Kerala, India.

***Corresponding author's E-mail:** manivannanresearch@gmail.com

Received: 15-11-2016; **Revised:** 10-01-2017; **Accepted:** 18-01-2017.

ABSTRACT

Rheumatoid arthritis is the commonest cause of chronic inflammatory joint disease. Due to the limitations and risks of conventional therapy, the past decade has witnessed use of herbal medicines as potent therapeutic agents. The aim of this study was to evaluate the anti-arthritic potential of the plant *Vitex-agnus castus* leaves in albino rats. *V. agnus-castus* leaves were collected and extracts were prepared using different solvents. Forty-two adult female albino Wistar rats (152-160g) were divided into seven groups of six rats each. The severity of adjuvant induced arthritis was quantified by measuring the volume of the hind paw using Plethysmometer. At the end of the study, hemoglobin, total WBC, ESR, and RBC count were estimated. Nitric oxide levels were quantified using Griess reagent. Evans Blue dye extra vasation technique was used to measure the permeability of capillaries. Phytochemical analysis of *V. agnus-castus* leaves showed the presence of various phytocompoments. The extract-treated rat groups showed significant ($P<0.05$) reduction in paw edema from 7 to 28 days of the study. Freund's complete adjuvant (FCA) induced arthritic rats showed slight elevation in the total WBC count and reduction in RBC count at 28 days. Standard drug (Indomethacin) and extract-treated groups showed significant reduction in the level of nitric oxide in arthritic rats compared to the control group. Extract-treated groups showed significant ($P<0.001$) inhibition of Evans blue extra vasation in the arthritic joints compared to the control groups. These results show that leaf extract of *V. agnus-castus* can be a promising therapeutic agent in the treatment of rheumatoid arthritis.

Keywords: *Vitex agnus-castus*, arthritis, freund's complete adjuvant, paw volume, nitric oxide synthesis, and vascular permeability.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, autoimmune, inflammatory disease with an unknown etiology. It affects approximately 5 million people worldwide of which 50% are unable to work beyond 10 years of diagnosis. This disease can affect all synovial joints as well as extra-articular structure, but mostly manifests in the small joints of hands and feet. It is an illness with significant morbidity and mortality rates caused due to organ damage and failure[1, 2]. In many developing countries, traditional medicine is still the main source of health care for about 80% of the population because of its cultural acceptability, affordability and accessibility. In the last few years, there has been an upsurge of interest in the use of traditional medicine even in developed countries. An estimated 25% of all modern medicines are derived either directly or indirectly from medicinal plants, largely through the application of modern technology to traditional knowledge. As there are about 5,00,000 plant species occurring worldwide, of which barely 1% has been phytochemically scrutinized, there is great potential for discovering novel bioactive compounds[3]. About 60% of anti-tumoral and anti-microbial agents are being derived from medicinal plants[4, 5]. Nevertheless, research on safety and efficacy is of prime importance to the continued advancement of traditional medicines.

Adjuvant-induced arthritis is a chronic, crippling, skeletomuscular disorder having the nearest approximation to human rheumatoid arthritis for which there is presently no medicine available effecting a permanent cure. Although modern drugs, both steroidal and non-steroidal anti-inflammatory drugs, used for the treatment of RA have been developed in the past few decades, there is still an urgent need for more effective drugs with lower side effects. Unfortunately, there is still no effective known medicinal treatment that cures rheumatoid arthritis as modern medicine can only treat the symptoms such as pain and joint inflammation of this disease. In this context, it is also possible to use traditional herbs and plants in various forms to relieve arthritic symptoms[6].

Vitex agnus-castus L, commonly called "chaste berry", is a small deciduous shrub or tree up to 6 m tall, which grows usually along river beds, coastal areas and other places with altitudes of 0-600m. It is found throughout Asia, Europe (especially in Mediterranean region), and North America. The plant bears slender spikes of violet blue and flowers measuring about 8-10 cm. The stalked palmate leaves consist of 5 to 7 narrow pointed entire leaflets that are dark green in colour and glabrous above and white felted beneath[7]. Traditionally, it has been used as a digestive aid, sedative, anti-infective agent, and to treat premenstrual syndrome, fibroid cysts, infertility and acne in teenagers. Crude ethyl acetate extract of *V. agnus-castus* possesses secondary metabolites such as alkaloids, flavonoids, tannins, and phenols[8]. Flavonoids and

diterpenoids isolated from ethyl acetate extract of *V. agnus-castus* fruits have been reported to exhibit antioxidant activity[9]. Bicyclic terpenes isolated from *V. agnus-castus* fruits are used for the treatment of movement disorders. Caffeic and chlorogenic acids extracted from the leaves and fruits of *V. agnus-castus* exhibited potent antioxidant activity[10]. Folk and traditional use of *V. agnus-castus* leaves in crippling arthritis and frozen joints. However, there is no systematic study regarding the anti-arthritic activity of *V. agnus-castus* leaves. Hence, in the present study, an attempt has been made to evaluate the anti-arthritic potential of petroleum ether, methanol, ethanol, and aqueous extracts of *V. agnus-castus.*

MATERIALS AND METHODS

Plant Collection

V. agnus-castus leaves were collected from the University of Madras Botanical garden, Chennai, Tamil Nadu, India, during June-July 2013.

Plant Extract Preparation

The leaves of *V. agnus-castus* were washed in running tap water and allowed for natural drying under shade. Then the leaves were coarsely powdered in a blender and the powder was extracted with different solvents such as petroleum ether, methanol, ethanol, and water for 48 hrs by Soxhlet extraction process[11]. The extracts were concentrated under reduced pressure using a rotary flash evaporator and the residues were dried in a desiccator over sodium sulfite. All the extracts were stored at -20°C and used for preliminary phytochemical screening of secondary metabolites.

Experimental Animals

Albino Wistar rats weighing approximately 152-160g were used. All animals were housed in an animal room under standard laboratory conditions (12/12 hr light/dark cycle at 25°C ± 5°C). The animals were fed a commercial rat pallet diet (Lipton India Ltd., Mumbai, India) and water add libitum. The bedding material of the cages was changed every day. All the experiments in this study were approved by the institutional animal ethical committee in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Acute Toxicity Test

Acute toxicity study was performed according to the acute toxic classic method as per the Organization for Economic Co-operation and Development (OECD) guidelines[12]. A group of six albino rats were administered methanol extract with doses of 0.25, 0.5, 1.0, 1.5 and 2.0 mg/kg body weight, respectively. Rats were continuously monitored for mortality and behavioral responses for 48 hrs and once daily thereafter until the 14th day. If mortality was observed in two animals out of three, then the dose administered was assigned as toxic dose. If the mortality was observed in one animal, then the same dose was repeated to confirm the toxicity. If mortality was not observed, the procedure was repeated for further higher doses such as 50, 200, 500, 1000 and 2000 mg/kg body weight. The animals were observed for adverse symptoms such as behavioral changes, locomotion, convulsions and mortality for 72 hrs.

Complete Freund's adjuvant induced arthritis

Freund's complete adjuvant (FCA) (Difco Laboratories, Detroit, MI) induced arthritis model was used to assess the anti-arthritic activity in albino rats. The animals were randomly divided into seven groups of six animals each (n=6).

The animal groups are as follows:

Normal Group

Group - I: Treated with 5 ml/kg.p.o. Normal saline + Mineral oil.

Control Group

Group - II: Treated with 5 ml/kg.p.o. Normal saline + FCA.

Standard group:

Group - III: Treated with 10 mg/kg.i.p. Indomethacin + FCA.

Test Groups

Group - IV: Treated with 200 mg/kg.p.o. Petroleum ether extract + FCA.

Group - V: Treated with 200 mg/kg.p.o. Methanol extract + FCA.

Group - VI: Treated with 200 mg/kg.p.o. Ethanol extract + FCA.

Group - VII: Treated with 200 mg/kg.p.o. Aqueous extract + FCA.

Arthritis was induced by FCA, following the method described by New bould, 1963, with slight modification[13]. Adjuvant arthritis was induced by subcutaneous injection of 0.1 ml of FCA (a suspension of heat killed *Mycobacterium tuberculosis* in mineral oil) into sub plantar tissue of the right hind paw of each rat. The test group consisted of FCA injected rats challenged with the respective doses of the extract administered orally 30 min before FCA injection, while the control rats were injected with 0.1 ml of mineral oil (incomplete Freund's adjuvant) only. The drug treatment was continued till 21 days. The swelling in the injected and contralateral hind paw was monitored daily using mercury displacement plethysmometer. This study was designed as per the US-FDA guidelines for industrial preclinical evaluation of anti-arthritis.

Measurement of Rat Paw Edema

The severity of adjuvant arthritis was quantified by measuring the volume of the hind paw using

Plethysmometer[14]. Paw volume (ml) was measured at 0 day and thereafter at 3, 7, 14, 21, and 28 days of FCA post-inoculation. The mean changes in injected paw edema with respect to initial paw volume were calculated on respective days and the percentage of inhibition of paw edema with respect to untreated group (control) was calculated using the formula:

$$\% \text{ inhibition} = \frac{To - Tt}{To} \times 100$$

Where,

T_o is the paw thickness of rats of control group at the same time.

T_t is the thickness of paw of rats given test extract at corresponding time

Measurement of Body Weight

Body weights were measured at 0, 3, 7, 14, 21 and 28 days using a single pan weighing balance[15].

Hematological Assessment

On the 28th day, all the animals were anaesthetized and blood was collected from the retro-orbital plexus into a test tube containing anti-coagulant (5% EDTA) and hematological parameters were determined. Hemoglobin (Hb) content was estimated by the method of Austin and Drabkin[16]. Red blood cell (RBC), White blood cell (WBC) counts and Erythrocyte sedimentation rate was measured according to the method of Chesbrough and Mcarthur[17].

Nitric Oxide Synthesis

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH, which interacts with oxygen to produce nitrite ions, was quantified using Griess reagent About 3ml of 10mM sodium nitroprusside in phosphate buffer was added to 2ml of agnuside and the reference compound was taken in different concentrations (5, 10, 25, 50, and 100µg/ml). The resulting solutions were then incubated at 25°C for 60 min. A similar procedure was repeated with methanol as blank, which served as control (0% inhibition). To 5 ml of the incubated sample, 5 ml Griess reagent (1% sulphanilamide, 0.1% naphthyl ethylene diamine dihydro chloride in 2% H_3PO_4) was added. The absorbance of the chromophore formed was measured using spectrophotometer at 546 nm. All the tests were performed in triplicate. The percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test sample preparations. Benzohydroxamic acid (BHA) was used as reference material.

Assessment of Vascular Permeability

Evans Blue (EB) dye extravasation technique was used to measure the permeability of capillaries to albumin in the aortic tissue of anesthetized rats. This technique is based on the principle that EB dye avidly binds to intravascular albumin and is thus a reliable way to assess transvascular fluxes of macromolecules. This technique has been extensively validated and has been shown to be a reliable estimate of the extravasation and interstitial accumulation of albumin as previously described[18]. Briefly, Evans blue (50 mg/kg) was administered *via* the jugular vein into the anaesthetized rat. After 4hr, the anterior and posterior synovial capsules and fat pad were dissected from each ankle joint, which were small. The tissues obtained from four ankles were grouped into one sample. The samples were weighed, and the amount of EB dye in the sample was estimated using dye extraction technique. This entailed cutting of the capsule into smaller pieces and mixing them with acetone in 1% $NaSO_4$ in the ratio of 7:3. The samples were shaken gently and continuously for 24 hr at room temperature. The amount of EB dye extracted was determined spectrophotometrically at 620 nm. The results were calculated from an EB dye standard curve (0.5–25 mg/mL) and were expressed as µg of EB dye per 100mg of tissue dry weight.

Statistical Analysis

The values are expressed as mean ± SEM. The n represents the number of animals studied. Statistical differences between normal and control and control and extract treatments were analyzed by one-way analysis of variance (ANOVA) and were further analyzed using Dunnett's multiple comparison tests. *P* value less than 0.05 was considered as statistically significant.

RESULTS

Preliminary Phyto constituent Analysis

Phytochemical analysis of *V. agnus-castus* leaves various extracts in various solvents such as petroleum ether, methanol, ethanol, and aqueous revealed the presence of carbohydrates, proteins, amino acids, steroids, terpenoids, glycosides, flavonoids, alkaloids, tannins, phenolic components, fats, and oils (Table.1).

Acute Toxicity Study

No toxic effects were observed at a higher dose of 2000 mg/kg body weight of Wistar rats. Hence, 1/10th dose was taken as effective dose (therapeutic dose). The cut-off value of LD_{50} 200mg was selected for assessing the anti-arthritic activity. The extract was considered to be safe and non-toxic for further pharmacological screening.

Anti-Arthritic Activity

In the present study, efforts were made to elucidate the possible mechanism of action that has been claimed by folk and traditional use of *V. agnus-castus* leaves in crippling arthritis and frozen joints.

Table:1 Phytochemical investigation of extracts of *V. agnus castus* leaves

S. No	Phytocons-tituents	Name of the Extracts			
		Petroleum ether Extract	Methanol Extract	Ethanol Extract	Aqueous Extract
1.	Carbohydrates	-	+	+	+
2.	Proteins	-	+	+	-
3.	Amino acids	+	+	+	-
4.	Steroids	+	+	+	-
5.	Terpenoids	+	-	-	-
6.	Glycosides	-	+	+	+
7.	Flavanoids	-	-	-	+
8.	Alkaloids	+	+	+	-
9.	Tannins and phenolic components	+	-	-	+
10.	Fats and oils	+	-	-	-

+ Presence; - Absence

Paw Volume

Chronic inflammation in the ankle joint of the rat is manifested as progressive increase in the volume of FCA injected paw. It is note worthy that the inhibitory effects of *V. agnus-castus* leaves extracts on rat paw edema were observed in standard drug and extract-treated groups. In the control group, significant ($P<0.001$) progressive increase in paw edema was observed compared to the normal group. However, the standard group showed significant ($P<0.001$) reduction in rat paw edema on day 3 that lasted till day 28. Similarly, the *V. agnus-castus* extract-treated groups also showed significant ($P<0.05$) reduction in rat paw edema from the 7 to 28 days of the study except the methanol and aqueous treated groups (Fig.1).

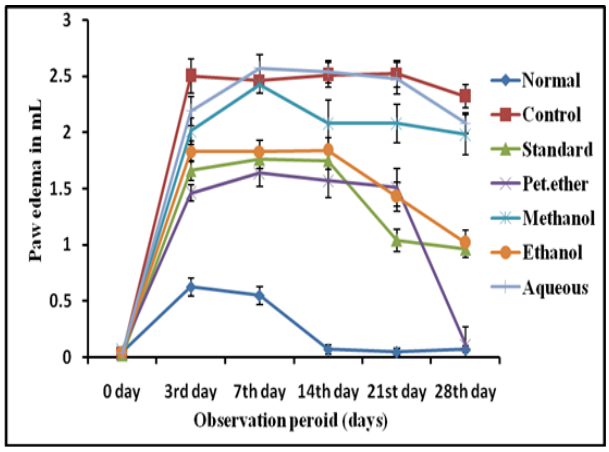

Figure 1: Effect of *V. agnus-castus* leaves extracts on FCA-induced changes in paw edema.

Changes in Body Weight

Bodyweights were almost identical in all groups of animals at 0 to 7 days and it was always declined in the control group from 14 to 28 days. In the normal group, increase in body weight was observed on subsequent days, whereas in the standard drug, petroleum ether, and ethanol extract groups a slight reduction was observed at 3 to 7 days; however, improvement in body weight was observed from 14th day to the last day of the experiment. On the other hand, the body weights of methanol and aqueous treated groups were almost similar to those of the control group rats (Table. 2).

Hematological Profile

FCA-induced arthritic rats at 28 days showed slight elevation in the total WBC count and reduction in RBC. Significant ($P<0.001$) increase in ESR level and significant reduction ($P<0.05$) in hemoglobin level were observed in the control group when compared with the normal group. In the standard drug, petroleum ether, and ethanol extract treated groups, recovery in RBC and WBC counts was observed, with a significant ($P<0.001$) recovery in Hb count and ESR level compared with the control group. Methanol and aqueous extract treated groups were not able to recover the hematological alterations induced by FCA in rats (Fig.2).

Nitric Oxide Synthesis

In the present study, serum nitric oxide (NO) levels were significantly ($P<0.001$) elevated to about twice the normal level in the control group compared to the levels in the normal group, which was considered as 100% (Fig.3). But, in contrast, the standard and extract treated groups showed significant reduction in the level of nitric oxide in arthritic rats compared to control group rats.

Table 2: Effect of *V.agnus-castus* leaves extracts on FCA-induced changes in body weight

BODY WEIGHT						
Treatment (mg/kg)	**0 Day**	**3rd day**	**7th day**	**14th day**	**21st day**	**28th day**
Group I (Normal)	153.8±3.1	157.3±4.1	157.4±4.2	158.5±4.1	163.3±4.5	163.2±4.6
Group II (Control)	158.8±6.1	156.4±6.0	157.5±5.8	152.1±5.7	153.4±5.3	148.2±5.6*
Group III (Indomethacin)	156.9±5.9	154.6±5.0	152.4±5.6	154.6±5.6	154.9±5.4	156.1±4.5
Group IV (Pet.ether Extract)	158.2±4.3	156.6±4.9	155.2±5.2	157.4±5.7	157.9±5.4	157.6±5.6
Group V (Methanol Extract)	154.4±4.2	154.2±4.4	151.4±4.5	138.6±5.1*	132.8±5.2*	128.8±5.2*
Group VI (Ethanol Extract)	152.2±5.6	152.1±4.8	148.9±4.7	152.6±4.6	154.4±4.7	158.3±4.9
Group VII (Aqueous Extract)	154.8±4.2	150.2±4.2	148.4±4.6	147.4±5.1	142.3±5.2	138.4±5.4*

Values are presented as mean ± SEM from six rats in each group. Each group compared with 0 day to 3, 7, 14, 21 and 28 days, *P* value less than 0.05 was considered as significant *$P<0.05$ significant.

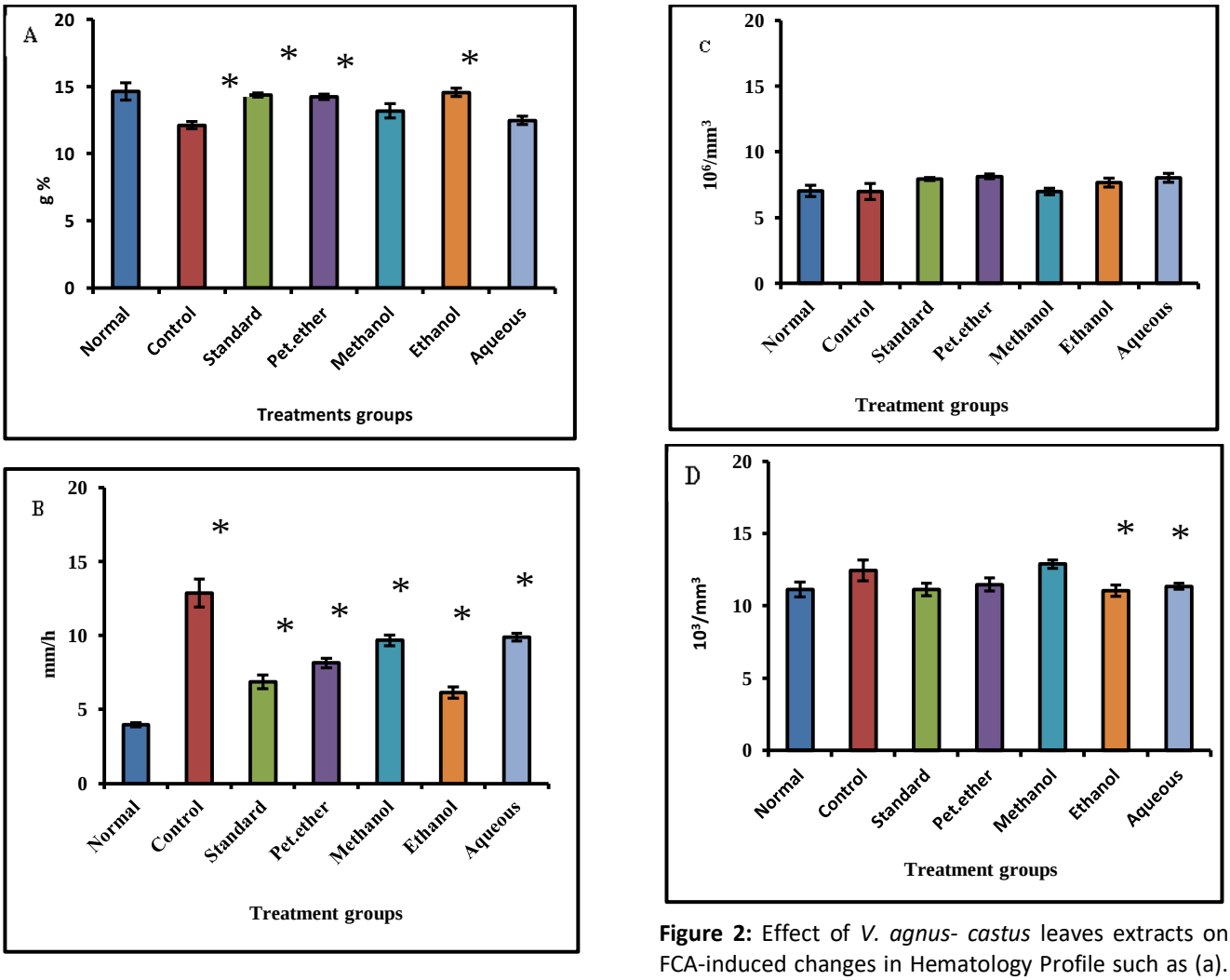

Figure 2: Effect of *V. agnus- castus* leaves extracts on FCA-induced changes in Hematology Profile such as (a). Hemoglobin (Hb), (b). ESR, (c). RBC, (d). WBC.

Values are presented as mean ± SEM from six rats in each group. Control group were compared with normal, standard drug, and extract treatment group. P value less than 0.05 was considered as significant. *P<0.05 significant.

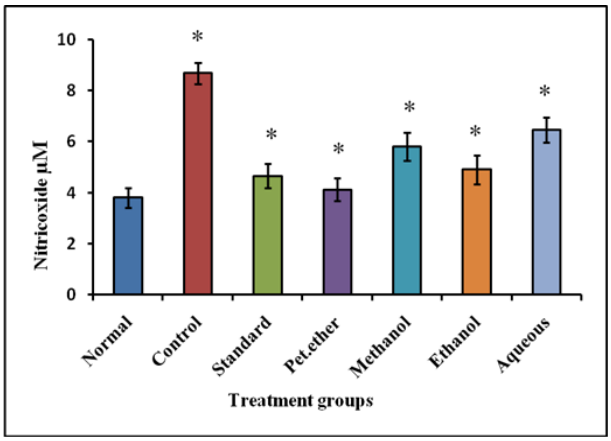

Figure 3: Effect of *V. agnus-castus* leaves extracts on FCA-induced serum nitric oxide synthesis.

Values are presented as mean ± SEM from six rats in each group. Control group were compared with normal, standard drug and extract treatment group. P value less than 0.05 was considered as significant. *P<0.05 significant.

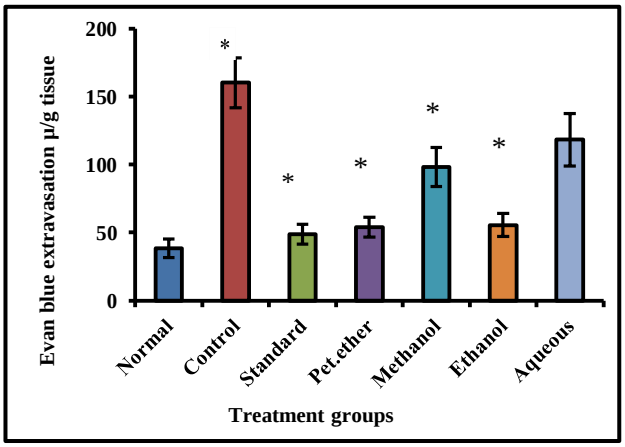

Figure 4: Effect of *V. agnus-castus* leaves extracts on FCA-induced vascular permeability

Values are presented as mean ± SEM from six rats in each group. Control group were compared with normal, standard drug and extract treatment group. P value less than 0.05 was considered as significant. *P<0.05 significant.

Vascular Permeability

Evans blue extravasation showed significant (P<0.001) augmentation in extravasations in FCA injected ankle joints of control group compared with normal group. However, all drug treated groups produced significant (P<0.001) inhibition of Evans blue extra vasation in arthritic joints of rats compared to the control group (Fig.4).

DISCUSSION

Animal models of rheumatoid arthritis are used extensively in research on pathogenesis arthritis. Adjuvant-induced arthritis in rats is recommended as a convenient model for preclinical studies of drugs used in the treatment of human arthritis, and has often been used to study the mechanism of action and preventive effects of a number of disease-modifying antirheumatic drugs. Freunds complete Adjuvant (FCA) was used to induce arthritis in rats to investigate the anti-arthritic effect of petroleum ether, methanol, ethanol and aqueous extracts of *V. agnus-castes* leaves in arthritic rats. This study was designed as per the US-FDA guideline for industrial preclinical evaluation of anti-arthritis[19]. In the present study, clinical aspects were considered for the evaluation of anti-arthritic activity which has been proposed as common animal model for rheumatoid arthritis. In the present study, the efforts were made to elucidate possible mechanism of action that has been claimed by flolk and traditional use of *V. agnus castes* leaves in crippling arthritis and frozen joints. The first phase of arthritis is associated with acute inflammation and systemic effect on liver from the 1st to 4th day. The second phase is from the 7th to 12th day with acute inflammation and per arthritis remission. The third phase (12th to 28 days) is marked with chronic inflammation, arthritis, and osteogenic activity[20].

Adjuvant-induced arthritis in rats is one of the most suitable test procedures to screen anti-arthritic agents since it closely resembles human arthritis. The development of FCA induced arthritis in the rat can be characterized by pronounced soft tissue swelling around the ankle joints during the development of arthritis, which is considered as edema of the particular tissues. As the disease progresses, a more diffused demineralization develops in the extremities[21]. The body weight of rats used as an indirect index in restoration of health suggests that the decrease in body weight during inflammation or disease condition is due to deficient absorption of nutrients through the intestine. Treatment with anti-inflammatory drugs normalizes the process of absorption[22]. The hematological profile in arthritic condition shows reduction in RBC, hemoglobin and lymphocytes whereas increase in WBC count and ESR level. It is proposed that the reduction in hemoglobin levels during arthritis was due to the reduced erythropoietin levels, owing to decreased response of the bone marrow and premature destruction of red blood cells. Similarly, an increase in the ESR is attributed to the accelerated formation of endogenous proteins such as fibrinogen and α/β globulin, which indicates an active, but obscure disease[23]. An indicator of infectious and inflammatory diseases, the WBC count was increased in arthritic rats[24].

Serum nitric oxide is produced by inducible nitric oxide synthase (iNOS), which has been observed in RA as well[25, 26]. McDougall et al reported that the several types of cells including macrophages, neutrophils, endothelial cells, chondrocytes and synovial fibroblasts produce nitric oxide. Increased levels of nitric oxide metabolic products such as nitrate and nitrite were detected in serum, urine and synovial fluid and their concentration was related to disease progression[27]. These findings confirm the presence of nitric oxide in RA and the inhibitory effect of treatment on nitric oxide synthesis would explain the possible mechanism of anti-arthritic activity.

In arthritic condition, a number of inflammatory mediators are released from the site of injury, which is caused by vasodilation. Evans blue has the capacity to pass through enlarged endothelial gaps from where it can escape into interstitial spaces. The amount of Evans blue dye present in synovial capsule can provide the relative index of vascular permeability. In the present study, a significant augmentation in extra vasation of Evans blue was observed in control group. The infiltration inhibitory effect of petroleum ether, methanol and ethanol extract of *V. agnus-castus* leaves was observed. However, aqueous extract showed no significant inhibitory effect[18, 26]. The anti-arthritic effect of the aqueous and petroleum ether, methanol and ethanol leaves extract of *V. agnus-castus* established in this study could be attributable to the presences of flavonoids, alkaloids, and sterols detected after phytochemical screening of the extracts. This assertion is supported by reports indicating that the presence of many biologically active phytochemicals, such as triterpenes, flavonoids, alkaloids, steroids, tannins, and glycosides, in various plant extracts may be responsible for their pharmacological properties[28].

CONCLUSSION

The results obtained from the present study clearly suggest that the petroleum ether and ethanol extracts of *V. agnus-castus* leaves have beneficial effects in long-lasting reduction of rat paw edema, recovery in hematological changes, inhibitory effects on nitric oxide synthesis and vascular permeability. Therefore, *V. agnus-castus* may be considered as a novel therapeutic agent in the treatment of rheumatoid arthritis.

Acknowledgements: The authors express their gratitude to Secretary and Principal, Bharath College of Science and Management, Thanjavur, Tamil Nadu, for providing the laboratory facilities and encouragement to conduct this study.

REFERENCES

1. Kaur A, Nain P, Nain J, Herbal plants used in treatment of Rheumatoid arthritis: A review, Int J Pharm Pharm Sci, 4, 2012, 44-57.
2. Murali Reddy B, Stalin C, Penchala Narasimhulu E, Gunashekar V, Evaluation of anti-arthritic activity of ethanolic extract of *Canthium dicoccum* in albino rats, International Journal of Novel aspects in Pharmaceutical Research, 1, 2013, 1-6.
3. Palombo EA, Phytochemicals from Traditional Medicinal Plants used in the Treatment of Diarrhoea; Modes of Action and Effects on Intestinal Function, Phytotherapy Research, 20, 2006, 717-724.
4. Sucher NJ, Carles MC, Genome-based approaches to the authentication of medicinal plants, Planta Medica, 74, 2008, 603-623.
5. Holtz C, Global health care: Issues and policies, Sudbury, MA, Jones & Bartlett, 59, 2008, 1-28.
6. Puratchikodi A, Yasodha, Siva Kumar A, VedhaHari BN, Preliminary phytochemical and anti-arthritic activity of an ayurvedic formulation-*Yogaraja gulgulu,* Journal of Phytology, 3, 2011, 31-36.
7. Mahady GB, Dietz B, Engle J, Michel J, Sagraves R, Chasteberry (*Vitex agnus-castus*) Encyclopedia of dietary supplements, Marcel Dekker, Inc, 28, 2005, 95-103.
8. Arokiyaraj S, Perinbam K, Vivek P, UdyaPrakash NK, Free radical scavenging and *in vitro* cytotoxicity activity of agnuside from *Vitex agnus-castus* (Verbenaceae), JPR, 5, 2012, 2548-2552.
9. Hajdu Z, Hohmann J, Forgo P, Martinek T,Dervarics M, Zupko I, Falkay G, Cossuta D, Mathe I, Diterpenoids and flavonoids from the fruits of *Vitex agnus –castus* and antiozidant activity of the fruit extracts and their constituents, Phytotherapy Research, 21, 2007, 391-394.
10. Sarer E, Gokbulut A, Determination of caffeic and chlorogenic acids in the leaves and fruits of *Vitex agnus-castus,* Turkish J Pharm. Sci, 5, 2008, 167-174.
11. Arokiyaraj S, Perinbam K, Agastian P Mohan Kumar R, Phytochemical analysis and antibacterial activity of *Vitex agnus-castus,* Int J Green Pharm, 3, 2009, 162-164.
12. Ecobichon DJ, The basis of toxicology testing, 2ed, CRC Press, New york, 1999.
13. Newbould BB, Chemotherapy of arthritis induced in rats by mycobacterial adjuvant, Br J Pharmacol Chemother, 21, 1963, 127-136.
14. De Castro Costa M, De Sutter P, Gybels J, Van Hees, J, Adjuvant-induced arthritis in rats: a possible animal model of chronic pain, Pain, 10, 1981, 173-185.
15. Jain AK, Manual of Practical Physiology, Arya Publications, New Delhi, India, 1998.
16. Austin JH, Drabkin DL, Estimation of Haemoglobin, J Biol Chem, 112, 1935, 67-88.
17. Chesbrough M, McArthur J, Determination of RBC and WBC count In a Laboratory Manual of Rural Tropical Hospitals, The English Language Book Society and Chruchill Livingstone, 1972, 145.
18. Demsar F, Van Dijke CF, Kirk BA, Kapila S, Peterfy CG, Roberts TP. et al, Mapping abnormal synovial vascular permeability in temporal mandibular joint arthritis in the rabbit using MRI, Br J Rheumatol,35, 1996, 23-25.

19. Butler SH, Godefroy F, Besson JM, Weil-Fugazza J, A limited arthritic model for chronic pain studies in the rat, Pain, 48, 1992, 73-81.

20. Begum VH, Sadique J, Long Term effect of herbal drug *withania somnifera* on adjuvant induced arthritis on arts, IJEB, 26, 1988, 877-882.

21. Patil KR, Patil CR, Jadhav RB, Mahajan VK, Patil PR, Gaikwad PS, Anti- arthritic activity of bartogenic acid isolated from fruits of *Barringtonia racemosa* Roxb Lecythdaceae), Evid Based Complement Alternat Med, 785245, 2011, 1-7.

22. Calvino B, Crepon-Bernard MO, Bars D, Parallel clinical and behavioural studies of adjuvant-induced arthritis in the rat: possible relationship with chronic pain, Behavioural Brain Research, 24, 1987, 11-29.

23. Maria M, Engeniusz M, Miroslaw K, Maria K, Iwona P, Adjuvant induced disease in rats, clinical findings and morphological and biochemical changes in the blood histological changes in internal organs, Rheumatology, 2, 1983, 231-245.

24. Fletcher DS, Widmer WR, Luell S, Christen A, Orevillo C, Shah S, Visco D, Therapeutic administration of a selective inhibitor of nitric oxide synthase does not ameliorate the chronic inflammation and tissue damage associated with adjuvant-induced arthritis in rats, JPET, 284, 1998, 714-721.

25. Huskicl J, Zaciragicl A, Babic N, Mulabegovic N, Nitric oxide in serum and renal during compensatory renal hypertrophy in rats, BJBMS, 6, 2006, 46-49.

26. Lam F, Wong HH, Ng ES, Time course and substance effects on the vascular and morphological changes in adjuvant-induced mono arthritic rats, Int Immunopharmacol, 2004, 299-310.

27. McDougall JJ, Karimian SM, Ferrell WR, Prolonged alteration of vasoconstrictor and vasodilator responses in rat knee joints by adjuvant monoarthritis, Exp Physiol, 80, 1995, 349-357.

28. Kyei S, Koffuor GA, Boampong JN, Antiarthritic effect of aqueous and ethanolic leaf extracts of *Pistia stratiotes* in adjuvant-induced arthritis in Sprague-dawley rats, J Exp Pharmacol, 4, 2012, 41-51.

Source of Support: Nil, **Conflict of Interest:** None.