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Investigation of *in vitro* anti-arthritic activity of aqueous extracts of leaves of *Vitex negundo* L. using methotrexate as DMARDs

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Abstract
The aim of the study was to investigate the aqueous extracts of *Vitex negundo* L. for its *in vitro* anti-arthritic activity by protein denaturation inhibition assay and membrane stabilization method. Methotrexate is one of the most popular and effective drug used worldwide for the treatment of inflammatory conditions like rheumatoid arthritis. The main cause of inflammation in rheumatoid arthritis is protein denaturation. Production of auto antigen in certain rheumatic disease was important for inflammation as well as arthritis. HRBC membrane stabilization was similar to lysosomal membrane which influences the process of inflammation. Methotrexate was used as a standard drug. The percentage of protein denaturation and membrane stabilization for aqueous extracts were done at different concentrations (100, 200, 400, 800, 1000 µg/ml). The maximum inhibition of protein denaturation and membrane stabilization of aqueous extracts of *Vitex negundo* was found to be 65.62 ±1.10 and 71.87 ±1.46 at dose of 1000 µg/ml respectively and standard inhibition of protein denaturation and membrane stabilization using methotrexate was found to be 87.50±7.02 and 81.25±3.51at 100 µg/ml respectively. The aqueous extracts of *Vitex negundo* L. showed significant activity at the highest concentration.

Keywords: *Vitex negundo*, methotrexate, protein denaturation assay, membrane stabilizing activity, anti-arthritic activity

Introduction
Inflammation is the reaction in living tissues which releases the lysosomal enzymes which produces a variety of disorders leading to tissue injury [1, 2]. The mechanism of inflammation is attributed in part to release of reactive oxygen species (ROS) from activated neutrophils and macrophages. Prolonged inflammation leads to rheumatoid arthritis, autoimmune disease and other infectious diseases [3].

Rheumatoid arthritis is a chronic inflammatory disease of joints that results in joint pain. It affects an estimated 1% population throughout the world. Progression of the disease results in joint destruction deformity and significant disability [4]. It is characterized by auto-antibody production, bone destruction, skeletal disorders and synovial inflammation [5].

The treatment of arthritis involves the use of different classes of drugs such as NSAIDs, corticosteroids and disease modifying anti-rheumatic drugs (DMARDs). In Indian traditional medicines literature describes parts of certain plants for treating pain and inflammatory conditions like arthritis. The anti-arthritic drugs showed severe side effects such as irritation of the gastric mucosa, gastric ulceration and bleeding, impair renal and hepatic functions [6]. As a result a search for other alternatives seems to be necessary which would be more beneficial

In comparison to other DMARDs methotrexate is well tolerated and used as major advancement of rheumatoid arthritis. Several studies showed that the combination of methotrexate plus other therapy was significantly better than monotherapy with methotrexate. Due to side effects and cost issues of other treatment with methotrexate it is necessary to find out other therapy which is having less side effects and cost effective. The low dose or weekly dose of methotrexate used as monotherapy or in combination with other drug is 10 to 25 mg/wk.

World's most population relies on traditional medicine for primary healthcare needs and involves use of plant extracts or their components .Arthritic conditions are treated with traditional medicine with considerable success. Although various modern drugs are used to treat these types of disorder their prolonged usage may cause severe side effects. So, there is urging to develop new herbal therapeutic agents with minimum side effects. Plants are excellent sources of antioxidants, anti-arthritic and anti-inflammatory agents [7].

Vitex negundo L. (family-Lamiaceae) is large aromatic shrub commonly available in Maharashtra and is claimed to possess anti-arthritic, anti-leprotic, anti-inflammatory, anti-spasmodic, laxative and useful in many other disease [8, 9].

Protein lose its structure or become denatured when there is activation of various enzymes, migration of tissues and break down of tissues occurs [10]. The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane. Therefore, as membrane stabilizes that interfere in the release and or action of mediators like histamine, serotonin, prostaglandins, leutcotriens etc.

The present study is aimed to investigate anti-arthritic activity of aqueous extract of *Vitex negundo* L.by *in vitro* methods using methotrexate as a standard drug.

Material and Method

Collection of Plant

The *Vitex negund*o was collected from local area of Sangli districts. The plant was identified and authenticated by Dr. V. B. Awale, an approved Botanist, Dept. of Botany, Dr. Patangrao kadam Mahavidyalaya, Sangli. A specimen voucher no. (ACOP/SNG/101 to 106) has been deposited in Department of Pharmacognosy, Adarsh College of Pharmacy, Vita, Sangli, MS, India.

Extraction

The leaves of *Vitex negundo* L. were cleaned and any dust particles were removed. It was then dried at room temperature. After that it was pulverized and passed through sieve no.40.The obtained powdered material then macerated for 7 days with water containing chloroform in the ratio 16:1.The filtered extract was concentrated and stored for further use.

Method: *In vitro* anti -arthritic activity [11]

Inhibition of protein denaturation [12]

The method was adopted from the previous article published by Pavithra *et al*. 2015 with some modification. The reaction mixture consisted of the 100µl aqueous extracts of *Vitex negundo* L. (final concentration 100, 200, 400, 800, 1000 µg/ml) and 100µl of 5% aqueous solution of bovine serum albumin (BSA). The sample was incubated at 37 °C for 20 min and then temperature was increased to 70 °C in hot water bath for 10 min. The mixture was allowed to cool under tap water for 10 min after which turbidity was measured at 660 nm. The blank comprised of the distilled water. The results were compared with methotrexate.

Formula: The percentage inhibition of protein denaturation can be calculated as:

$$\text{Percentage inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \text{ X } 100$$

Effect on Membrane Stabilization [13]

The method used was from procedure adopted by Vallabh Deshpande *et al*. with slight modifications. Blood from healthy human volunteers who did not consumed NSAID for two weeks was obtained. The reaction mixtures 4.5ml consists of 2ml hypotonic saline (0.25% NaCl) + 1ml 0.15M phosphate buffer (pH 7.4) + 1ml test solution (100-1000µg/ml) in normal saline + 0.5ml of 10% HRBC in normal saline. The mixture was incubated at 56 ºC for 30 min. The test tubes were cooled under running tap water. After that reaction mixture was centrifuged for 3000rpm for 10min and the absorbance of the supernatant was measured at 560nm. Methotrexate was used as standard drug.

Formula: The percentage membrane stabilization activity was calculated by the following formula

$$\% \text{ protection} = \frac{100 - \text{ OD of sample}}{\text{OD of control}} \text{X } 100$$

Table 1: The percentage inhibition of different concentration of aq. extracts of *Vitex negundo* by Protein denaturation method (BSA)

Plant name	Conc (µg/ml)	OD	% inhibition	IC_{50}
Blank		0.64		
Vitex negundo Linn	100	0.53	17.18 ±1.03	
	200	0.51	20.31 ±0.98	
	400	0.43	32.81 ±0.96	820.85
	800	0.39	39.06 ±1.06	
	1000	0.22	65.62 ±1.10	

Values represent in the results are mean±SD of three replicates; linear regression analysis was used to calculate IC_{50} value

Table 2: The percentage inhibition of different concentration of Methotrexate by Protein denaturation method (BSA)

Drug	Conc (µg/ml)	OD	% inhibition	IC_{50} (µg/ml)
Blank		0.64		
Standard Methotrexate	10	0.48	25.00±6.50	
	20	0.31	51.56±4.04	
	40	0.29	54.68±5.56	35.53
	80	0.17	73.43±8.08	
	100	0.08	87.50±7.02	

Values represent in the results are mean±SD of three replicates; linear regression analysis was used to calculate IC_{50} value

Table 3: The percentage stabilization of different concentration of aq. extracts of *Vitex negundo* L. by Membrane Stabilization method.

Plant name	Conc (µg/ml)	OD	% inhibition	IC_{50}
Blank		0.32		
Vitex negundo Linn	100	0.23	28.12 ±1.01	
	200	0.19	40.62 ±0.68	
	400	0.11	56.62 ±1.05	437.77
	800	0.10	68.75 ±1.50	
	1000	0.09	71.87 ±1.46	

Values represent in the results are mean±SD of three replicates; linear regression analysis was used to calculate IC_{50} value

Table 4: The percentage inhibition of different concentration of Methotrexate by Membrane Stabilization method.

Drug	Conc (µg/ml)	OD	% inhibition	IC_{50} (µg/ml)
Blank		0.32		
Standard (methotrexate)	10	0.15	53.12±4.50	
	20	0.12	62.50±5.00	
	40	0.10	68.75±4.50	>100
	80	0.08	75.00±3.51	
	100	0.06	81.25±3.51	

Values represent in the results are mean±SD of three replicates; linear regression analysis was used to calculate IC_{50} value

Results and Discussion

The *in vitro* anti-arthritic activity of various concentrations of aqueous extract of *Vitex negundo* L. was studied by protein

denaturation and HRBC membrane stabilization method respectively using methotrexate as standard drug.

The inhibition of protein denaturation and inhibition of hypotonicity induced membrane stabilization was taken as a measure of the anti-arthritic activity. The percentage of inhibition of protein denaturation and percentage of membrane stabilization for aqueous extract of *Vitex negundo* L. and methotrexate were done at concentration (100, 200, 400, 800, 1000 µg/ml) and (10, 20, 40, 80, 100 µg/ml**)** respectively. Aqueous extracts of *Vitex negundo* L. are effective in inhibiting protein denaturation at different concentrations. It showed that maximum inhibition 65.62% ±1.10 at 1000 µg/ml of aqueous extract and 87.50% at 100 µg/m**l** of methotrexate. The inhibition of hypotonicity induced HRBC membrane lysis i.e., stabilization of HRBC membrane was taken as a measure of the anti-arthritic activity. Aqueous extracts of *Vitex negundo* are effective in inhibiting the heat indued hemolysis of HRBC at different concentrations. It showed that maximum inhibition 71.87 ±1.46 at 1000 µg/ml of aqueous extract and 81.25% at 100 µg/ml of methotrexate. With increasing `concentration, the inhibition of protein denaturation and membrane stabilization and protection is also increased. Hence anti-arthritic activity of extracts was concentration dependent.

Protein denaturation which is identified as a cause of inflammation occurs when living tissue are injured. Denaturation of protein occurs due to disruption of electrostatic hydrogen disulphide bonds. The compounds which prevent these changes are having potential anti-arthritic activity. HRBC membrane stabilization was similar to lysosomal membrane which influences the process of inflammation. The inflammatory response of lysosomal membrane was important because it helps the inhibition process by inhibiting the release of lysosomal constituents of activated neutrophil. The aqueous extract of Vitex negundo may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation.

Conclusion

Inhibition of protein denaturation and stabilization of HRBC membrane by hypotonicity induced membrane lysis was carried out and demonstrate the reduction in inflammation when compared with methotrexate. The presence of active principle's in the aqueous extracts may be responsible for this activity. Hence aqueous extract of leaves of *Vitex negundo* L. *can* be used as a potent antiarthritic agent.

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