Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(6): 318-322
Received: 07-09-2018
Accepted: 09-10-2018

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In-vitro anti-inflammatory and anti-arthritic activity of arthrito

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Abstract
The study was done to evaluate the efficiency of Arthrito at different concentration for anti-inflammatory and anti-arthritic activity by HRBC membrane stabilization method and inhibition of protein denaturation method. HRBC membrane stabilization method was chosen for this experiment because of its simple, reliable, less toxic and less time consuming. HRBC membrane stabilization was similar to lysosomal membrane which influences the process of inflammation. In this experiment arthrito shows its membrane stabilizing activity over all concentration that was important for inflammation. Diclofenac sodium which was used as a standard drug in the experiment shows maximum membrane stabilization as well as protein denaturation property. To limiting the inflammatory response lysosomal membrane was important because it helps the inhibition process by inhibit the release of lysosomal constituents of activated neutrophil. The extracellular release of bactericidal enzymes and protease which are the active constituents of neutrophil can cause tissue inflammation and damage. The stabilization of erythrocyte membrane implies that that arthrito can also stabilize lysosomal membrane. Protein denaturation was the main causes of inflammation in rheumatoid arthritis condition. Production of auto antigen in certain rheumatic disease was important for inflammation as well as arthritis. Arthrito was capable to control the production of auto antigen and inhibit the denaturation of protein. Thus, Arthrito is very important for anti-inflammatory and anti-arthritic activity.

Keywords: Anti-inflammatory, anti-arthritic, HRBC membrane sedimentation, lysosomal membrane, rheumatoid arthritis

Introduction
Herbal medicines have been used for the relief of pain throughout the history. India is one of the largest producers of medicinal plants in the world. Herbal medicines are the synthesis of therapeutic experience of generations of practicing physicians of indigenous systems of medicine for over hundreds of years. Herbal medicines are widely used in the developed as well as in developing countries for primary health care because of their efficacy, safety and lesser side effects. “Save plants to save life”, was the call given by WHO a few years ago to stress on to the role of medicinal plants in achieving the goal of “health for all”. Plant derived drugs serve as a prototype to develop more effective and less toxic medicines. The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites such as alkaloids, flavonoids, phenols, saponins, sterols etc. There is a growing attention in correlating the phytochemicals of a medicinal plant with its pharmacological activity [1, 2, 3].

Inflammation is usually a local and protective response of living mammalian tissues to the injury intended to eliminate the initial cause of cell injury. It is the part of host defense mechanism of which includes several mechanisms and complex array of enzyme activation, mediator release, cell migration, tissue break down and repair. During inflammation, release of histamine, bradykinin, 5HT, prostaglandins, leukotriens, platelet activating factor, nitrogen and oxygen radicals play pivotal role [4, 5]. Rheumatoid arthritis is a chronic, systemic inflammatory disease usually affects joint on both sides of the body equally where wrist, fingers, knees, feet and ankles are the most commonly affected. It can occur at any stage and women are affected more than men [6]. The term rheuma indicates a flow of pain through the joints of the body. It is a systemic inflammatory disease of unknown etiology affecting both articular tissues and extra articular organs, characterized by pain, stiffness and swelling of joints which can lead to significant morbidity and increased mortality [7]. In RA, the synovial membrane becomes highly vascularised, synovial fibroblasts proliferate and the inflammatory cells release a numerous cytokines like interleukin-1, interleukin-6, interleukin-18, tumour necrosis factor – α (TNF-α), granulocyte macrophage colony stimulating factor etc into the joint. These agents subsequently cause synovial cells to release proteolytic enzymes, resulting in destruction of

bone and cartilage [8, 9, 10]. The clinical treatment of inflammatory disease is dependent on drugs which either to the non-steroidal or steroidal chemical therapeutics. The use of steroidal as well as non-steroidal anti-inflammatory drugs becoming highly controversial due to their multiple side effects. Therefore, a need arises for the Development of newer anti-inflammatory agents from natural sources having powerful activity with lesser side effects as substitutes for chemical therapeutics [11, 12]. Even though most of the synthetic anti-inflammatory drugs are available in the market, due to their well-known side effects, toxic effects and production cost, presently people are search for natural anti-inflammatory drugs without any adverse effects [13]. A systemic study of anti-inflammatory effects of Indian medicinal plants began by Gujral and his associates in 1956 and they screened a number of plants for their anti-arthritic effects. Subsequently, various workers from different laboratories in India have made significant contribution [14]. Since last one decade India has seen a tremendous growth in herbal drug market which has resulted in the development of numerous proprietary herbal drug formulations by various manufacturers, majority of them comprising of polyherbal formulations where it has been observed that one phytoconstituent synergizes the action of other one.

Arthrito, a polyherbal capsule formulation, is an Ayurvedic proprietary medicine indicated for arthritis and arthritis related inflammation, which is manufactured by Rumi Herbals Pvt. Ltd. It consists of fifteen herbal ingredients showed in Table 1. Most of the herbs are known for their anti-inflammatory and anti-nociceptive activities [15, 16]. The formulation of arthrito and its capsules are given in Fig.1.

Table 1: Most of the herbs are known for their anti-inflammatory and anti-nociceptive activities

Botanical name	Common name	Family	Parts used
Alpinia galangal	Siamese ginger	*Zingiberaceae*	Seed
Asteracantha longifolia	Kulikhara	*Acanthaceae*	Rhizomes
Boswellia serrata	Shallaki, Indian olibanum tree	*Burseraceae*	Dried-gum resin
Cassia fistula	Cascara, Golden Shower, Indian Labur num	*Caesalpiniaceae*	Dried pulp
Commiphora mukul	Guggul	*Burseraceae*	Dried-gum resin
Eclipta alba	Bhringaraj	*Compositae*	Whole plant
Elettaria caradamomum	Elaichi	*Zingiberaceae*	Fruits
Ricinus communis	Castor oil	*Euphorbia ceae*	Roots
Emblica officinalis	Amla	*Euphorbiaceae*	Fruits
Saussurea lappa	Costus	*Composita*	Rhizomes
Sida cordifolia	Indian ephedra, Jangli medhi	*Malvaceae*	Whole plant
Terminalia belerica	Myrobalan, Hardad	*Combretaceae*	Fruits
Terminalia chebula	Black my robalan or chebulic myrobalan	*Combretaceae*	Fruits
Vitex negundo	Nirgudi	*Verbenaceae*	Aerial parts
Withania somnifera	Ashwagandha	*Solanaceae*	Roots

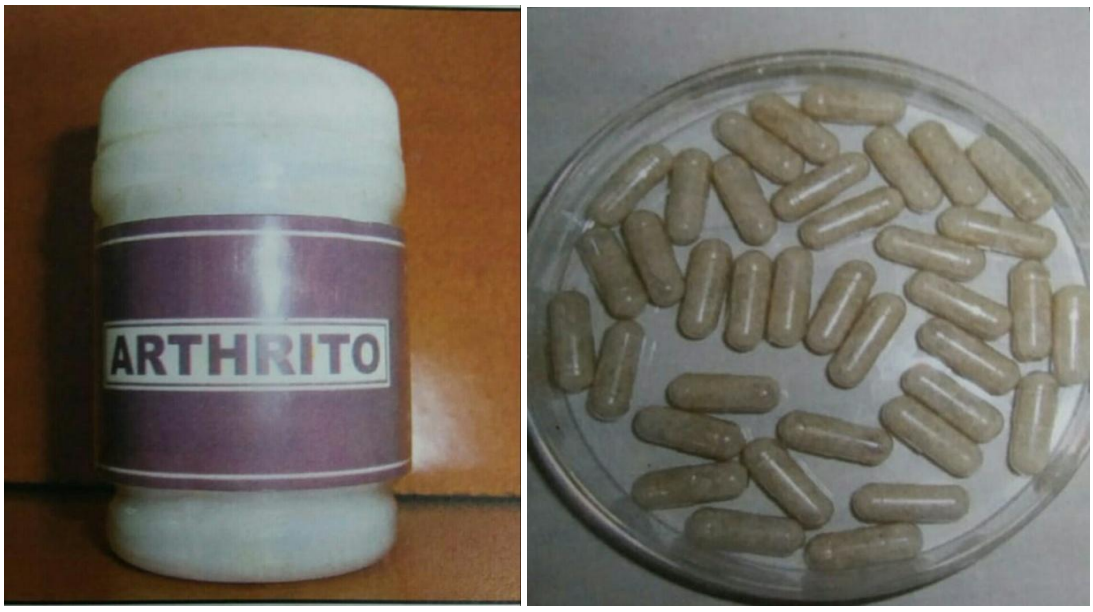

Fig 1: Arthrito and Arthrito capsule

Experimental Method

Human Red Blood Corpuscle (Hrbc) Membrane Stabilization Method [17]

Sample: Arthrito

Preparation of HRBC Suspension

4ml of venous blood was collected from healthy volunteers and mixed with equal volumes of Alsever's solution and centrifuge at 300 rpm and the packed cells were washed with isoline and a 10% v/v suspension was made with isoline.

To determine the anti-inflammatory activity by HBRC membrane stabilization method, the following four solution were used,

1. **Test Solution (4.5ml):** It consists of 2 ml hypotonic saline (0.25%w/v), 1 ml of phosphate buffer (pH7.4), 1 ml of sample extract (50, 100, 200, 400, 800, 1000 µg/mL) in normal saline and 0.5 ml of 10 % w/v human red blood cells in isotonic saline.

2. **Product Control (4.5 ml):** It consists of 2 ml of hypotonic saline (0.25% w/v), 1 ml of phosphate buffer (pH 7.4), 1 ml of sample extract (10, 50, 100,200, 400, 800, 1000 µg/mL) in normal saline and 0.5 ml of 10% w/v human red blood cells in isotonic saline.
3. **Test Control (4.5 ml):** It consists of 2 ml of hypotonic saline (0.25% w/v), 1 ml of phosphate buffer (pH 7.4), 1 ml of isotonic solution saline and 0.5 ml of 10% w/v human red blood cells in isotonic saline.
4. **Standard Solution (4.5 ml):** It consists of 2 ml of hypotonic saline (0.25% w/v), 1 ml of phosphate buffer (pH 7.4), 1 ml of diclofenac solution (200 µg/mL) in normal saline and 0.5 ml of 10% w/v human red blood cells in isotonic saline.

The above four solutions were included at 56ºC for 30 min. The tubes were then cooled under running tap water for 20 min. After that they were centrifuged. The supernatant liquid was separated and absorbance of supernatant solution was measured at 560 nm by UV spectrophotometer. The percentage stabilization activity was calculated as follows,

$$\text{Percent Stabilization} = \frac{(\text{O. D. of Test solution} - \text{O. D. of Product Control})}{\text{O. D. of Test Control}} \times 100$$

The test control represents 100% lysis. The results were compared with standard solution.

Determination of in-vitro anti-arthritic activity by inhibition of protein denaturation method [18, 19]

Chemical/ solution: Bovine serum albumin, pH 7.0 (Himedia) 1N HCl, Phosphate buffer (pH 6.3)

Instruments: incubator, UV spectrophotometer

To determine the anti-arthritic activity (In-vitro) by Inhibition of Protein Denaturation method, following four solutions were used.

1. **Test solution (0.5 ml):** It consists of 0.45 ml of bovine serum albumin (5% aqueous solution) and 0.05 ml of test solution (100, 200, 400, 800, 1000 µg/mL) and pH was adjusted to 6.3 by using small amount of 1N HCl. The samples were incubated at 37ºC for 20 minutes and heated at 57ºC for 20 minutes. After cooling, 2.5 ml of phosphate buffer (pH 6.3) was added.
2. **Test Control Solution (0.5 ml):** It consists of 0.45 ml of bovine serum albumin (5% aqueous solution) and 0.05 ml of distilled water and pH was adjusted to 6.3 by using small amount of 1N HCl. The sample was incubated at 37ºC for 20 minutes and heated at 57ºC for 3 minutes. After cooling, 2.5 ml of phosphate buffer (pH 6.3) was added.
3. **Product Control (0.5 ml):** It consists of 0.45 ml of distilled water and 0.05 ml of test extract solution (100, 200, 400, 800, 1000 µg/mL) and pH was adjusted to 6.3 by using small amount of 1N HCl. The samples were incubated at 37ºC for 20 minutes and heated at 57ºC for 3 minutes. After cooling, 2.5 ml of phosphate buffer (pH 6.3) was added.
4. **Standard Solution (0.5 ml):** It consists of 0.45 ml of bovine serum albumin (5% aqueous solution) and 0.05 ml of diclofenac sodium solution and pH was adjusted to 6.3 by using small amount of 1N HCl. The sample was incubated at 37ºC for 20 minutes and heated at 57ºC for 3 minutes. After cooling, 2.5 ml of phosphate buffer (pH 6.3) was added.

The percentage inhibition of protein denaturation was calculated as follows,

$$\text{Percent Denaturation} = \frac{(\text{O. D. of Test solution} - \text{O. D. of Product Control})}{\text{O. D. of Test Control}} \times 100$$

The control represents 100% protein denaturation. The results were compared with diclofenac sodium standard solution.

Result

Table 2: The percentage stabilization of different concentration of 'ARTHITO' by HRBC Membrane stabilization method

Concentration of Sample (µg/ml)	Percentage Stabilization of the sample (%)	Percentage Stabilization of Diclofenac Sodium (%)
50	49.4	
100	60.32	
200	69.79	95.88
400	78.50	
800	88.12	
1000	92.47	

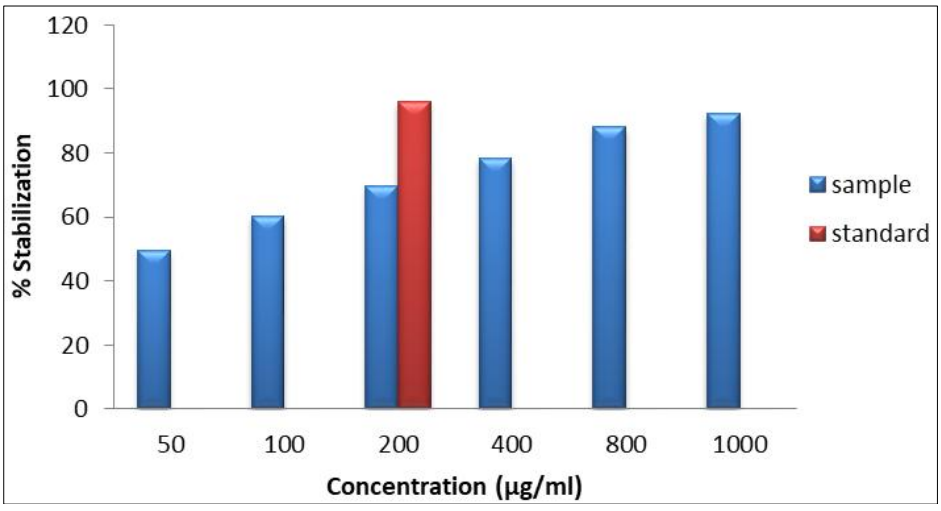

Fig 2: HRBC Membrane Stabilization Method

Table 3: The percentage Inhibition of different concentration of Arthrito by Protein Denaturation Method using Bovine Serum Albumin

Concentration of Sample (µg/ml)	Percentage Inhibition of protein denaturation of sample	Percentage Inhibition of protein denaturation of Diclofenac Sodium (%)
50	35.29	
100	60.00	
200	73.19	96.66
400	78.86	
800	86.33	
1000	91.26	

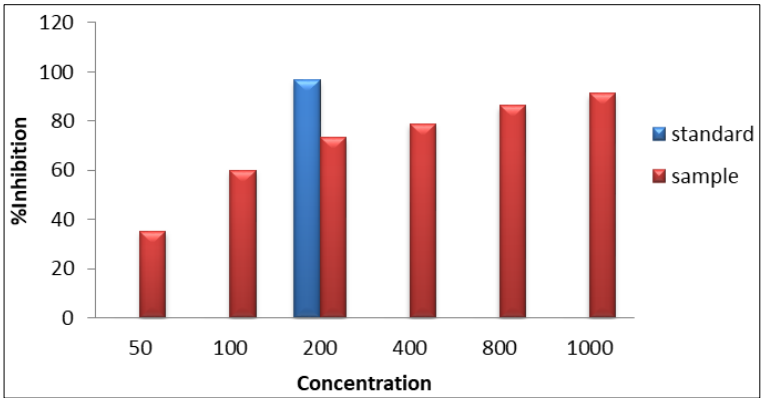

Fig 3: Protein Denaturation Method

The *in-vitro* anti-inflammatory and anti-arthritic activity of various concentrations of Arthrito was studied by HRBC membrane stabilization method and protein denaturation method respectively.

From the study it has been found that Arthrito was effective in inhibiting the heat induced hemolysis of HRBCs at different concentrations (50 to 1000µg/ml). Arthrito showed dose dependent membrane stabilizing activity over all the concentration ranges with the maximum RBC membrane stabilization 92.47% at 1000µg/ml. Arthrito showed significant effectiveness in inhibition of heat induced albumin denaturation as well. The maximum percentage inhibition of protein denaturation was 91.26% at 1000 µg/ml of Arthrito. Diclofenac sodium at 200µg/ml was used as a standard drug which showed 95.88% membrane stabilization and 96.66% inhibition of protein denaturation. Results were presented in Table No.2 and 3 and graphical representations were given in Fig.1 and Fig.2.

Discussion

Stabilization of lysosomal membrane is important in limiting the inflammatory response by inhibiting the release of lysosomal constituents of activated neutrophil. These neutrophil lysosomal constituents include bactericidal enzymes and protease, which upon extracellular release cause further tissue inflammation and damage [20]. The erythrocyte membrane is analogous to the lysosomal membrane 13 and its stabilization implies that the extracts may as well stabilize lysosomal membrane. Arthrito may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. Protein denaturation is one of well documented causes of inflammation in conditions like rheumatoid arthritis [21]. Production of auto-antigens in certain rheumatic diseases may be due to *in vivo* denaturation of proteins [22, 23]. Some anti-inflammatory drugs have shown dose dependent ability to inhibit protein denaturation. Similar results were observed from many reports from plant extract [24].

Mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding. From the results of present study it can be stated that Arthrito is capable of controlling the production of auto antigen and inhibits denaturation of protein in rheumatic disease.

Conclusion

The anti-inflammatory activity of arthrito may be due to the presence of many phytochemicals in the formulation. However, further studies are required to identify the lead molecule in the extract and to study the action of mechanism. Further and detailed studies are needed for the isolation and identification of active principles responsible for this property and determination of possible mechanism of anti-inflammatory and anti-arthritic activity.

Acknowledgement

Authors are very thank full to BCDA College of Pharmacy and Technology for their continuous support to performing this research work.

Conflict of interest: Authors have no conflict of interest.

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