

The Journal of Phytopharmacology

(Pharmacognosy and phytomedicine Research)

Research Article

ISSN 2320-480X
 JPHYTO 2018; 7(1): 60-65
 January- February
 Received: 17-09-2017
 Accepted: 02-11-2017
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Investigation of anti-arthritic activity (*in-vitro* models) of *Hibiscus hispidissimus* Griffith

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ABSTRACT

Aim of the experiment: The present study was designed to investigate the anti-arthritic potential of the plant *Hibiscus hispidissimus*. **Materials and Methods:** The aerial parts of the plant was collected, dried and extracted (maceration) with ethanol. Preliminary phytochemical studies were carried out. All the *in-vitro* models i.e. inhibition of protein denaturation, membrane stabilization and proteinase inhibition were carried out with standard reference drug diclofenac sodium. **Result:** Dose dependent and significant ($p < 0.05$) anti arthritic activity in *in-vitro* models were found. **Conclusion:** The results reveal promising anti arthritic potential of the plant. However further pharmacological investigation using isolated active ingredients can be carried out to confirm its efficacy and mechanism of action.

Keywords: Anti-arthritic activity, *Hibiscus hispidissimus*, inhibition of protein denaturation, membrane stabilization, proteinase inhibition, *in-vitro* studies.

INTRODUCTION

The plants provide foodstuff, attire, shelter and medicine. Most of the herbal benefits seem to have been developed through observation of wild animals and by trial and error methods. As time goes on, people started to find and to utilize more herbs having medicinal power. They systematically brought together information on herbs and developed to well-defined herbal pharmacopoeias i.e. traditional medicinal system [1]. Traditional use of medicine is identified as a way to learn about potential future medicines. Because of wide biological and medicinal values, high safety margins and lesser cost of herbal medicine, it has great demand and used as source of basic health care in both developed and developing countries [2, 3]. WHO notes that around 200 pharmaceutical medicines are derived from the plant, in modern medicinal system around 74% of which are used in ways that can directly correlated to their ancient medicinal uses [4]. Arthritis is one of the most common chronic inflammatory disorders, foremost cause of disability in world wide. There are more than 100 different types of arthritis and related conditions. Out of which rheumatoid arthritis and osteoarthritis are the major ones. Most of the diseases of joints affect synovial joints. Symptoms of one type arthritis are unlike other type. Some people may show mild but some are with strong symptoms. Some of the common symptoms are: Pain, Edema of Joints, Rigidity, Tenderness, Redness, Warmth, Loss of Flexibility, Limping, Bone Spurs, Discomfort when Standing or Walking, Fatigue (feeling tired) [5, 6, 7].

Rheumatoid arthritis is a common autoimmune disease that is associated with progressive disability, systemic complications, early death and socioeconomic costs. Rheumatoid arthritis is characterized by synovial inflammation and hyperplasia ("swelling"), autoantibody production [rheumatoid factor and anti-citrullinated protein antibody (ACPA)], cartilage and bone destruction (deformity), systemic features, including cardiovascular, pulmonary, psychological, and skeletal disorders [6, 7].

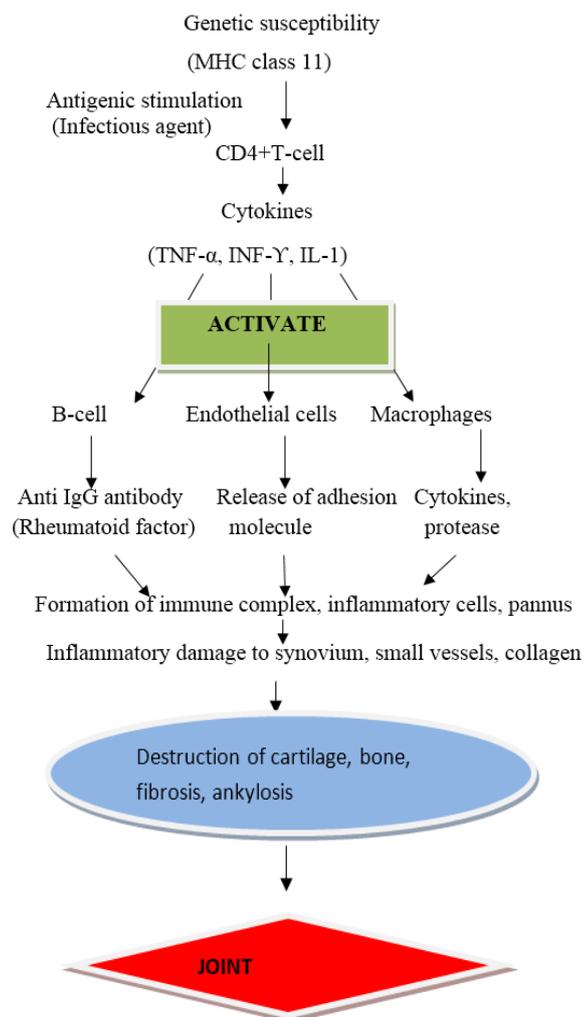
The body's natural defenses are part of the problem in rheumatoid arthritis. Due to some unknown reason, the immune system starts attacking certain parts of the body instead of protecting it [7].

Pathogenesis of rheumatoid arthritis [6, 7, 8]:

- ❖ In response to antigenic exposure in a genetically predisposed individual (HLA-DR), CD4+T-Cell are activated.
- ❖ These cells elaborate cytokines like tumour necrosis factor α , interleukin IL-1 and IL-6.
- ❖ These cytokines activates endothelial cells, B lymphocytes and macrophages.
- ❖ Activated B-cells releases IgM antibody against IgG – this molecule is termed as rheumatoid factor

- ❖ IgG and IgM immune complexes trigger inflammatory damage to the synovium, small blood vessels and collagen.
- ❖ Activated endothelial cell express adhesion molecules which stimulate inflammatory cells.
- ❖ Activation of macrophages releases more cytokines which cause damage to joint tissue and vascularisation of cartilage called as pannus formation.

Damage and destruction of bone and cartilage followed by fibrosis and ankylosis result in joint deformities.



Arthritis can be treated by using drugs such as non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, immunosuppressant, disease modifying anti-rheumatic drugs (DMARD) and newer biological agents such as TNF- α and monoclonal antibodies. But limitation is their side effects. Hence, there is a need to explore complementary and alternative medicines which are safe, potent, less toxic and cost effective [9].

Hibiscus hispidissimus is an annual or biennial suffrutescent trailing prickly herb found throughout India as under growths in forest up to 900 m³. The plant *Hibiscus hispidissimus* belongs to the family Malvaceae (Mallow family). Synonyms for the plant are *Hibiscus furcatus* DC Non Wild and *Hibiscus aculeatus* Roxb Non Walter. The common names of this plant are wild hibiscus, comfort root and big thicket hibiscus. The meaning of the plant *Hibiscus hispidissimus*, Hye-biskus: rose mallow, Hiss-pid-ISS-ih-mus: most bristly [10, 11, 12].

Anti oxidant and anti inflammatory effects of *Hibiscus hispidissimus* are reported. But no specific studies are done on anti arthritic activity of the plant. So an attempt was made to investigate anti arthritic potential of extract of aerial parts of *Hibiscus hispidissimus*. Aerial parts of the plant *Hibiscus hispidissimus* like leaf, stem, flower and flower blossoms contain flavonoids, terpenoids, steroids, tannins, glycosides and saponins. Anti-arthritic activity of many plants are attributed to terpenoids, steroids, alkaloids, flavonoids, tannins and phenols [10, 11]".

MATERIALS AND METHODS

Collection of Plant Material and Extraction

The aerial parts of *Hibiscus hispidissimus* Griffith was collected from the local areas in and around Kalmadka (Sullia), Karnataka. The plant material was collected during April - May. The plant was authenticated by Dr. K V Nagalaxamma, associate professor, HOD, department of botany, St. Aloysius College, Mangalore.

Preparation of Extract

The aerial parts of the plant *Hibiscus hispidissimus* was collected, washed and dried at room temperature. The dried plant material was grounded into coarse powder. This coarse powder was macerated using ethanol as a solvent.

Preliminary qualitative phytochemical investigation

The ethanolic extract of *Hibiscus hispidissimus* was subjected to qualitative phytochemical analysis for identifying the phyto constituents present in the extract. The tests were carried out by standard phyto chemical tests [13].

Assessment of *In-vitro* anti-arthritic activity

Inhibition of protein denaturation [14, 15]

In this model assessment of anti arthritic activity of ethanolic extract of the plant *Hibiscus hispidissimus*, was done for assessing the % inhibition of denaturation. The experiment was carried out by taking both bovine serum albumin and egg albumin. The reaction mixture 0.5ml consists of (0.45ml bovine serum albumin/egg albumin of 5% aqueous solution + 0.05ml of 100-500 μ g/ml of extract). The pH (6.3) was adjusted by using 1N HCl. Samples were incubated at 37 $^{\circ}$ C for 20min and then heated at 57 $^{\circ}$ C for 3min. After cooling the samples, 2.5ml of phosphate buffer saline (pH 6.3) was added to each tube. The

pH of the buffer saline was adjusted to 6.3 using 1N HCl. Absorbance was measured spectrophotometrically at 660nm.

Percentage inhibition of protein denaturation was calculated using the formula

$$\% \text{Inhibition} = \frac{(\text{Abs Control} - \text{Abs Sample})}{\text{Abs control}} \times 100$$

IC₅₀ value of extract and standard were also calculated.

Effect on Membrane Stabilization ^[14, 16]

In this model percentage membrane stabilizing activity was used to assess the anti inflammatory activity. The standard drug used was Diclofenac sodium.

The reaction mixtures 4.5ml consists of 2ml hypotonic saline (0.25% NaCl) + 1ml 0.15M phosphate buffer (pH 7.4) + 1ml test solution (100-500µg/ml) in normal saline + 0.5ml of 10% rat RBC in normal saline. The mixture was incubated at 56°C for 30 minutes. The tubes were cooled under running tap water for 20 minutes. The mixture was centrifuged for 3000rpm for 10min and the absorbance of the supernatant was measured at 560nm.

Percentage membrane stabilizing activity was calculated based on the formula ^[15]

$$\% \text{ Membrane stabilization} = \frac{(\text{Abs of Control} - \text{Abs of Sample})}{\text{Abs of control}} \times 100$$

Microscopical observation of RBC was done.

IC₅₀ value of extract as well as standard for inhibition of haemolysis was also calculated.

Preparation of 10% rat RBC: Blood was collected through the retro-orbital route into heparinised tube. To this blood, normal saline was added and then centrifuged for 3000rpm for 10min, repeated for 3 to 4 times. After this to 1ml of blood, 9ml of normal saline was added to prepare 10% of RBC.

Proteinase inhibitory action ^[14, 17]

Here proteinase inhibitory activity of the ethanolic extract of the plant *Hibiscus hispidissimus* was assessed along with standard drug Diclofenac sodium. The reaction mixtures 2.0ml consists of 250µl of trypsin + 1.0ml 25 mM tris-HCl buffer (pH 7.4) and 1.0ml aqueous solution of extract (100-500 µg/ml).

The mixture was incubated at 37 °C for 5 minutes. 1.0ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 minutes. 2.0ml of 70% (v/v) perchloric acid was added to terminate the reaction. The cloudy suspension was centrifuged. The absorbance of the supernatant at 280nm was measured.

The percentage inhibition was calculated using the formula ^[15]

$$\% \text{Inhibition} = \frac{(\text{Abs Control} - \text{Abs Sample})}{\text{Abs control}} \times 100$$

IC₅₀ value of extract as well as standard was calculated.

RESULTS

Evaluation of anti arthritic activity using *in-vitro* model

Effect on protein denaturation

Investigation of anti-arthritic activity of ethanolic extract of *Hibiscus hispidissimus* using this model was done in 5 different concentrations (100-500µg/ml). The detailed results are tabulated below

Table 1: Effect of ethanolic extract of *Hibiscus hispidissimus* on heat induced protein denaturation

Concentration (µg/ml)	% Inhibition			
	Diclofenac + BSA	Diclofenac + EGG	Extract + BSA	Extract + EGG
100	13.10±0.85	26.27±1.37	19.25±0.66	36.49±1.49
200	23.23±0.75	46.20±1.08	23.23±1.9	53.99±2.70
300	34.8±1.07	71.02±1.61	36.17±0.67	65.16±1.66
400	43.43±0.6	82.77±2.48	43.36±0.6	75.85±1.63
500	73.33±1.7	96.12±0.641	63.50±1.32	81.27±1.50

All the values are expressed as Mean± SEM (n=3),
All the values are significant when compared to control (p<0.05)

Table 2: IC₅₀ of standard and extract in protein denaturation

Group	IC ₅₀ value with BSA (µg/ml)	IC ₅₀ value with egg albumin (µg/ml)
Standard (Diclofenac sodium)	432	216
Extract	446	196

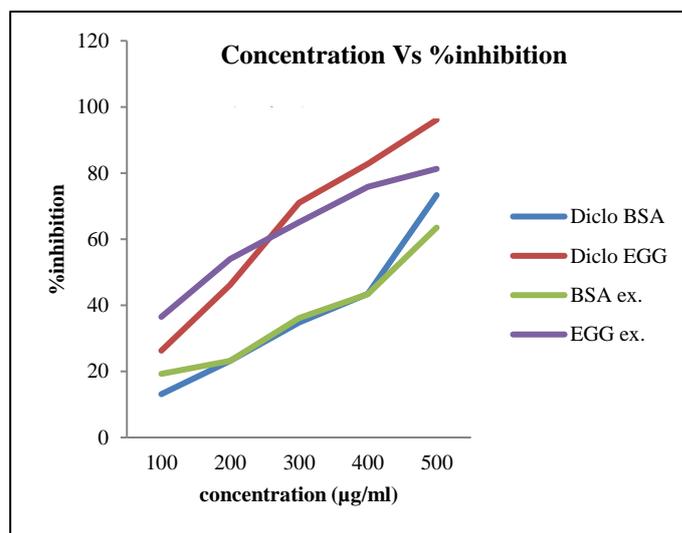


Figure 1: Inhibition of protein denaturation

Effect on Membrane Stabilization

The effect of ethanolic extract of plant and standard in 100, 200, 300, 400, 500µg/ml concentration was found to be significant (p<0.05) when compared to control. Detailed results are tabulated below

Table 3: Membrane stabilizing effect of ethanolic extract of *Hibiscus hispidissimus*

Treatment	Concentration (µg/ml)	% of membrane stabilisation
Standard	100	27.88±0.93
	200	37.45±1.30
	300	59.61±0.516
	400	74.057±0.990
	500	84.04±0.511
Extract	100	11.29±0.628
	200	23.36±1.26
	300	34.89±1.016
	400	50.65±1.182
	500	72.64±0.765

All the values are expressed as Mean± SEM (n=3).

All the values are significant when compared to control p<0.05

Table 4: IC₅₀ of standard and extract for membrane stabilizing effect.

Group	IC ₅₀ value (µg/ml)
Standard (Diclofenac sodium)	252
Extract	400

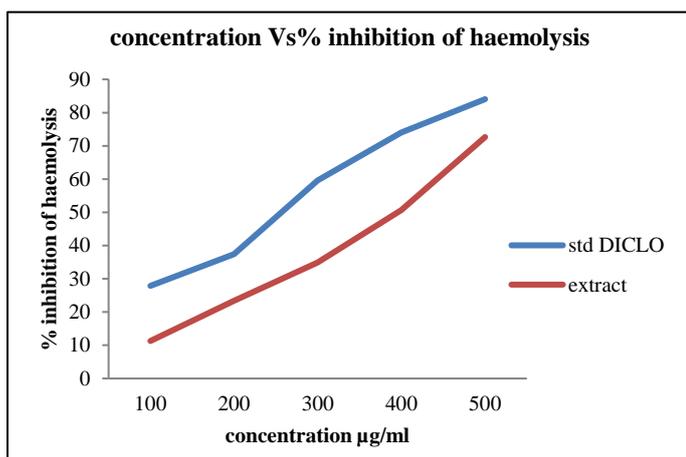
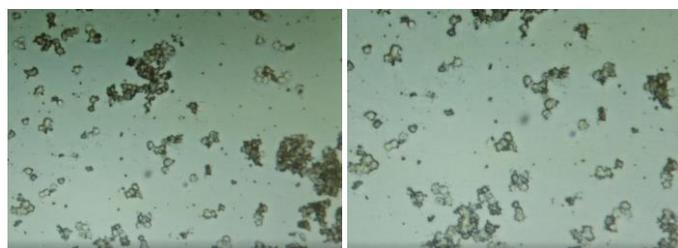
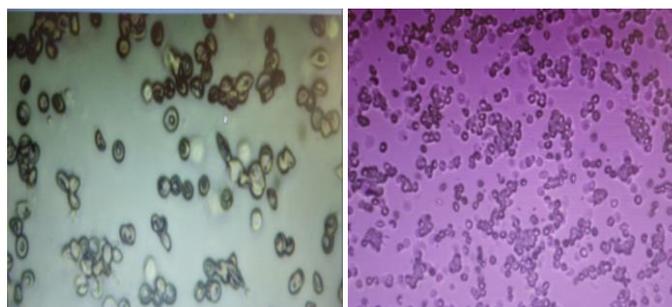


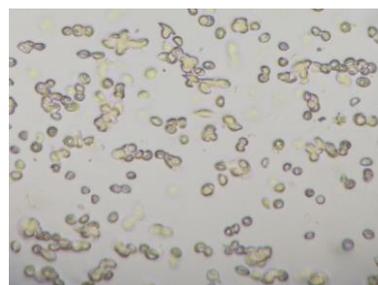
Figure 2: Inhibition of haemolysis



A: Ruptured RBC in control group.



B: Stabilised RBC membrane in standard diclofenac (500µg/ml) group.



C: RBC in extract (500µg/ml) treated group (reduction in membrane lysis compared to positive control)

Figure 3: Microscopically observed RBC.

Proteinase inhibitory action

Results are tabulated below.

Table 5: Effect of ethanolic extract of *Hibiscus hispidissimus* on Proteinase

Treatment	Concentration(µg/ml)	% inhibition of Proteinase activity
Standard(Indomethacin)	100	18.068±0.48
	200	32.45±3.16
	300	45.61±3.16
	400	58.76±3.15
	500	78.15±3.89
Extract <i>H. hispidissimus</i>	100	16.57±2.33
	200	28.419±1.64
	300	41.840±3.76
	400	51.049±1.85
	500	71.22±1.673

All the values are expressed as Mean± SEM (n=3), All the values are significant when compared to control p<0.05 except extract of concentration 100µg/ml

Table 6: IC₅₀ of standard and extract in Proteinase inhibitory action

Group	IC ₅₀ value (µg/ml)
Standard (Indomethacin)	335
Extract	383

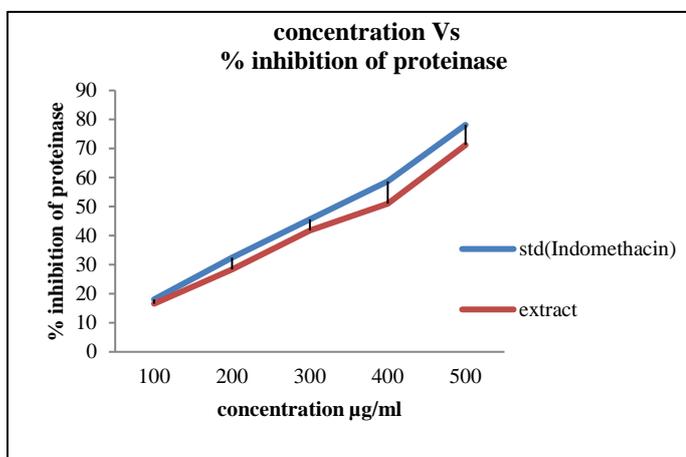


Figure 4: Inhibition of proteinase enzyme

DISCUSSION

In the present study investigation of anti-arthritic activity of ethanolic extract of *Hibiscus hispidissimus* was determined by the *in-vitro* models viz. inhibition of protein denaturation, membrane stabilization and Proteinase (trypsin) inhibition.

Denaturation of the protein involves the disruption of secondary, tertiary and quaternary structure of the molecules and finally leads to cell death, it occurs due to stress like a high level of salt, high temperature and high level of acidity. The mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding. Denaturation of proteins is well documented as contributing to inflammatory conditions like RA, diabetes and cancer. Most of the investigators have reported that denaturation of protein is one of the causes of RA due to the production of auto-antigens in certain rheumatic diseases. In the present study there was significant ($p < 0.05$) inhibition of protein denaturation in both standard and extract treated group and % inhibition of protein denaturation produced by extract at concentrations 100 and 300µg/ml showed better activity than standard (diclofenac sodium). From the results of the present study it can be stated that *H. hispidissimus* may control the production of auto-antigens by preventing *in-vivo* denaturation of proteins in rheumatic diseases [14].

Membrane stabilization: Stabilizing effect on heat and saline induced erythrocyte lysis is very good index of anti-inflammatory activity and there by anti-arthritic activity. The membrane of RBC is similar to that of lysosomal membrane. In inflammatory condition stabilising the lysosomal membrane helps to prevent the release of lysosomal constituents [14, 16] of activated neutrophil such as proteases and bactericidal enzymes which cause further tissue inflammation and damage upon extra cellular release. Though the exact mechanism of the membrane stabilization by the extract is not known yet, it showed significant membrane protection when compared to control group.

Proteinase inhibitory action: Proteinases have been implicated in arthritic reactions. Rich sources of proteinase are neutrophil lysosomal

granules. The proteases act enzymatically to degrade the collagen and proteoglycan matrix of bone and cartilage. It was previously reported that leucocyte proteinases play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors. As per the earlier reports, in certain forms of rheumatoid arthritis trypsin is activated hence in present experiment trypsin was used [17]. Based on the result ethanolic extract of *H. hispidissimus* showed significant inhibition of proteinase activity.

Preliminary phytochemical analysis revealed that the plant extract contains active constituents like saponins, alkaloids, tannins, flavonoids, glycosides, reducing sugars, tri terpenoids and steroids. These may exert anti-inflammatory activity by inhibiting the 5-lipoxygenase pathway, along with the COX-2 pathway. Tri terpenoids are known to inhibit the histamine release and exerts anti-inflammatory activity. Histamine is very important in producing and maintaining inflammation [18, 19]. Saponins and alkaloids are known to inhibit articular swelling, decrease arthritic index, and regulate down the content of IL-1B and TNF-α in the inflammatory tissues of arthritic rats [19, 20]. Flavonoids like hibiscatin and gossyptrin shows antioxidant effect against free radicals. Therefore, it can be proposed that the anti-arthritic activity of ethanolic extract of the plant could be due to combined effect of flavonoids, saponins, steroids, tannins, tri terpenoids and alkaloids, which are present in the aerial parts of the plant.

CONCLUSION

The following conclusion is drawn from the results obtained from the investigation of anti-arthritic activity of *Hibiscus hispidissimus* Griffith.

Denaturation of the protein is one of the main causes of RA due to the production of auto antigens. From the results it may be concluded that the plant extract may control the production of auto antigens by preventing protein denaturation. Preventing the lysosomal membrane lysis is one of the contributions to anti-inflammatory activity. In the study, plant extract showed protective effect against heat and hypotonicity induced RBC membrane lysis. Hence it can be concluded that the plant extract may also stabilize the lysosomal membrane which is similar to the RBC membrane and there by induce anti-inflammatory effect. Increased levels of trypsin have been implicated in certain arthritic conditions. Significant inhibition of proteinase activity was shown by plant extract.

The results of the present study suggest that *Hibiscus hispidissimus* could to be a potential anti arthritic drug. For confirmation of the activity we are continuing the investigation using *in-vivo* models. Further studies using isolated active ingredients and molecular level pharmacological investigations can also be carried out to confirm its efficacy and mechanism of action.

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HOW TO CITE THIS ARTICLE

Shilpa K, Chacko N, Shetty P. Investigation of anti-arthritis activity (*in-vitro* models) of *Hibiscus hispidissimus* Griffith. J Phytopharmacol 2018; 7(1):60-65.