

In-vitro Anti-arthritic Potential of *Syzygium caryophyllatum* (L) Alston Leaf Extract

Sandhya Savithri A^{*1}, Nimmy Chacko², Prerana Shetty³, Shilpa K⁴

^{1,2,4}Department of Pharmacology, ³Department of Biochemistry

Nitte Gulabi Shetty Memorial Institute of Pharmaceutical Sciences, Nitte University, Mangalore, Karnataka India

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*Corresponding author

Sandhya Savithri A

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Abstract: The present study was aimed to evaluate *in vitro* anti-arthritic activity of methanolic leaf extract of *Syzygium caryophyllatum* (L.)Alston. Materials and Methods: The anti-arthritic activity of the leaf extract was evaluated by the *in-vitro* studies viz. effect on membrane stabilization, protein denaturation and proteinase inhibitory activity.

Keywords: Rheumatoid arthritis, protein denaturation.

INTRODUCTION

Rheumatoid arthritis is a chronic, progressive, systemic inflammatory autoimmune disorder characterized by synovitis, autoantibody production and destruction of joint, resulting in deformity and disability. The cause of rheumatoid arthritis remains unclear. Rheumatoid arthritis involves a complex interplay among genotype, environmental trigger and chance [1]. In joint destruction there are multiple chemical mediators produced from rheumatoid synovium. These include arachidonic acids metabolites such as prostaglandins, leukotrienes, vasoactive amines, kinins, endothelins, complement fragments, reactive oxygens, neutral proteinases and cytokines. Among cytokines, interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor (TNF) and platelet-derived growth factor (PDGF) are most common [2-5]. The presently available synthetic drugs in the market are not only economical exploitation but also associated with adverse effects. Hence complementary and alternative medicines must be sought for. Traditionally *Syzygium caryophyllatum* is used to treat diabetes mellitus, diarrhoea, dysentery, leucorrhoea, menorrhagia, piles, fever, skin diseases, and general debility [6]. It is also used as antiemetic, anthelmintic, laxative, antioxidant and anti-inflammatory agent.

Review of literature revealed that *Syzygium caryophyllatum* possess anti-hyperglycaemic [7], antibacterial [8,9] and anticancer[10] activities. Free radicals are released during inflammation; they act as inflammatory mediators to sustain inflammatory process and their neutralisation by antioxidants and radical scavengers can attenuate inflammation. It was reported that methanolic leaf extract of *Syzygium caryophyllatum* showed potent antioxidant activity[9]. Based on the literature survey it was found that anti arthritic activity of many plant is due to the presence of phytoconstituents like terpenoids, steroids, alkaloids, flavanoids, tannins and phenols. The presence of alkaloids, flavanoids, tannins, phenols, and triterpenoids from *Syzygium caryophyllatum* leaves extract was reported but no substantial anti arthritic work have been reported on leaves of *Syzygium caryophyllatum*. Hence, the present study is attempted to screen the methanolic extract of leaves of *Syzygissum caryophyllatum* for its anti-arthritic potential using *in-vitro* models.

MATERIALS AND METHODS

Plant materials

The plant material was collected from the surroundings of Alangaru village, Dakshina Kannada District, Karnataka, India during the month of June 2016 and was authenticated by Dr. K. V. Nagalakshamma, HOD of Botany, St. Aloysius College (Autonomous), Mangalore, Dakshina Kannada, Karnataka.

Preparation of extract

The fresh leaves of *Syzygium caryophyllatum* was washed under running tap water to remove the adhered dust and other material and then shade dried for 10 days. The shade dried leaves were powdered using electric grinder to get coarse powder of leaves. The grounded leaves were subjected to maceration. Methanol was used as macerating solvent. The coarse powdered leaves were soaked in methanol and kept aside for 7 days with occasional stirring. After 7 days,

methanolic layer was filtered using a muslin cloth. The filtrate was kept aside for a day and the fine particles get settled down. The clear upper methanolic layer was decanted. The solvent from the extract was distilled off and then concentrated on a temperature controlled water bath to obtain dry powder of extract and stored in desiccators until further use.

Preliminary qualitative phytochemical analysis

Various chemical tests were performed using dried methanolic extract to detect the presence of phytoconstituents like alkaloids, reducing sugars, flavanoids, tannins, saponins, steroids, triterpenoids, phenols, glycosides and proteins.

In vitro anti-arthritic activity

Inhibition of protein denaturation [11,12]

The anti-arthritic activity of methanolic leaf extract of *Syzygium caryophyllatum* was assessed by the ability of leaf extract to inhibit the heat induced denaturation of egg albumin and bovine serum albumin.

The activity was evaluated by using bovine serum albumin and egg albumin. The different concentrations of leaf extracts ranging from 100-500µg/ml were prepared. Reaction mixture for each concentration was prepared which consists of 5% aqueous solution of 0.45ml bovine serum / egg albumin (got from fresh hen's egg) and 0.05ml of leaf extract. These reaction mixtures were incubated at 37 °C for 20 minutes and then heated at 57 °C for 3 minutes to induce denaturation of protein. The samples were cooled and then added 2.5ml of phosphate buffer (pH 6.3). The absorbance was measured spectrophotometrically at 660nm (SHIMADZU, UV 3600) by taking buffer as blank. For control, 0.05ml 0.6% sodium CMC solution was used instead of extract. The standard drug diclofenac sodium at concentrations 100, 200, 300, 400 and 500µg/ml were treated similarly and the absorbance was determined.

Evaluation

The turbidity obtained is measured spectrophotometrically at 660nm. The percentage inhibition of protein denaturation was calculated by using following equation:

$$\% \text{ inhibition} = \frac{V_c - V_t}{V_t} \times 100$$

Where V_t = absorbance of test samples
 V_c = absorbance of control

Effect on Membrane Stabilization[11,13]

The *in vitro* anti-arthritic activity of plant extract was evaluated based on the ability of the leaf extract to inhibit the hypotonicity induced lysis of RBC membrane.

Preparation of red blood cells suspension

The blood was collected from healthy *Wistar* rats into an EDTA tubes and transferred to the centrifuge tubes and added 0.9% saline solution allowing 1cm gap at the top of the tubes. Then tubes were centrifuged at 3000rpm for 10minutes and were washed three times by using equal volume 0.9% saline solution. The volume of blood was measured and re-constituted as 10% v/v suspension with normal saline.

The different concentrations (100, 200, 300, 400 and 500µg/ml) of *Syzygium caryophyllatum* extract were mixed with 2 ml hypotonic saline (0.25% NaCl), 1 ml 0.15 M phosphate buffer (pH 7.4). 0.5ml 10% RBCs suspension was added to above mixture and incubated at 56°C for 30minutes. After incubation, the tubes were cooled under running tap water and subjected to centrifugation at 2500rpm for 5minutes. The supernatant liquid was decanted and absorbance was measured spectrophotometrically at 560nm (SHIMADZU, UV 3600) by taking buffer as blank. For control, 1ml isotonic saline solution was used instead of extract.

The standard drug diclofenac sodium at concentrations 100, 200, 300, 400 and 500µg/ml were treated similarly and the absorbance was determined.

Evaluation

The haemoglobin content in the supernatant solution was estimated spectrophotometrically at 560nm. The percentage RBC membrane stabilisation was calculated by using formula:

$$\% \text{ inhibition} = \frac{V_c - V_t}{V_t} \times 100$$

Where V_t = absorbance of test samples

V_c = absorbance of control

Microscopical examination of RBCs was also carried out.

Proteinase inhibitory action [11,14]

The *in vitro* anti-arthritic activity of *Syzygium caryophyllatum* extract was assessed on the basis of anti-proteinase efficacy according to the established procedure.

The different concentrations (100, 200, 300, 400 and 500µg/ml) of *Syzygium caryophyllatum* extract were mixed with 0.06mg of trypsin, 1ml 25mM tris hydrochloric buffer (pH 7.4). The mixture was incubated at 37°C for 5 minutes and added 1ml (0.8% w/v) casein and again incubated for an additional 20 minutes. The reaction was arrested by adding 1ml of 70% v/v perchloric acid. The cloudy suspension was centrifuged at 3000rpm for 10 minutes, supernatant liquid was decanted and absorbance was measured spectrophotometrically at 280nm (SHIMADZU, UV 3600) by taking buffer as blank. For control, 0.05ml 0.6% sodium CMC solution was used instead of extract. The standard drug diclofenac sodium at concentrations

100, 200, 300, 400 and 500µg/ml were treated similarly and the absorbance was determined

Evaluation

The absorbance of supernatant solution was measured spectrophotometrically at 280nm and percentage inhibition was calculated using the formula

$$\% \text{ inhibition} = \frac{V_c - V_t}{V_t} \times 100$$

Where Vt = absorbance of test samples
Vc= absorbance of control

Statistical Analysis

All the data were represented as, Mean ± SEM. The data obtained were subjected to one-way Analysis of Variance (ANOVA) test, followed by Post hoc Scheffe’s test using SPSS computer software version 10. P value less than 0.05 was considered as statistically significant.

RESULTS

Preliminary qualitative phytochemical analysis

Preliminary phytochemical analysis revealed

the presence of alkaloids, reducing sugars, flavanoids, tannins, saponins, steroids, triterpenoids, phenols and glycosides.

Effect on protein denaturation

The percentage inhibition by the extract and standard drug at different concentrations are given in table no. 1 and fig. 1. The leaf extract at concentrations 100-500µg/ml significantly (p<0.05) inhibited the heat induced denaturation of bovine serum albumin (BSA) and egg albumin (EA) when compared to control. % inhibition of control was considered as zero, which showed highest absorbance value indicating complete denaturation. The activity was found to be in concentration dependent manner and hence maximum % inhibition of protein denaturation was observed at 500µg/ml concentration. The standard drug diclofenac sodium showed higher activity at all concentrations. The IC50 values of the diclofenac and leaf extract were found to be 195.19µg/ml and 303.33µg/ml respectively in heat induced denaturation of egg albumin. The IC50 values of the diclofenac and leaf extract was found to be 437.54µg/ml and 480.58µg/ml respectively in heat induced denaturation of bovine serum albumin.

Table-1: Percentage inhibition of protein denaturation by standard drug (diclofenac sodium) and leaf extract in BSA and EA

Concentration (µg/ml)	% inhibition of protein denaturation			
	Diclofenac sodium + BSA	Diclofenac sodium + EA	Leaf extract + BSA	Leaf extract + EA
100	13.32±0.2140	25.28±0.1804	9.95±0.0500	16.42±0.1113
200	20.14±0.0991	51.23±0.1072	17.30±0.2490	30.41±0.2777
300	34.21±0.1001	71.09±0.0548	25.03±0.0882	49.45±0.1040
400	45.71±0.1021	84.23±0.1053	33.42±0.4132	62.15±0.2550
500	70.67±0.1211	95.18±0.0986	52.02±0.2027	77.25±0.2300

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

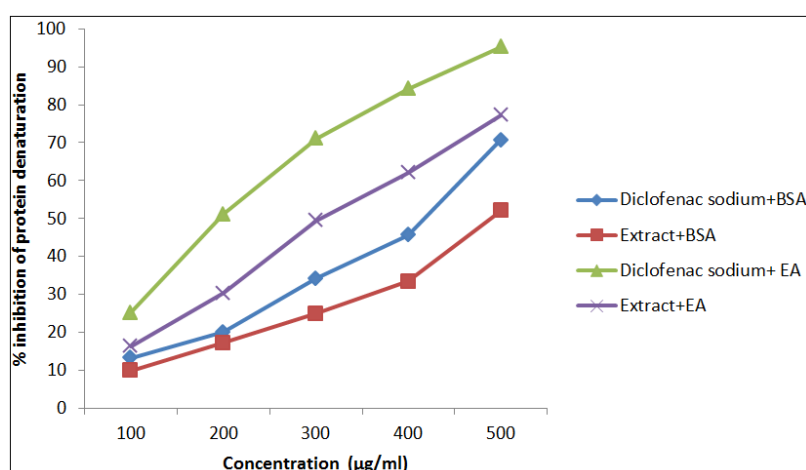


Fig-1: Effect of methanolic extract of *Syzygium caryophyllatum* leaves on Inhibition of Protein Denaturation in BSA and EA

Effect on membrane stabilisation

The protective effect of methanolic leaf extract of *Syzygium caryophyllatum* on hypotonicity induced

lysis of RBC membrane was studied by using rat RBCs. The percentage protection against haemolysis by the extract and standard drug at different concentrations are

given in table no. 2 and fig. 2. The leaf extract at concentration 100-500µg/ml showed significant ($p<0.05$) protection of rat RBCs membrane rupture when compared to control. % membrane stabilization by control was considered as zero, which showed highest absorbance value indicating complete haemolysis. Percentage protection of RBCs membrane increased as the concentration of extract increased. Hence concentration 500µg/ml of extract exhibited maximum % stabilisation (69.97 ± 0.0250). The standard

drug diclofenac sodium exhibited higher membrane stabilising activity at all concentration and 80.78 ± 0.0369 percentage protection was observed at 500µg/ml concentration. At concentration of 280.58µg/ml diclofenac showed 50% membrane stabilisation, whereas at concentration of 346.18µg/ml leaf extract exhibited 50% membrane stabilisation. Further the results were supported by microscopical observation of RBC cells.

Table-2: Percentage RBC Membrane Stabilisation by standard drug (diclofenac sodium) and leaf extract on hypotonicity induced haemolysis

Testing Material	Concentration µg/ml	% RBC Membrane Stabilisation	IC ₅₀
Diclofenac Sodium	100	29.56 ± 0.0305^b	280.58 µg/ml
	200	39.66 ± 0.0881^b	
	300	53.46 ± 0.0689^b	
	400	68.97 ± 0.0431^b	
	500	80.78 ± 0.0369^b	
<i>Syzygiumcaryophyllatum</i>	100	18.80 ± 0.1527^b	346.18 µg/ml
	200	29.27 ± 0.2141^b	
	300	41.25 ± 0.1443^b	
	400	57.64 ± 0.3218^{bc}	
	500	69.97 ± 0.0250^{bc}	

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

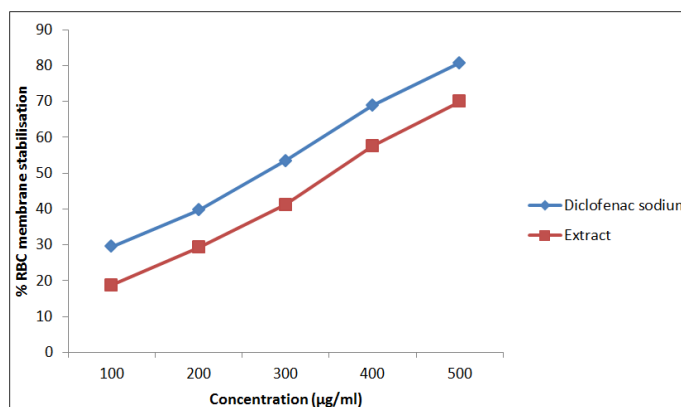


Fig-2: Effect of methanolic extract of *Syzygium caryophyllatum* leaves on RBC Membrane Stabilisation

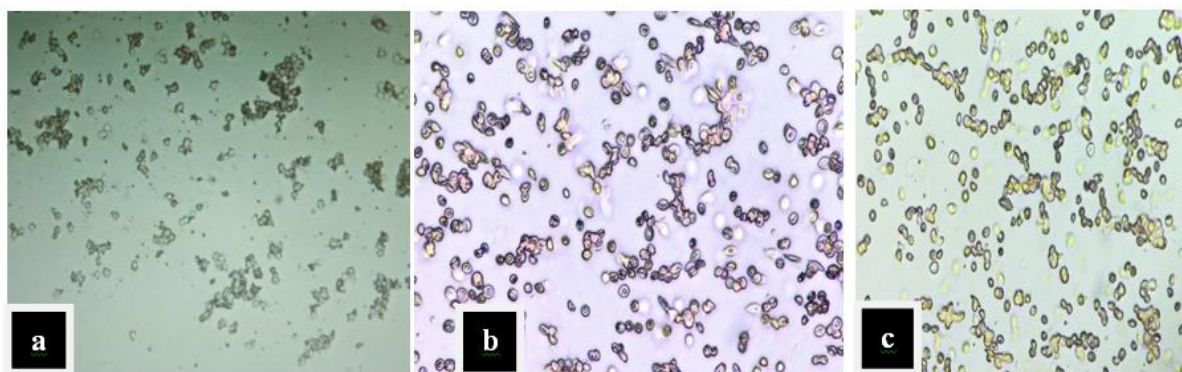


Fig-3: Ruptured RBC in control group (a), Stabilised RBC membrane in standard diclofenac (500µg/ml) group (b) and RBC in extract (500µg/ml) treated group (c)

Proteinase inhibitory action

The percentage inhibition of proteinase enzyme by the extract and standard drug at different concentrations are given in table no. 3 and fig. 4. At concentrations 100-500µg/ml, the leaf extract significantly ($p<0.05$) inhibited proteinase enzyme compared to control. % inhibition of proteinase of control was considered as zero, which showed highest absorbance value. Percentage inhibition was increased

as the concentration of extract increased. Hence concentration 500µg/ml of extract exhibited maximum % inhibition (61.93 ± 0.1474). The standard drug diclofenac sodium exhibited higher inhibition activity at all concentration and 72.23 ± 0.1742 percentage inhibition was observed at 500µg/ml concentration. The IC₅₀ values of the diclofenac and leaf extract was found to be $303.95\mu\text{g/ml}$ and $407.74\mu\text{g/ml}$ respectively.

Table-3: Percentage inhibition of proteinase activity by standard drug (diclofenac sodium) and leaf extract

Testing Material	Concentration µg/ml	% Inhibition	IC ₅₀
Diclofenac Sodium	100	21.41±0.1975	303.95 µg/ml
	200	32.05±0.0655	
	300	49.35±0.2500	
	400	62.01±0.0696	
	500	72.23±0.1742	
<i>Syzygiumcaryophyllatum</i>	100	9.32±0.0696	407.74 µg/ml
	200	21.08±0.1133	
	300	34.53±0.2905	
	400	49.05±0.0851	
	500	61.93±0.1474	

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

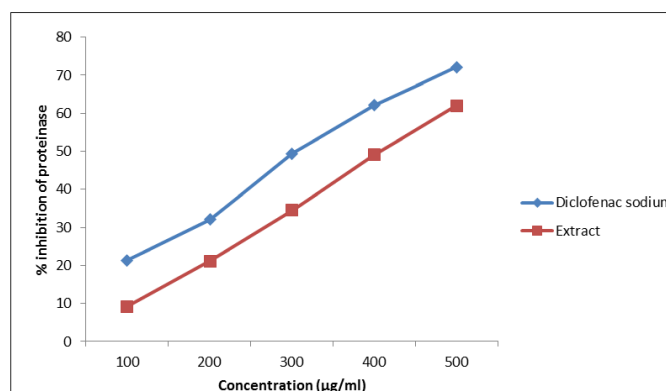


Fig-4: Inhibition of Proteinase by methanolic extract of *Syzygium caryophyllatum* leaves

DISCUSSIONS

Protein denaturation results in loss of secondary, tertiary or quaternary structure of proteins. It occurs due to stress like a high level of salt, high temperature and high level of acidity. Alteration of hydrogen, hydrophobic, disulphide and electrostatic bonds in proteins are the possible mechanisms by which denaturation of protein can occur. Denaturation of protein is a well-documented cause of inflammation in conditions like rheumatoid arthritis and other inflammatory diseases. In some rheumatic diseases, *in vivo* denaturation of proteins resulted in production of auto antigens[15,16]. The leaf extract inhibited thermal induced denaturation of protein i.e. Egg albumin and BSA in a concentration dependent manner compared to control. Based on the results it can be inferred that methanolic leaf extract offer protection against heat

induced protein denaturation and thereby prevent auto antigen production.

Any agent exhibiting protective effect against heat and hypotonic saline induced rupture of RBC membrane is considered to have anti-inflammatory activity. Since the RBC membrane is structurally similar to lysosomal membrane. Lyses of lysosomal membrane results in release of lysosomal constituents which results in chronic inflammation [17]. The leaf extract stabilised the hypotonic saline induced breakdown of RBC membrane in a concentration dependent manner when compared to control. RBC in hypotonic saline solution will bulge and thereby breakdown of membrane results in release of haemoglobin. The decrease in absorbance of haemoglobin indicates stabilisation of RBC membrane.

This implies that the extract may as well stabilise the lysosomal membrane.

Proteinases have been involved in arthritic conditions. Serine containing proteinases are abundantly present in neutrophils and are localised at granules of lysosomes. It was previously reported that during inflammatory reactions, leukocyte proteinases play a major role in development of damage to tissue⁵. The proteases act enzymatically to degrade the collagen and proteoglycan matrix of bone and cartilage [18,19]. As per the earlier reports in certain forms of rheumatoid arthritis trypsin is activated hence in present experiment trypsin was taken[20]. The methanolic leaf extract exhibited antiproteinase activity in a concentration dependent manner.

Rheumatoid arthritis involves inflammation of synovial membrane of joint. The inflamed joint contains variety of pro inflammatory cytokines, interleukins, TNF- α and macrophages. Findings have revealed that macrophages have the ability to generate reactive oxygen species. Free radical cause oxidative damage which worsens inflammation. Anti-oxidants and radical scavenger helps in attenuation of inflammation. It has been reported that methanolic leaf extract of *Syzygium caryophyllatum* possess potent antioxidant activity. Alkaloids, flavanoids, steroids, phenols and tannins play major role in inhibiting the enzymes involved in production of inflammatory mediators[21] and steroids have the ability to reduce activity of immune system[22]. Saponins and alkaloids are known to down regulate the elevated levels of IL-1B and TNF- α in inflammatory tissue of arthritic rats[23,24]. So the possible anti-arthritic activity of leaf extract could be attributed to inhibition of pro-inflammatory mediators. The presence of protective phytoconstituents and potent anti-oxidant activity might be the contributing factors.

CONCLUSIONS

From the results of study it can be concluded methanolic leaf extract of *Syzygium caryophyllatum* possessed anti-arthritic potential. The presence of polyphenolic compounds such as alkaloids, flavonoids, tannins, steroids, and phenols are contributing for the anti-arthritic activity. And also the extract fraction serves as potent anti-oxidant and free radical scavenger, also inhibited heat induced protein denaturation, stabilized the membrane in cells may be inhibiting the release of various inflammatory mediators and inhibited proteinase enzyme activity, there by inhibiting tissue damage.

However, further studies using isolated phytoconstituents and pharmacological studies at molecular level are essential to confirm the mechanism of action of the plant extract as an anti-arthritis drug.

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