

Preliminary phytochemical screening and *in vitro* evaluation of anti-inflammatory, antiarthritic, and thrombolytic activities of ethanolic leaf extract of *Bauhinia purpurea*

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Abstract

Aim: The aim of the present study is to carry out the preliminary phytochemical screening followed by an investigation of the *in vitro* anti-inflammatory, antiarthritic, and thrombolytic activity of ethanolic leaf extract of *Bauhinia purpurea*. **Methods:** Phytochemical screening was done to find the presence of various secondary metabolites of the plant. The anti-inflammatory activity was evaluated by human red blood cell (HRBC) method. Using hypotonic solution-induced human erythrocyte lysis model, membrane-stabilizing activity was examined by considering aspirin as standard. Thrombolytic activity was evaluated using the *in vitro* clot lysis model. Egg albumin and bovine serum albumin (BSA) were used to evaluate the antiarthritic potential. **Results and Discussion:** Phytochemical tests of ethanolic leaf extract of *B. purpurea* indicated the presence of flavonoids, alkaloids, steroids, terpenoids, lignin's, carbohydrates, proteins, tannins, saponins, and glycosides. In case of anti-inflammatory activity, the maximum percentage stabilization of HRBC membrane was observed as 59.2% at 500 µg/ml concentration. The maximum percentage inhibition by BSA method and egg albumin method was observed as 82.2% and 94%, respectively, at 500 µg/ml concentration for antiarthritic activity. During assay for thrombolytic activity, it revealed that $91.02 \pm 2.6\%$ lysis of clot, while standard streptokinase and water used as positive and negative controls, demonstrated $72.835 \pm 5.702\%$ and $2.725 \pm 0.983\%$ lysis of clot, respectively. **Conclusion:** The present outcomes highlight the role of ethanolic leaf extract of *B. purpurea* for its anti-inflammatory, antiarthritic, and thrombolytic activities. It reveals that the phytochemical constituents are responsible for these activities.

Key words: Antiarthritic, anti-inflammatory, *Bauhinia purpurea*, bovine serum albumin, egg albumin, human red blood cell method, protein denaturation, thrombolytic

INTRODUCTION

Arthritis is an autoimmune disorder characterized by pain, swelling, and stiffness. Its prevalence depends on age. It occurs more frequently in women than in men. It is an inflammation of synovial joint due to immune-mediated response. They do not suppress T-cell- and B-cell-mediated response because all anti-inflammatory drugs are not antiarthritic. Rheumatoid arthritis (RA) is an autoimmune disorder specified by the destruction of cartilage and bone, inflammation, subsequent destruction such as deformity of joints or synovial proliferation.^[1] RA is the most common inflammatory joint disease in humans and has long been classified among

the autoimmune diseases in which skeletal complications start with focal erosion of cartilage followed by marginal and subchondral bone loss. Extended joint destruction with ankylosis and generalized bone loss is characteristic for late complications.^[2] These long-term skeletal complications have serious consequences as they can lead not only to painful joint deformities but also to progressive functional disability

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and increased mortality rates.^[3] The *in vivo* denaturation of proteins may cause the production of autoantigens in certain arthritic diseases.^[4] The alteration in electrostatic, hydrogen, hydrophobic, and disulfide bonding is included in the mechanism of denaturation.^[5] The antiarthritic activity occurs by inhibiting denaturation of protein and membrane lysis and controlling the production of autoantigen in rheumatic disease. Hence, in *in vitro* antiarthritic activity, the inhibition of protein denaturation and membrane lysis was considered.^[6] Formation of blood clots is one of the vital reasons of blood circulation problem. Depriving tissues of normal blood flow and oxygen by lodging thrombi or emboli blood vessel and block the flow of blood in that location. As a result, it causes damage, destruction (infarction), or even death of the tissues (necrosis) in that area.^[7] A blood clot (thrombus) is formed from fibrinogen by thrombin and is lysed by plasmin which is activated from plasminogen by tissue plasminogen activator (tPA). Fibrinolytic drugs have been used to dissolve thrombi in acutely occluded coronary arteries, thereby restoring blood supply to ischemic myocardium to limit necrosis and to improve prognosis.^[8] Streptokinase (SK) is an antigenic thrombolytic agent used for the treatment of acute myocardial infarction. The advantages of being much less expensive and it reduces mortality as effectively as the nonantigenic alteplase in most infarct patients. Urokinase or SK-type activators are less preferred and less safer than tissue-type plasminogen activator (tPA). The large doses to be maximally effective are significant shortcomings for all available thrombolytic agents in need for limited fibrin specificity and a significant associated bleeding tendency. The attempts are underway to develop improved recombinant variants of these drugs because of the shortcomings of the available thrombolytic drugs.^[9] Only a small fraction of plants with medicinal activity has been assayed and nearly 50% of drugs used in medicine. Therefore, the higher plants having ethnobotanical information which are associated with phytochemical investigations are devoted to the current research. The different types of biological activity like thrombolytic potentials are isolated by phytochemicals.^[10] Herbal preparations are used potential source of medicine since ancient times to maintain health and regain healthy state of mind. Thrombolytic activity of some herbs has been studied and some significant observations have been reported.^[11]

The ornamental plant *Bauhinia purpurea* Linn. (*Caesalpinaceae*) is found subtropical, India, North and South America, Nepal, Australia, Africa, and the United Kingdom. The use of plant species and has higher plant species around 17,000 species, of which 7500 are medicinal plants are found in India.^[12] The plant is commonly known as Mandarai in Tamil and Khairwal in Hindi. Flowers are broadly known and used for their beauty as well as the color they radiate. Medicinal plants find its application in the treatment of diseases since the dawn of world in the form of traditional medicine.^[13]

MATERIALS AND METHODS

Collection of Plant Material

B. purpurea leaves were collected from Peddapuram area of East Godavari district of Andhra Pradesh. The plant authentication was done by Dr. T. Raghuram Taxonomist, Maharani College, Peddapuram.

Extract Preparation

Leaves of *B. purpurea* were undergone shade drying at room temperature for 4–5 days. The dried leaves were then powdered in a mixture. The powder was taken and weighed. From the obtained fine powder, 100 g powder was taken and it is macerated in 200 ml of ethanol for 3 days. The hot percolation process is carried out for about 3 h. later on the filtration was done and distillation is performed to get concentrated product.

Phytochemical Analysis

The powdered leaf was evaluated for qualitative determination of major phytoconstituents, i.e., alkaloids, carbohydrates, glycosides, phenolic compounds, tannins, saponins, steroids, and flavonoids.^[14]

Chemicals and Instruments

1. Drugs used in the present study include sodium hydroxide, potassium chloride, dextrose, dimethylformamide, acetylsalicylic acid, bovine serum albumin (BSA), ethanol, potassium dihydrogen phosphate, sodium chloride, disodium hydrogen phosphate, hydrochloric acid, and sodium citrate.
2. Instruments used were digital photoactometer, P^H m, and ultraviolet (UV) spectrophotometer.

Evaluation of Antiarthritic Activity

BSA method

Test solution (0.5 ml) consists of 0.45 ml of BSA (5% w/v aqueous solution) and 0.05 ml of test samples of different concentrations (50 µg/ml, 100 µg/ml, 300 µg/ml, and 500 µg/ml). Control solution of test (0.5 ml) mainly consists of BSA of 0.45 ml (5% w/v aqueous solution) and 0.05 ml of distilled water. Product control solution (0.5 ml) consists of 0.45 ml of distilled water and 0.05 ml of test samples of different concentrations (50 µg/ml, 100 µg/ml, 300 µg/ml, and 500 µg/ml). Standard solution consists of 0.45 ml of serum albumin (5% w/v aqueous solution) and 0.05 ml of diclofenac sodium of concentrations 100 µg/ml and 200 µg/ml. The above solutions were adjusted to a pH of 6.3 using 1 N HCl. The samples were incubated for 20 min at 37°C and the temperature was raised to 57°C for 3 min.

After cooling, 2.5 ml of phosphate buffer was added to the above solutions.^[14,15] The absorbance was measured using UV-visible spectrophotometer at 255 nm. The percentage inhibition of protein denaturation was calculated as,

% Inhibition of protein denaturation = $100 - \left[\frac{\text{O.D of test solution} - \text{O.D of product control}}{\text{O.D of test control}} \times 100 \right]$

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

Egg albumin denaturation

About 0.2 ml of eggs albumin (from hen's egg) was comprised 5 ml of reaction mixture, 2 ml of varying concentrations of extract, and 2.8 ml of phosphate-buffered saline (PBS, pH 6.4). The control was served as similar volume of double distilled water. Then, the mixture was incubated at 37°C in biochemical oxygen demand incubator for about 15 min and then heated at 70°C for 5 min. After cooling, their absorbance was measured at 660 nm using pure blank. Diclofenac sodium (standard drug) was used as reference drug and treated as such for the determination of absorbance.^[16] The percentage inhibition of protein denaturation was calculated as below:

Percent inhibition = $\frac{\text{Abs control} - \text{Abs treated}}{\text{Abs treated}} \times 100$

Evaluation of *in vitro* Anti-inflammatory Activity

Human red blood cell (HRBC) membrane stabilization method

The extrapolation to the stabilization of lysosomal membrane is leading to the effect of drugs on the stabilization of erythrocyte resembling erythrocyte membrane. Therefore, the reasons for inflammation are release and/or action of mediators such as histamine, serotonin, prostaglandins, and leukotrienes, which interfere with the membrane stabilizes.^[17,18] The prevention of HRBC membrane lysis which is induced by hypotonicity is taken as a measure of anti-inflammatory activity.

Preparation of HRBC Suspension

Fresh whole human blood was collected and mixed with equal volume of sterilized Alsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% sodium chloride in water). The blood was centrifuged at 3000 rpm for 10 min, and packed cells were washed 3 times with isosaline (0.85%, pH 7.2). The blood volume is measured and reconstituted with isosaline as 10% v/v suspension.

Procedure (Hypotonic Solution-induced Hemolysis)

The reaction mixture (4.5 ml) consists of 2 ml of hyposaline (0.25% w/v NaCl), 1 ml of 0.15 M phosphate buffer (pH 7.4), and 1 ml of test solution (50 µg/ml, 100 µg/ml, 300 µg/ml,

and 500 µg/ml) in isosaline, 0.5 ml of 10% HRBC in isosaline was added. 1 ml of distilled water used instead of hyposaline (to produce 100% hemolysis) for test control, while product control lacked red blood cells. The solutions were incubated for 30 min at 37°C and centrifuged at 3000 rpm 20 min. Diclofenac sodium was used as the reference drug. In the suspension, the hemoglobin content was estimated using a spectrophotometer at 560 nm. Percentage membrane-stabilizing activity was calculated as follows:

% Membrane stabilization = $\frac{\text{Abs control} - \text{Abs treated}}{\text{Abs treated}} \times 100$

Thrombolytic Activity

The thrombolytic activity of this extractive was evaluated by the *in vitro* thrombolytic test^[19] using SK as standard. The dried crude extract (10 mg) was suspended in 10 ml of distilled water, and it was kept overnight. Then, the soluble supernatant was decanted and filtered. The blood was withdrawn from healthy volunteers and was distributed into five different pre-weighed microcentrifuge tubes and is incubated at 37°C and then the serum was removed completely without disturbing the clot, then tubes with clot were weighed to determine clot weight (clot weight = weight of clot containing tube-weight of tube alone). To each pre-weighed clot in microcentrifuge tube, along with the crude extract and 100 µl aqueous solution of different partitionates was added separately. 100 µl of distilled water was separately added to the control tubes. As a positive control 100 µl of SK was used. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, the released of fluid was removed, and tubes were again weighed to observe the difference in weight after clot disruption. The percentage of clot lysis is shown.

% of clot lysis = $(\text{weight of released clot} / \text{clot weight}) \times 100$

RESULTS

Phytochemical Screening

Various chemical tests are carried out and tabulated in Table 1; preliminary phytochemical tests showed the presence of flavonoids, alkaloids, steroids, terpenoids, lignin's, carbohydrates, proteins, tannins, saponins, and glycosides.

Antiarthritic Activity [Tables 2 and Figures 1-2]

Table 2 shown as Inhibition of Ethanolic leaf extract of *Bauhinia purpurea* on Protein Denaturation.

Anti-inflammatory Activity [Tables 3 and Figures 3]

Table 3 shown as Percentage Stabilisation of Ethanolic leaf extract of *Bauhinia purpurea* using HRBC Method.

Table 1: Phytochemical screening of ethanolic leaf extract of *B. purpurea*

Chemical constituents	Chemical tests	Ethanolic extract
Alkaloids	Dragendorff's test	+
	Mayer's test	+
	Hager's test	+
Flavonoids	Shinoda's test	+
	Zinc chloride test	+
	Alkaline reagent test	+
Steroids and terpenoids	Salkowski test	+
	Liebermann–Burchard's test	+
Lignin's	Thionine test	+
Carbohydrates	Molisch's test	+
	Benedict's test	+
	Fehling's test	+
	Barfoed's test	+
Proteins	Xanthoproteic test	+
	Million's test	+
Saponins	Froth formation test	+
Glycosides	General test	+
	Mod. Borntrager's test	+

+Indicates positive. *B. purpurea*: *Bauhinia purpurea*

Table 2: Inhibition of Ethanolic leaf extract of *B. purpurea* on protein denaturation

Extract	Concentration ($\mu\text{g/ml}$)	BSA method		Egg albumin method	
		Absorbance at 255 nm	% of inhibition	Absorbance at 660 nm	% of inhibition
Control	-	0.90 \pm 0.13	-	0.5 \pm 0.16	-
<i>B. purpurea</i>	50	0.191 \pm 0.23	78.7	0.18 \pm 0.21	64
	100	0.173 \pm 0.21	80.7	0.076 \pm 0.24	82
	300	0.172 \pm 0.25	80.8	0.071 \pm 0.22	85
	500	0.168 \pm 0.22	82.2	0.03 \pm 0.20	94
Diclofenac sodium	400	0.14 \pm 0.23	84.4	0.02 \pm 0.23	96

Absorbance values are expressed as mean \pm SEM, $n=3$. *B. purpurea*: *Bauhinia purpurea*, BSA: Bovine serum albumin, SEM: Standard error of mean

Thrombolytic Activity [Table 4]

Table 4 shown as Thrombolytic activity of Ethanolic leaf extract of *Bauhinia purpurea*.

DISCUSSION

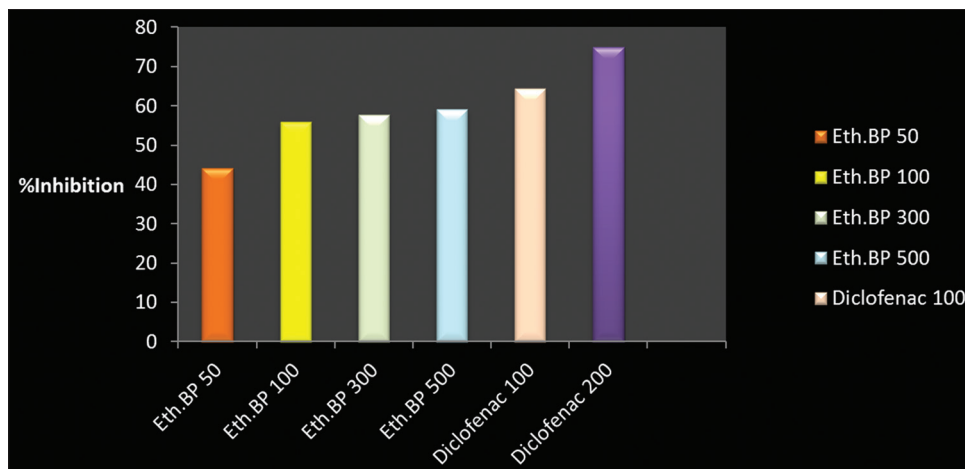
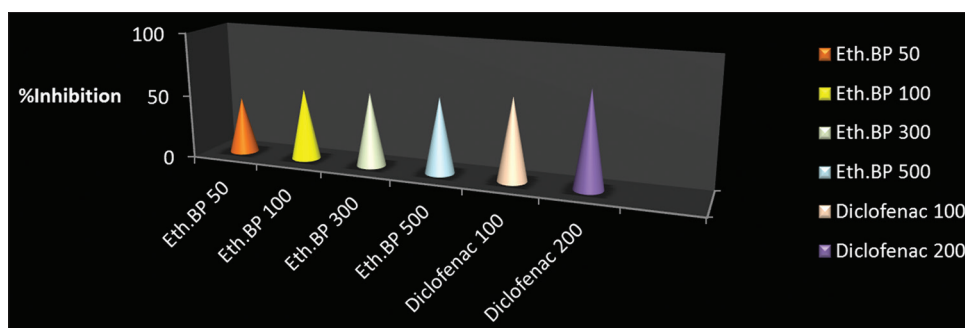
Plants have been used due to various phytochemicals synthesized as secondary metabolites plant extracts and phytochemicals can be of great significance in therapeutic treatments. The compounds are known by their active substances such as phenols, alkaloids, and tannins.^[20] The leaf extract neither showed any seizure nor organ malfunction, concluding that the

extract was safe. The route of administration of the extract played an important role. Phytochemical tests of ethanolic leaf extract of *B. purpurea* showed the presence of flavonoids, alkaloids, steroids, terpenoids, lignin's, carbohydrates, proteins, tannins, saponins, and glycosides. They may lead to their quantitative estimation and also in locating the source of pharmacologically active chemical compounds by preliminary phytochemical tests which are significant and helpful in finding chemical constituents in the plant material. The cause of RA was documented by denaturation of protein. The denaturation of protein may be due to the production of autoantigen in certain arthritic disease. The alteration of electrostatic hydrogen, hydrophobic, and disulfide bonding occurs in mechanism of denaturation. From the result of the present study, it can be stated that all the extracts of *B. purpurea* leaves are capable to inhibit the denaturation of

Table 3: Percentage stabilization of ethanolic leaf extract of *B. purpurea* using HRBC method

Extract	Concentration ($\mu\text{g/ml}$)	Absorbance at 560 nm	% Stabilization
Control	-	1.52	-
<i>B. purpurea</i>	50	0.85 \pm 0.23	44.2
	100	0.67 \pm 0.25	55.9
	300	0.64 \pm 0.24	57.8
	500	0.62 \pm 0.22	59.2
Diclofenac sodium	100	0.54 \pm 0.25	64.47
	200	0.38 \pm 0.23	75

Absorbance values are expressed as mean \pm SEM, $n=3$. *B. purpurea*: *Bauhinia purpurea*, HRBC: Human red blood cell

**Figure 1:** Inhibition of ethanolic leaf extract of *Bauhinia purpurea* on protein denaturation**Figure 2:** Inhibition of ethanolic leaf extract of *Bauhinia purpurea* on albumin denaturation

proteins, controlling the production of autoantigen and thereby it and its effect was compared with the standard drug diclofenac sodium. The percentage protection was found to be diclofenac (84.4%). The extract exhibited dose-dependent response. This effect may be due to the presence of steroids, alkaloids, and flavonoids presents in various fractions.^[21] Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound such as strong acid or base, a concentrated inorganic salt, an organic solvent, or heat. Most biological proteins lose their biological function when denatured. Denaturation of proteins is a well-documented cause of inflammation. As part of the investigation on the mechanism of the antiarthritic activity, ability of plant extract to inhibit protein denaturation

was studied. In inhibiting heat-induced albumin denaturation, it was effective. Maximum inhibition at 500 $\mu\text{g/ml}$ was observed as 94%, a standard anti-inflammatory drug showed the maximum inhibition of 68% at the concentration of 100 $\mu\text{g/ml}$ which was compared with control. Hence, from the results of our study reveal that the ethanolic leaf extract of *B. purpurea* is capable of controlling the production of autoantigens and also inhibits protein denaturation, albumin denaturation, and membrane lysis in rheumatic disease. Our present studies indicate that extract of *B. purpurea* exhibits strong antiarthritic property could be potential source of antiarthritic property. The inhibition of protein denaturation, albumin denaturation, and membrane stabilization was studied to establish the mechanism of antiarthritic activity of *B. purpurea*. Therefore,

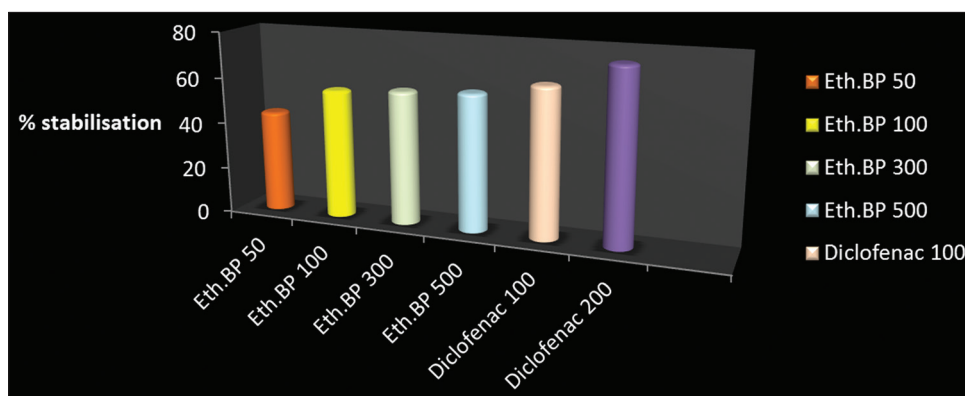


Figure 3: Percentage stabilization of ethanolic leaf extract of *Bauhinia purpurea* using human red blood cell method

Table 4: Thrombolytic activity of ethanolic leaf extract of *B. purpurea*

Extracts/drugs	Mean±SD (% clot lysis)
Water (negative control)	2.725±0.983%
SK (positive control)	72.835±5.702%***
Ethanolic extract of <i>B. purpurea</i>	91.02±2.6%***

*Values are expressed as mean±SEM, n=3. *B. purpurea*: *Bauhinia purpurea*, SK: Streptokinase, SD: Standard deviation, SEM: Standard error of mean

our *in vitro* studies on extract of *B. purpurea* demonstrate the significant antiarthritic activity. Hence, the result shows that the extracts of *B. purpurea* exhibited antiarthritic activities might be due to the presence of active principles such as polyphenolic content, triterpenoids, alkaloids, and flavonoids. The HRBC membrane stabilization has been used because the membrane of erythrocytes is analogous to the membrane of lysosomes,^[22,23] and the stabilization of erythrocyte membrane implies that our extract may well stabilize the lysosomal membranes. Stabilization of lysosomal membrane is one of the important steps for preventing the activated neutrophil release such as bacterial enzymes and proteases, which causes tissue inflammation and damage the extracellular release. Various disorders are produced during inflammation by the lysosomal enzymes and are related to be acute or chronic inflammation by their extracellular activity. Either by stabilizing the lysosomal membrane or by inhibiting these lysosomal enzymes the action of nonsteroidal drugs takes place.^[24] Addition of 100 µl SK (Durakinase, Dongkook Phama. Co. Ltd., South Korea), a positive control (30,000 I.U.) to the clots along with 90 min incubation at 37°C, showed 72.83% clot lysis. On the other hand, with 100 µl sterile distilled water (negative control), it showed negligible clot lysis which was only 2.72%. The mean difference in percentage clot lysis between positive and negative control was found to be very statistically significant (***) ($P < 0.001$). However, when 100 µl sample formulation was added to three different clots, 91.02% clot lysis was obtained and when compared with the negative control (water) the mean clot lysis percentage difference was found to be statistically significant (***) ($P < 0.001$).

CONCLUSION

From the result of the study, it can be concluded that the ethanolic leaf extract of *B. purpurea* possessed anti-inflammatory, antiarthritic, and thrombolytic activities. However, one should try to further figure out extract more as having much better activity in quest of active candidate or chemical molecule that is mainly responsible for this activity through detailed experimentation.

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