

IN VITRO ANTIHEMOLYTIC AND ANTI-ARTHRITIC ACTIVITIES OF *ARISTOLOCHIA BRACTEATA* (Lam.)

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ABSTRACT

Background: *Aristolochia bracteata* (Lam.) is described in Ayurveda for the treatment of painful inflammations, cardiac disorders.

Objectives: The present study was aimed to investigate the phytochemical, *in vitro* antihemolytic and anti-arthritic activities of ethanolic extracts of leaf, stem and root of *A. bracteata*. The phytochemical, *in vitro* antihemolytic and anti-arthritic activities of different parts of *A. bracteata* by standard protocols.

Results: The various bioactive phytochemicals such as carbohydrates, flavonoids, saponins, steroids and coumarins present in leaf, stem and roots of *A. bracteata*. The maximum protection of RBC membrane lysis was observed in ethanolic extract of *A. bracteata*. The ethanolic extracts of stem exhibited significant protection of membrane denaturation and proteinase inhibitory activities.

Conclusion: So, from this result indicates *A. bracteata* may be used in designing various pharmacological drugs and treatment of rheumatoid arthritis.

Keywords: *Aristolochia bracteata*, Antihemolytic, Anti-arthritic, Protein denaturation, ethanolic extract.

INTRODUCTION

Herbal medicine has been commonly used over the years for treatment and prevention of diseases and health promotion and quality of life. However, there is a lack of a systematic approach to assess their safety and effectiveness. The holistic approach to health care makes herbal medicine very attractive to many people in worldwide. Herbal extracts may be contaminated, adulterated and may contain toxic compounds. The quality control of herbal medicines has a direct impact on their safety and efficacy¹. The medicinal value of these plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body². Some of the most important bioactive phytochemical constituents are alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, phenolic compounds etc³. Several herbal secondary metabolites such as flavonoids have been found to protect cells from oxidative damage. These compounds have been evidenced to stabilize RBC membrane by scavenging free radicals and reducing lipid peroxidation⁴. These natural compounds formed the foundations of modern prescription drugs⁵.

Hemolysis is a destruction of erythrocytes with liberation of hemoglobin in plasma. Hemolysis occurs in a variety of pathological conditions such as autoimmunity against RBC-surface antigen, mechanical disruption of RBC, malaria/clostridium infection, thalassemia and sickle cell disease⁶. Erythrocytes are exclusive blood cells that deliver oxygen to our body⁷ and participate in detoxification of a great variety of toxic xenobiotics⁸. Oxidative damage and hemolysis caused by reactive oxygen species (ROS) have a major role in the development of diseases such as thalassemia, glucose-6-phosphate dehydrogenase deficiency and sickle cell anemia. Red blood cells (RBCs) are the primary targets of free radicals, owing to their high membrane concentrations of polyunsaturated fatty acids (linoleic and arachidonic acids) O₂ transport associated with redox active hemoglobin molecules, which are potent promoters of ROS. Furthermore, RBCs are very susceptible to oxidative stress due to high cellular concentration of oxygen and hemoglobin, high polyunsaturated fatty acid content

while oxidative stress on RBC is implicated to hemolysis⁹. Erythrocytes, which are the most abundant cells in the human body, possessing desirable physiological and morphological characteristics, are exploited extensively in drug delivery¹⁰. Oxidative damage to the erythrocyte membrane (lipid/protein) may be implicated in hemolytic associated with some hemoglobinopathies, oxidative drugs, transition metal excess, radiation and deficiencies in some erythrocyte antioxidant systems¹¹. Many plants contain chemical substances that might have a hemolytic or antihemolytic effect on human erythrocytes. Several reports indicate that the membranes of human erythrocytes from blood types have varying stability as determined from the mean corpuscular fragility¹². Plant extracts can positively affect the red cell membrane¹³ and many plants have serious adverse effects, which include induction of hemolytic anemia. Therefore, many of the commonly used plants need to be evaluated for their potential hemolytic activity.

Arthritis represents one of the most prevalent chronic health problems and is a leading cause of disability¹⁴. Arthritis affected 43 million U.S. adults in 2002 and the year 2020, this number is expected to reach 60 million¹⁵. In Rheumatoid arthritis is the inflammation of a joint, osteoarthritis is the most common form of arthritis and associated with inflammation resulting from an overactive immune system. It is a degenerative disease characterized by damage to the articular cartilage, changes in subchondral and marginal bone, synovitis and capsular thickening, typically affecting weight bearing can include infiltration of inflammatory cells (monocytes), synovial hyperplasia, bone erosion and new bone formation, narrowing of the joint space and ankylosis of the joint¹⁶. Herbal plants and other traditional medicinal system for the treatment of various diseases. Hence, they play an important role as alternative medicine due to less side effects and low cost.

Aristolochia bracteata is a perennial herb. This plant belongs to the family Aristolochiaceae. This species which had been shown to be nephrotoxic, mutagenic and carcinogenic due to the cytotoxicity of the Aristolochic acid constituents. The leaves of the plant which are

used by native tribal and the villagers. It is commonly called as worm killer in English and aadutheendapaalai in Tamil. It is used in traditional medicines as a gastric stimulant and in the treatment of cancer, lung inflammation, dysentery and snakebites. The whole plant was used as a purgative, anti pyretic and anti inflammatory. The plant contain Aristolochic acid has many medicinal properties in various disease condition. The root part was used to treat syphilis, gonorrhoea and also used during labors to increase uterine contraction. The plant produced diverse range of bioactive molecules making them a rich source of different types of medicine¹⁷. There is no detailed on *in vitro* antihemolytic and antiarthritic activity of ethanolic extracts of leaf, stem and root of *A. bracteata* (Lam.).

MATERIALS AND METHODS

Collection of plant materials

The fresh leaves of *Aristolochia bracteata* (Lam.) were collected in November 2016 from the village of Thirunanriyur, Nagappattinam (district), Tamilnadu. The plant was identified by Dr. S. John Britto, Director, Rapinat Herbarium and Centre for Molecular Systematics, Department of Botany, St. Joseph's College, Tiruchirappalli, Tamilnadu, India. The collected leaves were cleaned well and air dried under shade at room temperature, the sample was powdered in an electric grinder, sieved with coarse powder and stored in air tight container.

Preparation of extracts

25 g of powdered materials were soaked in the 250 ml of ethanol and plug with cotton for 3 days and then filtered through muslin cloth followed by Whatmann No. 1 filter paper. The filtrates were concentrated by boiling water bath and then crude form of extracts was stored at 4 – 8 °C in air tight container.

Qualitative method of phytochemical screening

The preliminary chemical tests were carried out for the extracts of *Aristolochia bracteata* (leaf, stem and root) to identify the presence of various phytoconstituents by using standard protocol [18].

In vitro antihemolytic activity

Inhibitory effects on H₂O₂ induced erythrocyte hemolysis

The antihemolytic activity was examined by the method of Naim et al.¹⁹. The erythrocytes from cow blood were separated by centrifugation and washed with saline or isotonic sodium phosphate buffer (pH 7.4), until the supernatant was colorless. The erythrocytes were then diluted with saline or phosphate buffer to give a 4% suspension. Varying amounts of sample (200-1000 µg mL⁻¹) with saline or buffer were added to 2 mL of the suspension of erythrocytes and the volume was made up to 3.5 mL with saline or buffer. This mixture was preincubated for 5 min and then 0.5 mL of 0.3% H₂O₂ solutions of appropriate concentration in saline or buffer was added. The concentration of H₂O₂ in the reaction mixture was adjusted so as to bring about 90 % hemolysis of blood cells after 120 min incubation. Incubation was concluded after these time intervals by centrifugation at 1000 g for during 5 min. The extent of hemolysis was determined by measurement of the absorbance at 540 nm corresponding to hemoglobin liberation.

$$\% \text{ antihemolysis} = [(A_0 - A_1)/A_0] \times 100$$

In vitro anti-arthritis activities

Inhibition of protein denaturation

The inhibition of protein denaturation was carried out by the method of Eduardo and Tannia²⁰. The test solution consisted of 0.45 ml of Bovine serum albumin (5 % w/v aqueous solution) and 0.05 ml of EA (100 and 250 µg/ml) in DMSO. Test control (0.5 ml) consisted of 0.45 ml of bovine serum albumin and 0.05 ml of distilled water. Whereas product control (0.5 ml) consisted of 0.45 ml of distilled water and 0.05 ml of EA and standard consisted of 0.45 ml of bovine serum albumin and 0.05 ml of acetyl salicylic acid. The samples were incubated at 37 °C for 20 min and the temperature was increased to 57 °C for 3 min. After cooling, 2.5 ml of phosphate

buffer saline (pH 6.3) was added to each tube. The absorbance was measured spectrophotometrically at 660 nm. The control represented 100 % protein denaturation. The results were compared with acetyl salicylic acid. The percentage inhibition of protein denaturation was calculated as below:

Percent inhibition =

$$\frac{100 - (\text{OD of test solution} - \text{OD of product control}) \times 100}{\text{OD of test control}}$$

Proteinase Inhibitory activity

Proteinase inhibitory activity was carried out by the method of Chatterjee²¹. The test solution (2.0 ml) contained 0.06 mg trypsin 1.0 ml 25 mM tris-HCl buffer (pH 7.4) and 1.0 ml of EA (100 and 250 µg/ml) in DMSO and standard consisted of 0.5 ml Acetyl salicylic acid (250 µg/ml). The mixture was incubated at 37 °C for 5 min. Then 1.0 ml of 0.8 % (w/v) casein was added. The mixtures were incubated for an additional 20 min and 2.0 ml of 70 % perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged and absorbance of the supernatant was read at 280 nm against buffer as blank. The product control lacked trypsin in reaction mixture and test control represented 100 % inhibition. The results were compared with acetyl salicylic acid (250 µg/ml) treated samples. The percentage inhibition of protein was calculated as below:

Percent inhibition =

$$\frac{100 - (\text{OD of test solution} - \text{OD of product control}) \times 100}{\text{OD of test control}}$$

RESULTS

Phytochemical analysis

The phytochemicals of *A. bracteata* were qualitative analyses from leaf, stem and root separately and result is mention in Table 1. The present study revealed that the ethanolic extracts of leaf, stem and root of *A. bracteata* contains the presence of various bioactive phytochemicals were rich in carbohydrates, flavonoids, saponins, steroids and coumarins. Secondary metabolites such as phenols and terpenoids present in the leaf and stem extracts of *A. bracteata* where as phlobataninns and anthraquinones were absent in all the three parts (leaf, stem and roots) of *A. bracteata*.

Antihemolytic activity

The present study indicated that *in vitro* antihemolytic activity of ethanolic extracts of leaf, stem and root of *A. bracteata* (Lam.) shown in Table 2 and Fig. 1. *In vitro* RBC membrane destruction was induced by H₂O₂. The maximum protection of RBC membrane lysis (48.3 ± 1.34) was observed in ethanolic extract of *A. bracteata* leaf when compared to stem and root extracts of *A. bracteata*. These results were compared with standard drug aspirin (58.3 ± 0.96 at 1mg/ml). The minimum RBC protection (12.5 ± 0.95) was observed in root extract at 200 µg of *A. bracteata*. Antihemolytic activity was increased with increasing concentration of plant extracts (Plate 1, 2 and 3).

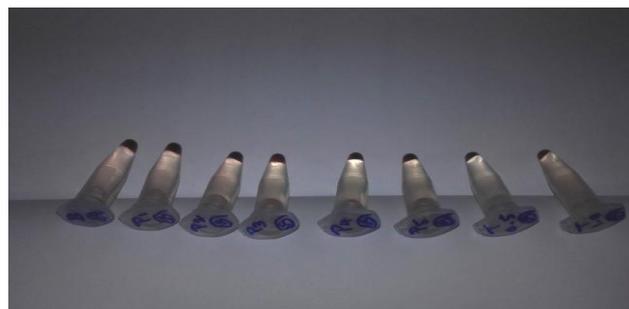
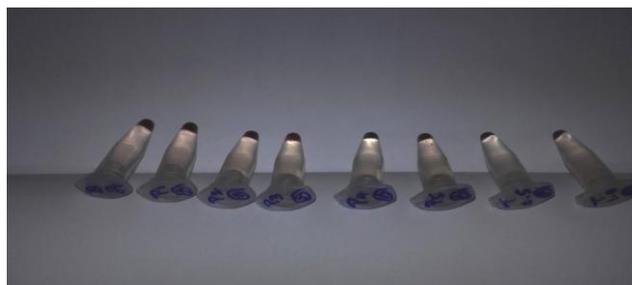


Plate 1: Antihemolytic activity of *A. bracteata* (Lam.) leaf

Plate 2: Antihemolytic activity of *A. bracteata* (Lam.) stemPlate 3: Antihemolytic activity of *A. bracteata* (Lam.) rootTable 1: The phytochemical analysis of ethanolic extracts of *A. bracteata* (Lam.)

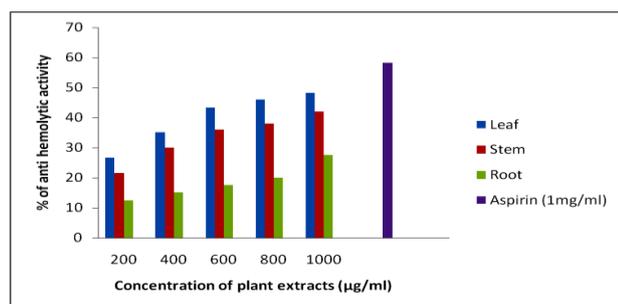
Name of the phytochemicals	Different parts of <i>A. bracteata</i>		
	Leaf	Stem	Root
Alkaloids	-	-	-
Carbohydrates	+++	+++	+++
Glycosides	+++	-	-
Proteins	+	+	+
Steroids	++	+++	+
Tannins	+	-	-
Phenols	+++	++	-
Flavonoids	++	++	+
Coumarins	++	++	+
Saponins	+	+++	++
Quinines	-	-	++
Terpenoids	+	++	-
Phlobatannins	-	-	-
Antraquinones	-	-	-

+++ highly; ++ moderate; + mild; - absent; + present.

Table 2: *In vitro* antihemolytic activity of ethanolic extracts of *A. bracteata* (Lam.)

Concentration of plant extracts (µg/ml)	% of anti hemolytic activity			
	Leaf	Stem	Root	Aspirin
200	26.6 ± 0.98	21.6 ± 0.95	12.5 ± 0.95	-
400	35 ± 0.94	30 ± 0.94	15 ± 0.94	-
600	43.3 ± 0.94	36 ± 2.41	17.5 ± 1.20	-
800	46 ± 0.94	38 ± 1.69	20 ± 1.41	-
1000	48.3 ± 1.34	42 ± 1.18	27.5 ± 1.20	58.3 ± 0.96

Values are expressed as mean ± S.E (n=3)



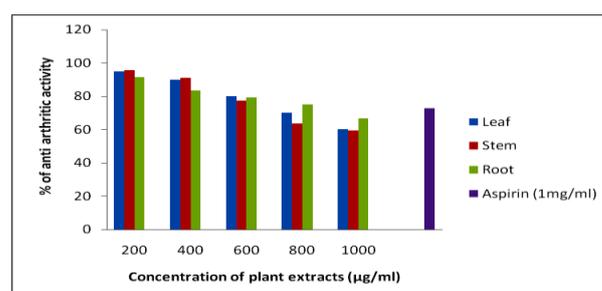
Values are expressed as mean ± S.E (n=3)

Fig. 1: *In vitro* antihemolytic activity of ethanolic extracts of *A. bracteata* (Lam.)

Anti-arthritis activity

The production of auto antigen in certain arthritic disease may be due to denaturation of protein activity. In the present study showed that the ethanol extracts of different parts of *A. bracteata* (Lam.) against albumin denaturation in Table 3 and Fig. 2. The anti-arthritis activity was significantly decreased at the increasing concentration of plant extracts. The highest inhibition of protein denaturation (95.5 ± 1.32) was observed in stem extract of *A. bracteata* at 200 µg and the minimum inhibition (59.4 ± 0.85) was observed in stem at

the concentration 1000 µg, which was compared to ethanolic extracts of leaf and root of *A. bracteata* and standard drug aspirin.



Values are expressed as mean ± S.E (n=3)

Fig. 2: *In vitro* protein denaturation of ethanolic extracts of *A. bracteata* (Lam.)

Proteinase inhibition

Proteinase that begins the hydrolytic breakdown of proteins by splitting into polypeptide chains. The maximum percentage of proteinase inhibitory activity was showed in all three parts of *A. bracteata* at 200 µg/ml as shown in Table 4 and Fig. 3. From this result, ethanolic extracts of stem has maximum proteinase inhibitory activity (88.8 ± 0.61) was observed in 200 µg, when compared to root extracts of *A. bracteata*. The minimum inhibition of protein lysis

(60 ± 0.47) was observed in leaf extracts of *A. bracteata*. The activity

was decreased at increasing concentration of plant extracts.

Table 3: *In vitro* protein denaturation of ethanolic extracts of *A. bracteata* (Lam.)

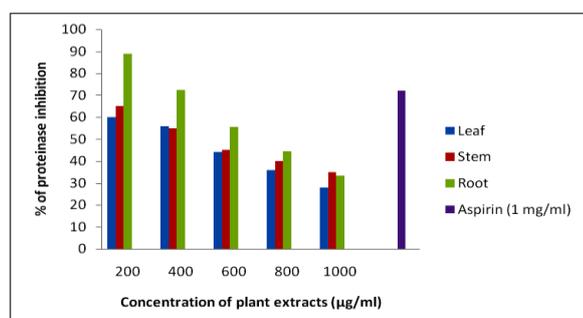
Concentration of plant extracts (µg/ml)	% of protein denaturation			
	Leaf	Stem	Root	Aspirin
200	95 ± 0.94	95.5 ± 1.32	91.6 ± 0.98	-
400	90 ± 1.70	90.9 ± 1.22	83.3 ± 1.23	-
600	80 ± 0.47	77.2 ± 0.99	79.1 ± 1.20	-
800	70 ± 0.94	63.7 ± 2.61	75 ± 1.41	-
1000	60 ± 1.88	59.4 ± 0.85	66.6 ± 1.50	72.8 ± 2.96

Values are expressed as mean ± S.E (n=3)

Table 4: *In vitro* proteinase inhibitory activity of ethanolic extracts of *A. bracteata* (Lam.)

Concentration of plant extracts (µg/ml)	% of Proteinase inhibition			
	Leaf	Stem	Root	Aspirin
200	60 ± 0.47	88.8 ± 0.61	65 ± 0.94	
400	56 ± 0.94	72.3 ± 1.06	55 ± 1.41	
600	44 ± 1.41	55.6 ± 1.08	45 ± 1.88	
800	36 ± 1.88	44.5 ± 1.55	40 ± 0.47	
1000	28 ± 0.94	33.4 ± 1.57	35 ± 0.94	72.2 ± 1.03

Values are expressed as mean ± S.E (n=3)



Values are expressed as mean ± S.E (n=3)

Fig. 3: *In vitro* proteinase inhibitory activity of ethanolic extracts of *A. bracteata* (Lam.)

DISCUSSION

The variation in type of phytochemicals present in different solvents as shown in the result of phytochemical screening might be attributed to the ability of the solvents to dissolve into solution specific type of secondary metabolites as reported by Yusha'u et al.²². Bharathajothi and Bhaaskaran²³ also reported the similar result were observed in ethanolic extracts of *Aristolochia bracteata* (Lam.), Deepa et al.²⁴ also reported the phytochemical analysis of aqueous and methanolic extracts of *Aristolochia bracteata* leaf. Phenolic compounds are a large and complex group of chemical constituents found in plants²⁵. They are plant secondary metabolites, and they have an important role as defense compounds. Phenolics exhibit several properties that are beneficial to humans, and its antioxidant properties are important in determining their role as protective agents against free radical mediated disease processes. Flavonoids are a phenolic structure containing one carbonyl group complexes with extracellular and soluble proteins and with bacterial cell wall²⁶. The phytochemical analysis of ethanolic extracts of leaf, stem and root of *A. bracteata* contains carbohydrates, phenols, tannins, flavonoids, saponins, glycosides, coumarins, terpenoids and steroids were highly present in leaf extract of plant, when compared to stem and root extracts of *A. bracteata*.

Hemolysis is due to red blood cells destruction which resulted from lysis of membrane lipid bilayer. This hemolysis relates to concentration and potency of plant extracts. The erythrocyte model has been widely used as a direct indication of toxicity of injectable formulations as well as general indication of membrane toxicity. Another advantage of erythrocytes model that blood is readily

available and RBC cells are easy to isolate from the blood; moreover, its membrane has similarities with other cell membranes²⁷. Erythrocytes have been used as a model system by a number of workers for the study of interaction of drugs with membranes²⁸.

During its lifetime of ~120 days, the red blood cell (RBC) is exposed to continuous oxidative stress. It is particularly susceptible to oxidative damage due to the high content of unsaturated fatty acid chains in the lipid bilayer²⁹ combined with high oxygen levels, as well as protein susceptibility to oxidative processes. Hydrogen peroxide generated during the autoxidation of oxyhemoglobin contributes to heme degradation thus the reactive oxygen species is a good model to study experimentally-induced erythrocyte damages³⁰. It has been suggested that phytochemicals can either protect erythrocytes or increase their resistance to oxidative reaction³¹.

Erythrocytes are considered as prime targets for free radical attack, owing to the presence of both high membrane concentration of polyunsaturated fatty acids (PUFA) and the O₂ transport associated with redox active hemoglobin molecules, which are potent promoters of reactive O₂ species, especially linoleic acid and arachidonic acid are targets of lipid peroxidation³². Erythrocytes are the most abundant cells in the human body possess desirable physiological and morphological characteristics¹⁰. The capability of different phenolic substances to scavenge various types of oxidation-initiating radicals has been reported in the polar phase³³. It was demonstrate that binding of the flavonoids to the RBC membranes significantly inhibits lipid peroxidation, and at the same time enhances their integrity against lyses³⁴. Scavenging of H₂O₂ by *A. bracteata* extract may be attributed to its phenolics and other active components which can donate electrons to H₂O₂, thus neutralizing it to water³⁵. The maximum protection of RBC membrane lysis was seen in leaf extract of *A. bracteata* may be due to the presence of flavonoids, phenolics and other phytochemicals are scavenging H₂O₂ induced the free radical formation in RBC.

RA is considered as a chronic systemic autoimmune disease with the main characteristic of chronic joint inflammation that ultimately leads to joint destruction. *In vitro* anti-arthritis activity when tested using standard methods. However, treatment with plant extracts although may have some unpredictability in the effectiveness; being non-toxic and less side effect as compared to other system of medicine, many medicinal plants have proven effects on arthritic symptoms as compared to that of conventional medicines³⁶.

Denaturation of tissue proteins is one of the well documented causes of inflammatory and arthritic diseases. Production of auto-antigens in certain arthritic diseases may be due to denaturation of proteins *in vivo*. The mechanism of denaturation probably involves alteration

of electrostatic hydrogen, hydrophobic and disulphide bonding. The increments in absorbance of plant extract and reference drug with respect to control indicated the stabilization of albumin protein. This anti-denaturation effect was further supported by the change in viscosities. It has been reported that the viscosities of protein solutions increase on denaturation³⁷. The ethanolic extracts of *A. bracteata* controlling the formation of auto antigen and inhibit the protein denaturation was observed in stem. The inhibition of protein denaturation was decreased at increasing concentration of plant extracts. Similarly, Shruthi et al.,³⁸ also observed in anti-arthritis activity of ellagic acid isolated from methanolic extracts of *Kirganelia reticulata* leaf.

In the present study, the ethanolic extract of *A. bracteata* contains several phytochemicals such as terpenoids, alkaloids, glycosides, flavonoids and steroids. The presence of these phytochemicals may be attributing to its anti-rheumatic activity through the anti-inflammatory activity as well as modifying the autoimmune system. It can be stated that ethanolic extracts are capable of controlling the production of auto antigen and inhibits denaturation of protein and membrane lysis in rheumatic disease³⁹.

Proteinases have been implicated in arthritic reactions. Neutrophils are known to be a source of proteinase, which carries in their lysosomal granules many serine proteinases. It was previously reported that leukocytes proteinase play important role in the development of tissue damage during in inflammatory reactions and significant level of protection was provided by proteinase inhibitors⁴⁰. Similarly, Shruthi et al.,³⁸ also observed in anti-arthritis activity of ellagic acid isolated from methanolic extracts of *Kirganelia reticulata* leaf. So we purified plant extracts and their isolated phytoconstituents can be very useful against rheumatoid arthritis.

CONCLUSION

In this study the phytochemical and *in vitro* antihemolytic and anti-arthritis activities of ethanolic extracts of *A. bracteata*. The phytochemical analysis of ethanolic extracts of leaf, stem and root of *A. bracteata* contains pharmacological active phytoconstituents were highly present in leaf extract of plant, when compared to stem and root extracts of *A. bracteata*. *In vitro* antihemolytic activity of ethanolic extracts of *A. bracteata* was evaluated against hydrogen peroxide-induced hemolysis of erythrocytes (RBC). The minimum lysis was observed in leaf extracts of *A. bracteata*. In this present study, the maximum inhibition of protein denaturation and proteinase inhibitory activities were showed in stem of *A. bracteata*, when compared to leaf and root of *A. bracteata*. These results showed that the presence of active principles in ethanolic extracts of different parts of *A. bracteata*. Therefore, further investigation is required to isolate the pure bioactive phytochemicals may be responsible for antihemolytic and anti-arthritis activities.

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CONFLICT OF INTERESTS

Declared none

ABBREVIATIONS

Red blood cell- RBC; Rheumatoid arthritis –RA; Hydrogen peroxide- H₂O₂

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