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PHYTOCHEMICAL ANALYSIS AND ANTI-INFLAMMATORY ACTIVITY OF MEDICINAL PLANT AQUEOUS AND ETHANOLIC EXTRACT OF *BOERHAAVIA DIFFUSA* LEAVES EXTRACTS

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ABSTRACT

In this study aqueous and ethanol extract of *Boerhaavia diffua* leaves was qualitatively assessed the presence of phytochemicals for its anti-inflammatory activity by *in-vitro* methods. Phytochemical analysis of aqueous and ethanol extract of leaves revealed the presence of tannins, flavonoids, terpenoids, phenols and alkaloids. Invitro anti-inflammatory activity was evaluated against albumin denaturation, proteinase activity, membrane stabilization, and lipoxygenase activity at different concentrations of aqueous and ethanol extract of leaves. The results showed that IC_{50} values for aqueous and ethanol extractat a concentration of 325.71±2.03 and 222.4±1.43µg/ml, respectively highly protects the heat induced protein denaturation. At the concentration of 394.31±1.93 and 279.43±2.77µg/ml, aqueous and ethanol extract showed significant 50% (IC_{50}) of proteinase inhibitory action. Heat induced haemolysis of erythrocyte was significantly 50% of inhibited atthe concentration of 300.47±2.57 and 238.97±2.03 µg/ml for aqueous and ethanol extract, respectively. Hypotonicity induced haemolysis and lipoxygenase activity were significantly 50% inhibited at the concentrationof309.87±2.69 and300.94±1.77µg/ml, respectively by the aqueous extract. Ethanol extract showed 50% of inhibition of hypotonicity induced haemolysis and lipoxygenase activity was found to be 205.94±2.07 and259.34±2.33µg/ml, respectively. The results obtained in the present study indicate that ethanol extracts of *B. diffusa* leaves can be a potential source of anti-inflammatory agents compared than aqueous extract.

KEYWORDS: *Boerhaavia diffua*, phytochemical analysis, Anti-inflammatory activity, Medicinal plant.

INTRODUCTION

Inflammation is a normal protective response to the infectious agents, antigen-antibody reactions, thermal, chemical, physical agents, and ischemia.[1] All inflammatory diseases have almost a common pathway of generation of disease, which involves generation of various inflammatory mediators at various stages. Inflammation can be classified as acute and chronic. Acute inflammation is the initial response to the harmful stimuli resulting in redness, pain in the area of infection or injury. Chronic inflammation is also known as prolonged inflammation involves the lymphocytes and macrophages resulting in fibrosis and tissue necrosis. This increases the development of diseases such as arthritis, heart disease, asthma etc.[2, 3]

Synthetic drugs for inflammation were has side effects and less effective so that, the new drug is necessary to search for new anti-inflammatory from alternative sources. Medicinal plants have secondary metabolites phytochemicals showing anti-inflammatory activities have the potential of filling the need of manufacture of new drugs.[4] However, medicinal plants may offer an alternative source for anti-inflammatory activity. Screening of phytochemicals present in the plants has potential to the discovery of new medicinal drugs which have efficient protection and treatment roles against various diseases.

Boerhaavia diffusa is an herbaceous member of the family Nyctaginaceae. It is widely distributed in the tropics andsubtropics. It has been used in Ayurvedic or natural herbal medicines.[5] The whole plant and preferably the roots are effectively used to cure several diseases including Jaundice.[6] The root and aerial parts of *Boerhaavia diffusa* were used in Ayurveda for the treatment of diabetes. Pharmacological studies have reported its diaphoretic, laxative, snake venom neutralizing, to cure stomach ache, anemia and expectorant properties.[7,8] Besides, the *B. diffusa* plant is reported to possess many pharmacological, clinical, and antimicrobial properties. This plant has been extensively used in the treatment of dyspepsia, jaundice, enlargement of liver, abdominal pain and as antistress agent.[9] The aims of the present investigations are as follows:(1) to screening the phytoconstituents from the aqueous and ethanolic extract of the *Bhorovia diffusa* plant leaves. (2) To evaluate the in-vitro Anti-inflammatory activity using albumin denaturation assay,

proteinase inhibitory activity, membrane stabilization, and anti-lipoxygenase activity at different concentrations of aqueous and ethanolic extract of *Bhorovia diffusa* leaves.

MATERIALS AND METHODS

Preparation of aqueous and ethanolic extract of *B. diffusa*

The leaves of *B. diffusa* medicinal plant were collected from…..and washed with sterile distilled water. Then shade dried at room temperature for 3 days and grind into fine powder. 10 g of shade dried powdered plant leaf sample was sequentially extracted in water and 80% ethanol and up to 24 h using Soxhlet's apparatus. The extracted samples were evaporated under reduced pressure at room temperature for complete distilled out the solvent. The dried extracts were weighed and preserved at 4 °C in refrigerator for further analysis.

Phytochemical screening of leaves extract

The aqueous and ethanolic extract of plant leaves was subjected to preliminary phytochemical analysis. The presence of various groups of phytoconstituents was analyzed by carried out using the standard methods described by Trease and Evans[10], Harborne[11] and Sazada et al.[12] The phytochemical analysis was carrying out for the following chemical analysis i.e. Alkaloids, Flavonoids, Glycosides, steroids, triterpenoids, quinone, phenols, Tannin, Saponin, protein and Reducing sugar.

Assessment of in-vitro anti-inflammatory activity

Inhibition of albumin denaturation

The anti-inflammatory activity of aqueous and ethanol extract of *B. diffusa* was studied by using inhibition of albumin denaturation technique which was studied according to Mizushima et al[13] and Sakat et al[14] with slight modifications. In this test assay the reaction mixture contains various concentrations (100 – 500µg/ml) of aqueous and ethanolic test extracts and 1% aqueous solution of bovine albumin fraction. The pH of the reaction mixture was adjusted using 1N HCl. The reaction mixtures were incubated at 37 ºC for 20 min and then heated to 51 ºC for 20 min, subsequently cooling the samples. The turbidity of the reaction mixture was measured at 660 nm using UV- Visible Spectrophotometer. The inhibition Percentage of protein denaturation was calculated as follows.

Percentage inhibition = (Abs Control –Abs Sample) X 100/ Abs control

Antiproteinase action

The test antiproteinase activity of both extracts was executed according to Oyedepo and Femurewa[15] and Sakat et al[14] with modifications. In this evaluation, 0.06 mg trypsin was mixed with 1 ml 20 mM Tris HCl buffer (pH 7.4) and separately1ml aqueous and ethanol extract test sample at different concentrations (100 – 500µg/ml). The mixture was incubated at 37^{o}C for 5 min and then 1 ml of 0.8% casein was added and incubated for 20 min. After that 2 ml of 70% perchloric acid was added to stop the reaction and the suspension was centrifuged. The absorbance of the supernatant was read at 210 nm against buffer as blank. The percentage inhibition of Proteinase activity was calculated as follows

Percentage inhibition (%) = (Abs control –Abs sample) X100/ Abs control

Membrane stabilization

Preparation of Red Blood cells (RBCs) suspension

Fresh human blood was collected from and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for10min and cells were washed three times with saline solution. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline according to Sakat et al[14] and Sadique et al.[16]

Heat induced haemolysis

To this assay, 1 ml of test sample of different concentrations of aqueous and ethanolic extract (100 - 500 µg/ml) mixed with 1 ml of 10% human RBCs suspension. The control test tube was maintained with only saline solution. Aspirin was used as a standard drug. All the centrifuge tubes were incubated in water bath at 56 ºC for 30min and the tubes were cooled and the reaction mixture was centrifuged at 2500 rpm for 5 min. The absorbance of the supernatants was measured on UV spectrophotometer at 560 nm.[14, 17] The Percentage inhibition of Haemolysis was calculated as follows.

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

Hypotonicity-induced haemolysis

In this study, the assay mixture contains 0.5 ml of different concentrations of extract (100-500µg/ml), 1ml of phosphate buffer (pH 7.4), 2ml of hyposaline and 0.5ml of HRBC suspension. Diclofenac sodium of different concentrations was used as a standard drug. All the reaction mixtures were incubated at 37^{0}C for 30minutes and centrifuged at 3000rpm for 20 min. The supernatant liquid was collected and the haemoglobin content was estimated by

using spectrophotometer at 560 nm.[18] The percentage of membrane stabilization was calculated as follows.

Percentage protection (%) = 100- (OD sample/OD control) x 100

Anti-lipoxygenase activity

Anti-Lipoxygenase activity of aqueous and ethanol extract was studied according the method of Shinde et al.[17] In this assay, linoleicacid was used as substrate which initiates the reaction and lipoxidase as enzyme. Different concentrations of test samples of both extracts (100-500µg/ml) were dissolved in 0.25ml of 2M borate buffer pH 9.0. To this, 0.25ml of lipoxidase enzyme solution (20,000U/ml) was added and they are incubated at 25^0C for 5 min. After which, 0.6 mM of 1.0 ml linoleic acid solution was added to initiate the reaction, mixed well and the absorbance of the reaction mixture was measured at 234nm. Indomethacin was used as reference standard.[17] The percent inhibition was calculated from the following equation,

% inhibition= [{Abs control- Abs sample}/Abs control] x 100

RESULTS AND DISCUSSION

Phytochemical screening

Preliminary phytochemical results showed the presence or absence of certain phytochemicals in aqueous and ethanolic extract of *B. diffusa* leaves. From Table 1, the results reveal that the phytoconstituents present in the ethanol extract of *B. diffusa* contains glycosides alkaloids, flavonoids, tannins, quinines, protein, reducing sugars and phenol. Whereas, the water extract contains glycosides, triterpenoids, quinines and reducing sugars. Alkaloids, tannins and saponins were absent in aqueous extract which are confirmed by the chemical tests. Similarly, some report resulted that the *B. diffusa* plant contains a large number of such compounds as flavonoids[19], and alkaloids[20], lipids, steroids, carbohydrates, proteins, and glycoproteins.[21, 22]

Anti-inflammatory activity

Inhibition of albumin denaturation

The ability of aqueous and ethanolic plant extract to inhibit protein denaturation was studied. Both extracts were effective in inhibiting heat induced albumin denaturation compared than standard. The inhibitory action increased in concentration dependent manner. Therefore, the result confirms that aqueous and ethanol extract is capable of controlling protein denaturation during inflammation (Figure 1). The 50% of protein denaturation inhibition concentration

(IC_{50}) of aqueous and ethanol extract was observed at 325.71±2.03 and 222.4µg/ml, respectively. Aspirin, a standard anti-inflammation drug showed the 50% of inhibition at the concentration of 241±2.05µg/ml (Table 2).

Proteinase Inhibitory Action

Aqueous and ethanol extract of *B. diffusa* leaves exhibited significant antiproteinase activity at different concentrations as shown in Table 3. It showed 50% of inhibition (IC_{50}) of aqueous and ethanol extracts at 394.31±1.93and 279.43±2.77 µg/ml, respectively. Aspirin showed the IC_{50} concentration is295±2.55µg/ml. When the concentration of extract was increased, the activity of inhibiting protein degrading enzyme was observed to increase (Figure2). It is evident that the *B. diffusa* leaves inhibits proteinase activity.

Membrane stabilization

The membrane stabilization of RBC has been used as a method to study the in-vitro anti-inflammatory activity of aqueous and ethanolic extract of *B. diffusa* leaves. The erythrocyte membrane is similar to the lysosomal membrane[23, 24] and its stabilization implies that the both extracts may well stabilize lysosomal membranes.

The aqueous and ethanol extract was effective in inhibiting the heat induced haemolysis at different concentrations. The results showed that IC_{50} value of aqueous and ethanol extract at concentration 300.47±2.57 and 238.97±2.03µg/ml, respectively protect significantly the erythrocyte membrane against heat induced lysis (Table 4). Aspirin shows198.21±2.72µg/ml offered a significant 50% protection against damaging effect of heat solution (Figure 3).

The results showed that aqueous and ethanol extract protect the erythrocyte membrane against lysis induced by hypotonic solution (Table 5). With the increasing concentration the membrane haemolyis inhibition increased. Hence anti-inflammatory activity of the extracts was concentration dependent. IC_{50} value of aqueous and ethanol extract at concentration 309.87±2.69 and205.94±2.07µg/ml, respectively, protect the erythrocyte membrane against lysis induced by heat (Figure 4). Diclofenac sodium shows 198.21±2.72µg/ml offered a significant 50% protection against damaging effect of heat solution.

Anti-lipoxygenase activity

The establishment of in-vitro testing method has stimulated the screening of plants aiming to find leads for the improvement of new drugs. From the result (Table 6), the 50% of inhibition

of aqueous and ethanol extract was obtained at concentration of 300.94±1.77 and 259.34± 2.33 µg/ml, respectively (Figure 5). The standard Indomethacin showed and 50% inhibition at a concentration of 292.47 µg/ml.

Ethanol extract of this plant has phenol, tannins, flavonoids compounds have been found to possess potent anti-inflammatory activity. Similarly, the flavonoids from plant extracts have been found to possess antioxidants, antimicrobial and anti-inflammatory properties in various studies.[25, 26] Strong presence of tannins and alkaloids in all extracts may explain its potent bioactivities as tannins are known to possess potent antimicrobial activities[27], antioxidants[28], and anti-inflammatory properties.[29]

Figure captions

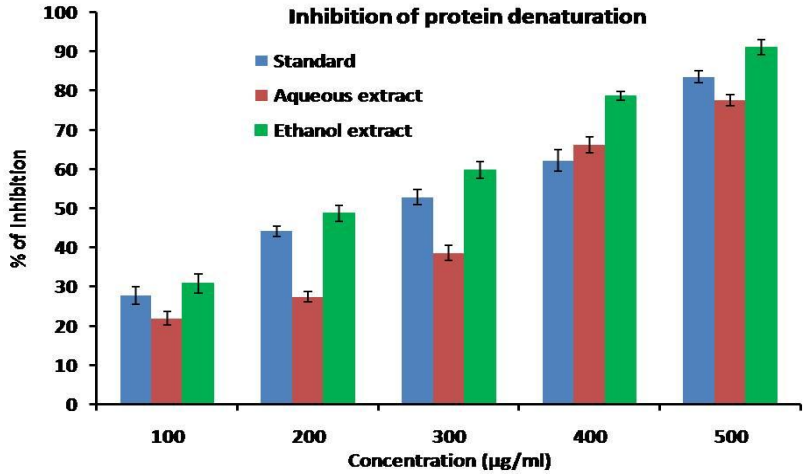

Figure1: Effect of different concentration of aqueous and ethanol extract on inhibition of protein denaturation.

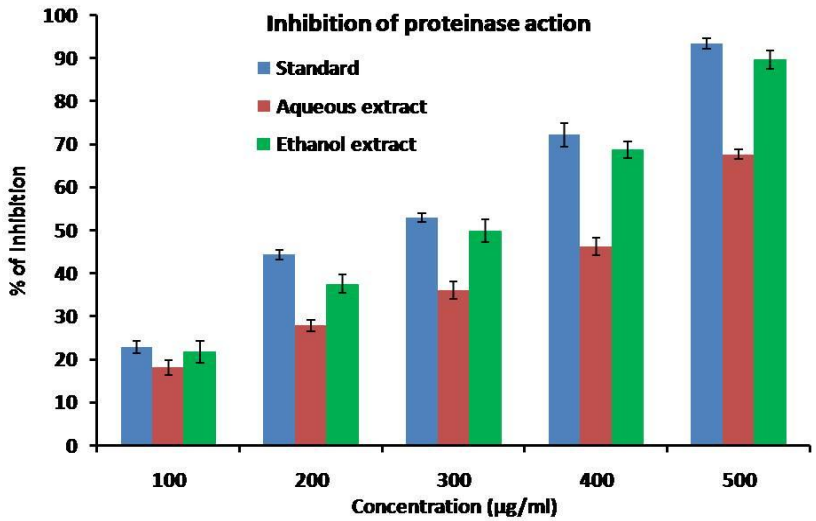

Figure 2: Effect of different concentration of aqueous and ethanol extract on inhibition of proteinase.

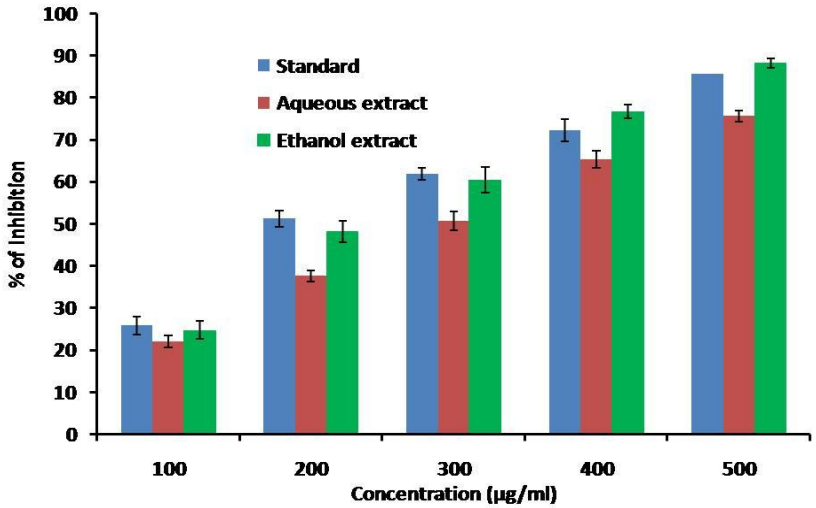

Figure 3: Effect of different concentration of aqueous and ethanol extract on inhibition of heat induced haemolysis.

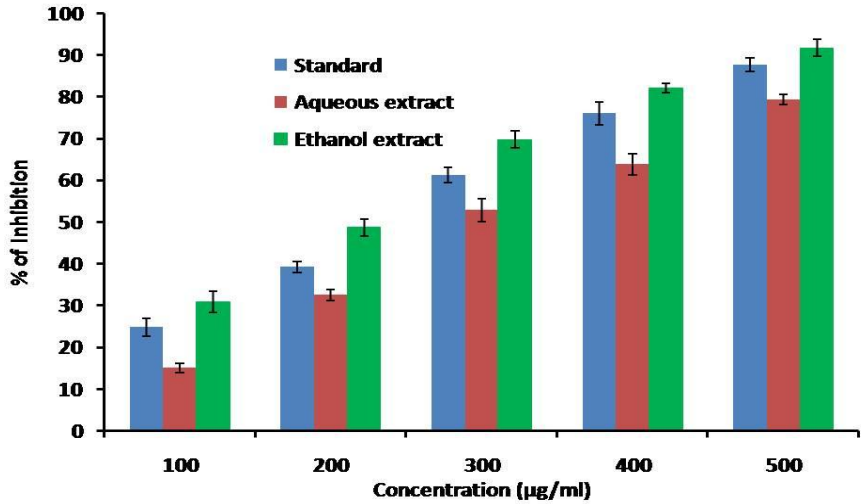

Figure 4: Effect of different concentration of aqueous and ethanol extract on inhibition of Hypotonicity Induced Haemolysis.

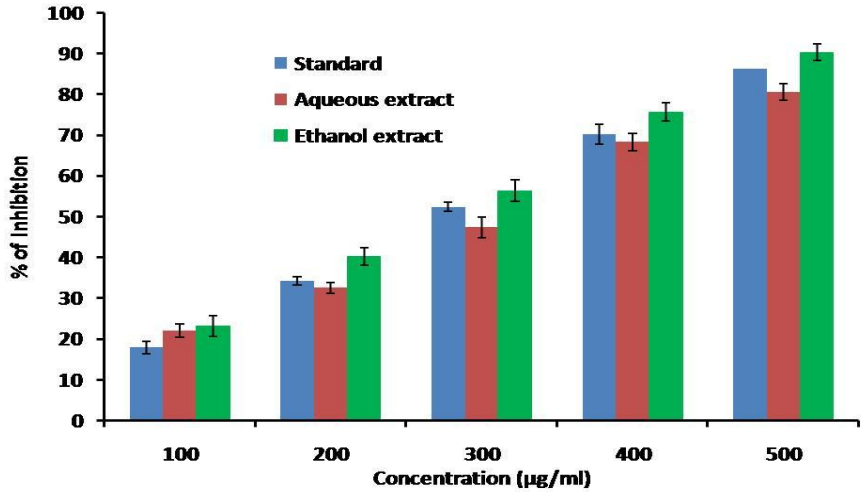

Figure 5: Effect of different concentrations of aqueous and ethanol extract on inhibition of lipoxygenase action.

Table 1: Phytochemical analysis of *B. diffusa* solvent extracts.

Test	Aqueous Extract	Methanol extract
Glycosides	+	+
Steroids	_	_
Alkaloids	_	+
Flavanoids	_	+
Triterpenoids	+	_
Tannins	_	+
Saponins	_	_
Quinones	+	+
Protein	_	+
Reducing sugars	+	-
Phenol	_	+

+ = presence, - = absent

Table 2: Effect of different concentration IC_{50} valuesof aqueous and ethanol extract on inhibition of protein denaturation.

Concentration (µg/ml)	% of inhibition of protein denaturation		
	Aqueous extract	Ethanol extract	Standard
100	22.01±1.69	30.83± 2.53	27.80±2.19
200	27.50±1.35	48.69±2.07	44.21±1.33
300	38.66±2.01	59.73 ± 2.09	52.86±1.89
400	66.21±2.09	78.66± 1.07	62.20±2.74
500	77.58±1.37	91.11 ± 2.01	83.60±1.59
IC_{50} value (µg/ml)	325.71±2.03	222.4±1.43	241.54±2.05

Table 3: Effect of different concentrations IC_{50} valuesof aqueous and ethanol extract on inhibition of proteinase action.

Concentration (µg/ml)	% of proteinase inhibition		
	Aqueous extract	Ethanol extract	Standard
100	18.07±1.69	21.65 ± 2.53	22.80±1.33
200	27.75±1.35	37.48 ±2.17	44.21±1.19
300	35.94±2.21	49.79 ± 2.69	52.86±1.09
400	46.21±2.45	68.66± 1.87	72.09±2.74
500	67.58±1.17	89.54 ± 2.09	93.33±1.29
IC_{50} value (µg/ml)	394.31±1.93	279.43 ±2.77	295.37±2.55

Table 4: Effect of different concentrations IC_{50} valuesof aqueous and ethanol extract on heat induced haemolysis of erythrocyte.

Concentration (µg/ml)	% of heat induced haemolysis		
	Aqueous extract	Ethanol extract	Standard
100	22.01±1.39	24.67 ± 2.13	25.80±2.09
200	37.50±1.33	48.09±2.57	51.21±1.93
300	50.66±2.17	60.45 ± 2.99	61.86±1.49
400	65.21±2.03	76.66± 1.67	72.20±2.67
500	75.58±1.37	88.11 ± 1.13	85.60±1.19
IC_{50} value (µg/ml)	300.47±2.57	238.97±2.03	198.21±2.72

Table 5: Effect of different concentrations and IC_{50} values of aqueous and ethanol extract on Hypotonicity induced haemolysis.

Concentration (µg/ml)	% of inhibition		
	Aqueous extract	Ethanol extract	Standard
100	15.01±1.69	30.83± 2.53	24.80±2.19
200	32.50±1.35	48.69±2.07	39.21±1.33
300	52.80±2.71	69.73 ± 2.07	61.26±1.89
400	63.76±2.59	82.06 ± 1.09	75.94±2.74
500	79.28±1.17	91.65 ± 2.03	87.60±1.59
IC_{50} value (µg/ml)	309.87 ±2.69	205.94±2.07	252.67±2.75

Table 6: Effect of different concentrations IC_{50} valuesof aqueous and ethanol extract on inhibition of lipoxygenase action.

Concentration (µg/ml)	% of inhibition of lipooxygenase		
	Aqueous extract	Ethanol extract	Standard
100	22.01±1.69	23.13± 2.59	17.80±1.49
200	32.50±1.35	40.19±2.07	34.21±1.03
300	47.26±2.51	56.33 ± 2.69	52.33±1.09
400	68.21±2.09	75.66± 2.27	70.20±2.45
500	80.58±2.07	90.32 ± 2.06	86.16±1.55
IC_{50} value (µg/ml)	300.94±1.77	259.34±2.33	292.47±2.49

CONCLUSION

In the present study results indicate that the aqueous and ethanol solvent extracts of *B. diffusa* leaves has interesting anti-inflammatory property. Phyto-chemical analysis revealed the presence of glycosides, triterpenoids, quinones and reducing sugars in aqueous extract and glycosides, alkaloids, flavonoids, tannins, quinones, phenols, and proteins in ethanolic extract. The water solvent extract exhibited a moderate inflammation inhibitory activity and

therefore may due to presence of water soluble bioactive constituents. Ethanol extract shows highly significant anti-inflammatory activity when compared than aqueous and standard drug. As a result, this medicinal plant by *in-vitro* results appear as interesting and promising and may be effective as potential sources of novel a anti-inflammatory drugs.

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