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RESEARCH ARTICLE

A COMPARATIVE "*IN-VITRO* ANTI-INFLAMMATORY ACTIVITY" STUDY OF AERIAL PARTS OF PLANT *HAMELIA PATENS*

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ABSTRACT

Background: Inflammation is a complex biological vascular tissues response to harmful stimuli which may including pathogens, irritants or damaged cells. Natural therapy is a key to overcome limitations of present NSAIDs used to treat inflammatory disorder and related symptoms. The plant *Hamelia patens* belongs to family Rubiaceae, which is rich in active phytochemicals like flavonoids and alkaloids. This Plant has been traditionally used for its beneficial effect in the treatment of various inflammatory disorder and related symptoms.
Aim: In the present communication *in-vitro* anti-inflammatory activity of aerial parts (leaf and stem) of plant *Hamelia patens* was assessed using different in-vitro anti-inflammatory activity assays.
Methods: The anti-inflammatory activity of different extracts i.e methanolic and aqueous extracts of leaf and stem of plant *Hamelia patens* was assessed using following three methods- membrane stabilization method (HRBC), protein (trypsin) inhibitory method and protein (albumin) denaturation method.
Results: Anti-inflammatory activity by HRBC membrane stabilization method was found to be best in methanolic stem extract with IC_{50} value of 508.28 µg/ml and least in methanolic leaf extract with IC_{50} value of 916.12µg/ml, anti-inflammatory activity by protein inhibitory method and protein albumin method was found to be highest in aqueous stem extract with IC_{50}value of 259.69 and 2.86µg/ml respectively while least IC_{50} value was observed in aqueous leaf extract i.e. 1153.29 µg/ml and 9.81 µg/ml by protein inhibitory method and protein albumin method respectively.
Conclusion: The result of the present study support the anti-inflammatory activity of plant *Hamelia patens*. Moreover, the potential anti-inflammatory effect of plants could be enhanced by extracting with methanol instead of water. Although plant proves its role in inflammation and can be used to reduce inflammatory injury and tissue damage but further detailed investigation need to be undertaken by following *in-vivo* study and then proper isolation and characterization techniques to identify responsible chemical constituent for anti-inflammatory activity.

Key words: *Hamelia patens*, Methanolic and Aqueous Extract, Anti-Inflammatory Activity, HRBC Membrane Stabilization Method, Protein (Trypisn) Inhibitory Method and Protein (Albumin) Denaturation Method.

INTRODUCTION

Inflammation can be defined as a reaction of living tissues towards injury and it comprises systemic and local responses in which part of the body becomes reddened, swollen and often painful (Nagaharika *et al*., 2013). When there is injury to any part of the human body, chemicals from the body's white blood cells are released into the blood or affected tissues to protect body from foreign substances. Vasoactive chemicals also increases the permeability (increase pore size) of arterioles which allows blood cells, chemical substance, blood proteins and fluid to accumulate in the injured region. This fluid accumulation results in swelling and may compress the nerves which causes pain (Apu *et al*., 2012). To reduce the inflammation processes involved with injury, substances possesses anti-inflammatory activity are used. Anti-inflammatory activity can be defined as the property of a substance that helps in the reduction of inflammation and associated symptoms. About half of analgesics are used as anti-inflammatory drugs that reduce pain by reducing inflammation.

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To date, pharmacotherapy of inflammatory conditions are based mainly on the use of non-steroidal anti-inflammatory drugs (NSAIDs), steroids and more recently tumor necrosis factor alpha (TNFα) inhibitors. Non-steroidal anti-inflammatory agents may act via single or combination of mechanism involving inhibition of arachidonic acid metabolism, inhibition of cyclo-oxygenase (COX)/inhibition of the prostaglandin (PG) synthesis, inhibition of lipoxygenase (LOX), inhibition of cytokines (IL, TNF, etc.), release of steroidal hormones from the adrenals, stabilization of lysosomal membrane, uncoupling of oxidative phosphorylation etc. In the same manner most of natural acting anti-inflammatory substances works (Kohli and Ali, 2005). Despite of effective treatment for inflammation with NSAIDs, the prolonged use of NSAIDs can cause serious gastrointestinal toxicity. Some studies claimed that NSAIDs have also been found to increase blood pressure, risk of congestive heart failure and occurrence of thrombosis. These findings illustrate the need to develop novel and safe anti-inflammatory medicines. Therefore, there is a worldwide search for new anti-inflammatory drugs as an alternative to NSAIDs. There are a number of herbs that possesses anti-inflammatory property that could help to achieve similar results as NSAIDs against

inflammation without causing harmful side effect (Low *et al*., 2015). The plant *Hamelia patens* belongs to family Rubiaceae, which is rich in active phytochemicals like flavonoids and alkaloids. The family Rubiaceae consists of several important medicinal plants with wide range of pharmacological and biological activities. Although *Hamelia paten* was widely used in ethno medicine for the treatment of inflammatory and related disorders, its anti□inflammatory properties have not yet been pharmacologically and systematically evaluated. Hence, the present study was undertaken to evaluate the anti-inflammatory activity of methanolic and aqueous leaf and stem extracts of plant *Hamelia patens* by *in-vitro* methods to explore its bioefficiency.

MATERIALS AND METHODS

Preparation of extracts

Air dried coarsely powdered leaves (400g) and stems (400g) of *Hamelia patens* were extracted with methanol and aqueous water separately by continuous hot percolation using soxhlet apparatus. It was continued for 48 hour until the whole drug was exhausted. Extracts was then concentrated using distillation unit followed by lyophilization. The dried extracts obtained after lyophilization were covered with paraffin foil and stored in vacuum desiccator at room temperature.

In-vitro anti-Inflammatory Activity

Anti-inflammatory activity was determined by the following three methods:

- HRBC Membrane Stabilization Method
- Protein (trypsin) Inhibitory Method
- Protein (albumin) Denaturation Method

HRBC Membrane Stabilization Method (Das *et al*., 2014; Oyedepo *et al*., 1995; Sakat *et al*., 2010)

Preparation of HRBC suspension

About 4 ml of venous blood was collected from healthy volunteer and mixed with equal volume of freshly prepared Alsever's solution and centrifuged at 3000 rpm, the packed cells obtained after centrifugation were washed with iso-saline three times, followed by the collection of supernatant after last wash and a 10% v/v suspension of collected supernatant was made with iso-saline.

Preparation of assay mixture

The assay mixture contains 1 ml of phosphate buffer (pH 7.4, 0.15M), 2ml hypo-saline (0.36%), 0.5ml HRBC suspension (10% v/v) and 0.5 ml of various extracts and standard (Diclofenac) of different concentration (100, 200, 400, 600, 800, 1000, 2000 µg/ml) prepared in methanol. The solution was incubated at 56°C for 30 minutes (min.). The tubes were then cooled under running tap water for 30 min. After that they were centrifuged and supernatant liquid was separated. Absorbance of collected supernatant liquid was measured at 560 nm by UV spectrophotometer.

Membrane stabilizing activity in Percentage (%) Inhibition was calculated by the following formula

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

From the curve plotted against concentration and % inhibition, IC_{50} value of extracts were calculated and compared with IC_{50} value of standard.

Protein (Trypsin) Inhibitory Method (Govindappa *et al*., 2011; Mizushima *et al*., 1968; Chandra *et al*., 2012)

The reaction mixture (2 ml) contained 0.06 mg trypsin, 1 ml of 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample (extracts)/standard (Diclofenac) of different concentrations (20, 40, 60, 80, 100, 200 µg/ml). The reaction mixture was incubated at 37°C for 5 min. and then 1ml of 0.8% (W/V) casein (prepared using sodium hydroxide) was added. The mixture was incubated for an additional 20 min., and then 2 ml of 70% perchloric acid was added to terminate the reaction. Cloudy suspension formed was centrifuged and the absorbance of the supernatant was taken at 210 nm against buffer as blank. The experiment was performed in triplicates.

The percentage (%) of inhibition of protein inhibitory activity was calculated from the following formula

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Protein (Albumin) Denaturation Method (Akhtar *et al*., 2012; Rauhaa *et al*., 2003)

To 1ml of different concentration (20, 40, 60, 80, 100, 200 µg/ml) of extracts and standard (Disprin), 1ml of 1% bovine albumin was added. The mixture solution was incubated at 27˚C for 15 minutes. Then the reaction mixture was heated at 70°C in water bath or hot air oven for 10 min., after cooling absorbance of reaction mixture was observed at 660 nm using distilled water as a blank.

Control: 1ml of 1% albumin + 1ml methanol.

$$\% \text{ Inhibition} = \frac{\text{Absorbance of test} - \text{Absorbance of control}}{\text{Absorbance of control}} \times 100$$

From the curve plotted against concentration and % inhibition, IC_{50} value of extracts were calculated and compared with IC_{50} value of standard.

RESULTS AND DISCUSSION

HRBC Membrane Stabilization Method

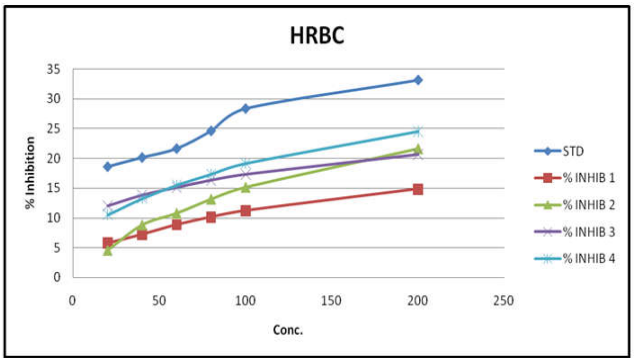
The HRBC membrane stabilization assay is used to study the *in-vitro* anti-inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extracts may well stabilize lysosomal membranes.

Table No. 1 Percentage Inhibition of Standard (Diclofenac) and extracts

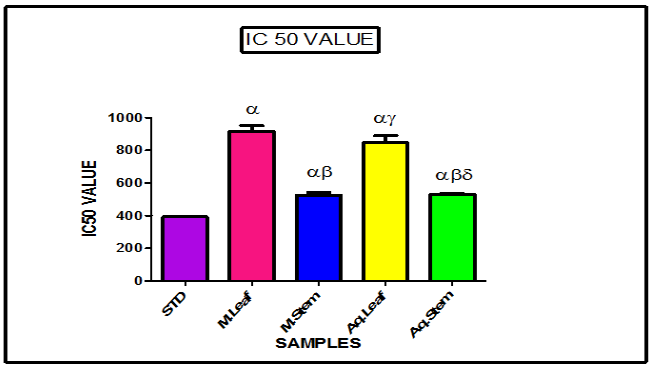
S. No.	Concentration (µg/ml)	% Inhibition (Mean ± SD)				
		Standard	M. Leaf	M. Stem	Aq. Leaf	Aq. Stem
1.	20	18.62 ± 0.49	5.746 ± 0.610	4.580 ± 0.504	12.046 ± 0.180	10.506 ± 0.362
2.	40	19.76 ± 0.54	7.223 ± 0.387	8.843 ± 0.448	13.846 ± 0.640	13.263 ± 0.400
3.	60	21.153 ± 0.32	8.856 ± 0.281	10.803 ± 0.446	15.136 ± 0.733	15.520 ± 0.610
4.	80	23.996 ± 0.17	10.146 ± 0.144	13.183 ± 0.511	16.366 ± 0.470	17.366 ± 0.510
5.	100	26.116 ± 0.22	11.220 ± 0.399	15.176 ± 0.883	17.340 ± 0.949	19.186 ± 0.558
6.	200	32.846 ± 0.30	14.856 ± 0.349	21.676 ± 0.555	20.673 ± 0.508	24.563 ± 0.398
IC_{50} Value (µg/ml)		408.067±1.11	916.126 ± 62.30	508.28 ± 11.86	847.263±74.33	529.506±13.709

Table No. 2 IC_{50} Value of Standard and Extracts (Graph 2)

S. No.	Standard/Extracts	IC_{50} Value (µg/ml)
1.	Diclofenac	408.067±1.11
2.	Methanolic Leaf	$916.126\pm62.305^{\alpha}$
3.	Methanolic Stem	$508.28\pm11.86^{\alpha\beta}$
4.	Aqueous Leaf	$847.263\pm74.33^{\alpha\gamma}$
5.	Aqueous Stem	$529.506\pm13.709^{\alpha\beta\delta}$



Graph 1. Percentage Inhibition vs. Concentration of Standard and Extracts



All values are Mean ± SD, $^{\alpha}p<0.05$ with STD, $^{\beta}p<0.05$ with M. leaf $^{\gamma}p<0.05$ with M. stem, $^{\delta}p<0.05$ with Aq. leaf.

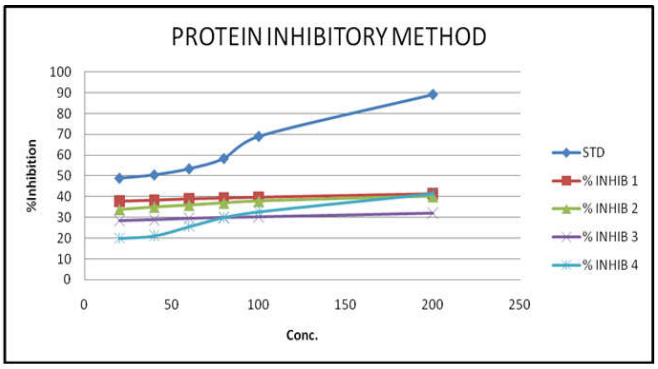
Graph 2. IC_{50} Values of Standard & Extracts by HRBC Method

Table No. 3 Percentage Inhibition of Standard (Diclofenac) and extracts

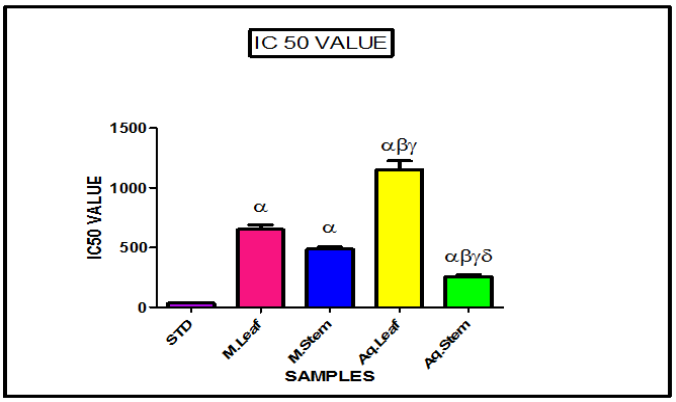
S. No.	Concentration (µg/ml)	% Inhibition (Mean ± SD)				
		Standard	M. Leaf	M. Stem	Aq. Leaf	Aq. Stem
1.	20	48.916 ± 0.292	37.667 ± 0.146	33.866 ± 0.255	28.52 ± 0.421	19.966 ± 0.935
2.	40	50.526 ± 0.292	38.183 ± 0.247	35.060 ± 0.108	29.02 ± 0.425	21.156 ± 0.635
3.	60	53.460 ± 0.364	38.870 ± 0.295	35.946 ± 0.060	29.616 ± 0.270	25.623 ± 0.671
4.	80	58.413 ± 0.162	39.223 ± 0.100	36.993 ± 0.156	30.06 ± 0.206	30.043 ± 1.130
5.	100	69.036 ± 0.390	39.646 ± 0.223	37.886 ± 0.123	30.340 ± 0.291	32.790 ± 1.613
6.	200	89.176 ± 0.336	41.360 ± 0.250	40.163 ± 0.325	32.110 ± 0.137	41.556 ± 1.536
IC_{50} Value (µg/ml)		34.590±1.090	651.343 ± 69.212	487.093 ± 31.843	1153.29 ± 128.1	259.69 ± 20.92

Table No. 4 IC_{50} Value of Standard and Extracts (Graph 4)

S. No.	Standard/Extracts	IC_{50} Value(µg/ml)
1.	Diclofenac	34.590±1.090
2.	Methanolic Leaf	$651.343±69.212^{\alpha}$
3.	Methanolic Stem	$487.093±31.843^{\alpha}$
4.	Aqueous Leaf	$1153.29±128.103^{\alpha\beta\gamma}$
5.	Aqueous Stem	$259.69±20.92^{\alpha\beta\gamma\delta}$



Graph 3. Percentage Inhibition vs. Concentration of Standard and Extracts



All values are Mean ± SD, $^{\alpha}p<0.05$ with STD, $^{\beta}p<0.05$ with M. leaf, $^{\gamma}p<0.05$ with M. stem, $^{\delta}p<0.05$ with Aq. leaf.

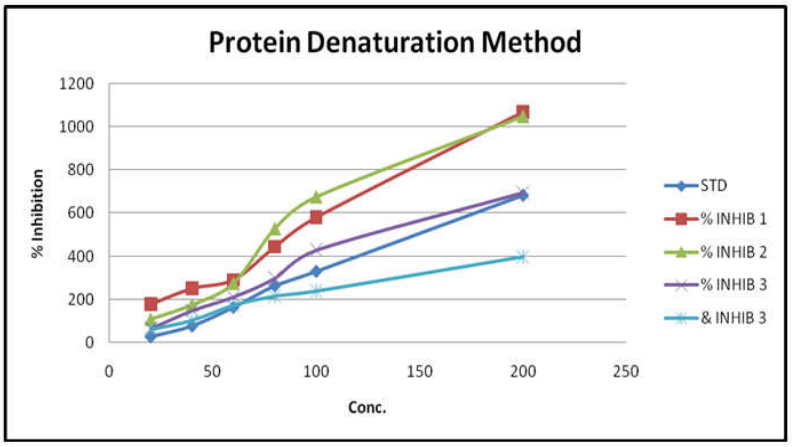
Graph 4: IC_{50} Values of Standard & Extracts by Protein Inhibitory Method

Table No. 5 Percentage Inhibition of Standard (Acetylsalicylic acid) and extracts

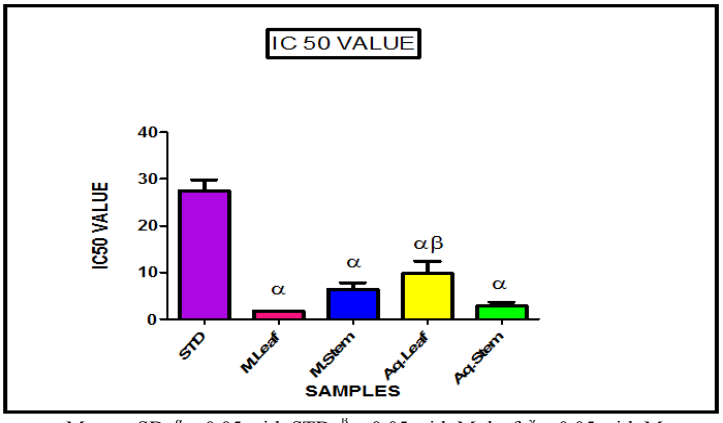
S. No.	Concentration (µg/ml)	% Inhibition (Mean ± SD)				
		Standard	M. Leaf	M. Stem	Aq. Leaf	Aq. Stem
1.	20	26.666 ± 15.275	176.66 ± 15.27	110 ± 10	66.666 ± 15.275	60 ± 10
2.	40	76.666 ± 15.275	250 ± 40	176.66 ± 15.27	150 ± 10	103.33 ± 5.773
3.	60	163.333 ± 28.867	290 ± 43.58	276.66 ± 25.16	213.33 ± 25.166	173.33 ± 15.275
4.	80	263.333 ± 11.547	443.33 ± 41.63	530 ± 20	300 ± 20	216.66 ± 15.275
5.	100	330 ± 26.457	580 ± 30	676.66 ± 35.118	430 ± 20	240 ± 10
6.	200	683.333 ± 30.550	1070 ± 72.11	1050 ± 30	696.66 ± 15.275	400 ± 10
IC_{50} Value (µg/ml)		27.513 ± 4.071	1.72 ± 0.13	6.38 ± 2.485	9.813 ± 4.501	2.863 ± 1.384

Table No. 6 IC_{50} Values of Standard and Extracts (Graph 6)

S. No.	Standard/Extracts	IC_{50} Value (µg/ml)
1.	Acetylsalicylic acid	27.513±4.071
2.	Methanolic Leaf	$1.72±0.13^{\alpha}$
3.	Methanolic Stem	$6.38±2.485^{\alpha}$
4.	Aqueous Leaf	$9.813±4.501^{\alpha\beta}$
5.	Aqueous Stem	$2.863±1.384^{\alpha}$



Graph 5. Percentage Inhibition vs. Concentration of Standard and Extracts



All values are Mean ± SD, $^{\alpha}p<0.05$ with STD, $^{\beta}p<0.05$ with M. leaf, $^{\gamma}p<0.05$ with M. stem.

Graph 6. IC_{50} values of Standard & Extracts by Protein Denaturation Method

Table No. 7 Comparison of IC_{50} Values Standard and Extracts

Standard and Extracts	Different methods used for Anti-Inflammatory Activity Determination		
	IC_{50} Value (µg/ml)		
	HRBC	Protein Inhibitory Method	Protein Denaturation Method
Standard	408.067 ± 1.11	34.590 ± 1.090	27.513 ± 4.071
M. Leaf	916.126 ± 62.305	651.343 ± 69.212	7.38 ± 2.485
M. Stem	508.28 ± 11.86	487.093 ± 31.843	6.38 ± 2.485
Aq. Leaf	847.263 ± 74.33	1153.29 ± 128.103	9.813 ± 4.501
Aq. Stem	529.506 ± 13.709	259.69 ± 20.92	2.863 ± 1.384

Stabilization of lysosomal is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage upon extra cellular release (Leelaprakash and Dass, 2011). The different extracts of *Hamelia patens* had showed significant membrane stabilizing activity which was comparable to the standard diclofenac (Table No. 2). The different extracts of *Hamelia patens* were effective in inhibiting the heat induced hemolysis of erythrocyte membrane and its effectiveness was dose-dependent. Methanolic stem extract showed minimum IC_{50} value of 508.28 µg/ml while the methanolic leaves extract showed the maximum IC_{50} value of 916 µg/ml in comparison to standard.

PROTEIN INHIBITORY METHOD

Neutrophils are known to be a rich source of serine proteinase and are localized at lysosomes. It was previously reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors.[13] The extracts of *Hamelia patens* exhibited significant anti-proteinase activity (Table No. 4). Aqueous stem extract showed the minimum IC_{50} Value of 259.69 µg/ml thus exhibited highest protein inhibitory activity while Aq. leaf extract showed the maximum IC_{50} Value of 1153.29µg/ml in comparison to standard Diclofenac.

PROTIEN (ALBUMIN) DENATURATION ASSAY

Most biological proteins disoriented their biological function when denatured. The different extracts of *Hamelia patens* had significant Protein Denaturation Inhibition Activity which was comparable to the standard acetylsalicylic acid (Table No. 6).The aqueous extract exhibited highest activity in inhibiting heat induced albumin denaturation expressed as the lowest amount of sample (µg/ml) needed for 50% inhibition (IC_{50}) i.e. 2.86 µg/ml, while aqueous leaves extract showed maximum IC_{50} value of 9.81µg/ml. The above table showing the correlation between IC_{50}values of standard and different extracts obtained by three different methods. There was no positive correlation seen between different IC_{50} values obtained by the different methods. But a positive correlation was seen between IC_{50} values obtained by Protein Inhibitory Method and Protein Denaturation Method. This difference might be due to the different mechanism of action followed by the assays to show anti-inflammatory activity. i.e. HRBC method based on membrane stabilisation and as using this method highest IC_{50} value was shown in methanolic stem extract thus it can be concluded that constituents present in methanolic stem extracts may have good potential to stabilize lysosomal membrane. Similarly constituents present in aqueous stem extract might be having good anti- protein denaturation activity and proteinase inhibition activity.

Conclusion

Hamelia patens methanolic leaf and stem extracts showed anti-inflammatory activity by all three mentioned methods. Among all the extracts tested the methanolic extract showed significant anti-inflammatory activity in a concentration dependent manner. This effect may be due to influence on inflammatory mediators and also on pathway of prostaglandins synthesis which may be due to the presence of Alkaloids, flavonoids, tannins, polyphenolic compounds and other secondary metabolites present in plant extract. Anti-inflammatory activity by HRBC membrane stabilization method was found to be best in methanolic stem extract with IC_{50} value of 508.28 µg/ml and least in methanolic leaf extract with IC_{50} value of 916.12µg/ml, anti-inflammatory activity by protein (trypsin) inhibitory method and protein (albumin) denaturation method was found to be highest in aqueous stem extract with IC_{50} value of 259.69 and 2.86 µg/ml respectively while least IC_{50} value was found in aqueous leaf extract i.e. 1153.29 µg/ml and 9.81 µg/ml respectively. Table 7 showed the correlation between IC50 values of standard and different extracts obtained by three different methods. There was no positive correlation seen between different IC50 values obtained by the different methods. But a positive correlation was seen between IC50 values obtained by Protein Inhibitory Method and Protein Denaturation Method. This difference might be due to the different mechanism of action followed by the assays to show anti-inflammatory activity. i.e. HRBC method based on membrane stabilisation and as using this method highest IC50 value was shown in methanolic stem extract thus it can be concluded that constituents present in methanolic stem extracts may have good potential to stabilize lysosomal membrane. Similarly constituents present in aqueous stem extract might be having good anti- protein denaturation activity and proteinase inhibition activity. However further investigational study is still required to study *in-vivo* activity and then further isolate and identify the chemical compounds and mechanism responsible for the pharmacological activities. A well characterized standardized *H.patens* extracts present an excellent opportunity to further investigate the advantages and disadvantages of the herbal vs. synthetic approach in the treatment of inflammatory conditions.

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Disclosure

The author declares that they have no competing interests.

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