Title: Phytochemical Screening and Investigation of the Central and Peripheral Analgesic and Anti-Inflammatory activity of ethanol extract of Hiptage Benghalensis (L) Kurz

Shehla Unaiza Hridi[1]*, Nafisa Ferdous[1] , MD.Fakhar Uddin Majumder[1], Dr.JMA Hannan[1]

[1] Department of Pharmacy, North South University, Plot -15, Block- B, Bashundhara R/A, Dhaka, Bangladesh .

7 ***Authors and Affiliation***

A. Shehla .U. Hridi* (Student, Research worker and Editor)
9 Department of Pharmacy, North South University,
10 Plot - 15, Block- B, Bashundhara
R/A, Dhaka, Bangladesh.

B.Nafisa Ferdous (Student, Research worker and Co-editor)
12 Department of Pharmacy, North South University,
Plot -15, Block- B, Bashundhara R/A, Dhaka, Bangladesh.

C.MD.Fakhar Uddin Majumder (Lab and Research Assistant)
15 Department of Pharmacy, North South University,
15 Plot -15, Block- B, Bashundhara R/A, Dhaka, Bangladesh.

D.Dr.JMA Hannan (Research Supervisor)
Department of Pharmacy, North South University, Plot -15, BlockB,
Bashundhara R/A, Dhaka, Bangladesh.

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ABSTRACT

Aims: Hiptage Benghalensis is used in the traditional system of medicine. The leaf is considered one of the important plant organs for the treatment of various diseases such as rheumatism, leprosy, wounds, ulcer, burning sensation, scabies, inflammation and cough. Hence, the present studies were carried out to evaluate chemical constituents and possible pharmacological activities of the leaf.

Study Design: Our present studies were focused on the assessment of probable analgesic and anti-inflammatory activities of ethanol extract of Hiptage Benghalensis in laboratory animals and the statistical significance of such effects.

Place and Duration of Study: The experiments were carried out in Pharmacology lab of Department of Pharmacy North South University Dhaka, Bangladesh during the period of June 2012-February 2013.

Methodology: Carrageenan induced Hind Paw Edema test in Long Evans rat was the experiment for anti-inflammatory activity of the ethanol extract of Hiptage Benghalensis while Hot Plate test and Acetic Acid induced Writhing method were was carried out to assess its

* Tel.: +88001741595373
E-mail Shehla_hridi89@hotmail.com.

analgesic activity in Swiss albino mice. At two different doses of the leaf extract, 250 and 500 mg/kg body weight, the analgesic test was evaluated on mice and the anti-inflammatory test was evaluated on rats.

Result: Phytochemical analysis of ethanol extract of Hiptage Benghalensis has indicated the presence of steroid, carbohydrate, flavonoid, alkaloid, tannin, phenol and, mangiferin and terpenoids-compounds.

The ethanol extract of Hiptage Benghalensis exhibited statistically significant ($p<0.05$) anti-inflammatory effect in Carrageenan induced Hind Paw Edema in Long Evans rats and incited significant analgesic response to Hot plate and acetic acid induced writhing in Swiss albino mice.

Conclusion: In conclusion, these observations provide evidence and possible mechanisms of action for the anti-inflammatory and analgesic properties of leaf of Hiptage Benghalensis claimed in Ayurveda medicine.

Keywords: Analgesic, Anti-inflammatory, Carrageenan, Hiptage Benghalensis, Phytochemical

1. INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many of which based on their use in traditional medicine. It has been noted that the original source of many important pharmaceuticals currently in use have been plants used by indigenous people [1]. Herbal medicine or phytomedicine refers to the use of any plant's seeds, berries, roots, leaves, bark, or flowers for medicinal purposes [2]. In this paper, we analyzed the analgesic and anti-inflammatory property of leaves of *H.Benghalensis*.

Pain has been defined by The International Association for the Study of Pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage [3].This process enables an individual to take protective measures, by providing with rapid awareness about threatening or potentially threatening injury [4].However, if the painful sensation remains after removal of the detectable stimulus, it calls for a regimen for pain management [5].

Hiptage Benghalensis (L) Kurz belongs to the family Malphigiaceae. The plant has strong therapeutic potential thus occasionally cultivated for medicinal purposes in the alternative medicine practice *Ayurveda*. The leaves of *H.benghalensis (L.)* Kurz are used in treating skin diseases in Burma and the bark is used to heal wounds in Indonesia. In India, *H.benghalensis (L.) Kurz* is widely used to treat cough, asthma, leprosy and also to quench thirst. According to some researches the therapeutic actions of this plant may be due to the presence of mangiferin, which is known to be anti-inflammatory, hepatoprotective, antioxidant, and antimicrobial.

2. MATERIAL AND METHODS

Hot Plate (Model – 35100, UGO BASILE, ITALY),Electronic Balance (Ohous manufacturer, Canada), Refrigerator (Butterfly Marketing Ltd,LG), Rotary evaporator (Eyela n 1000, Tokyo Rikaki Kai Co.Ltd, Rotary vacuum, Japan),Beakers, Petri dishes & glass wrought, Safety rat handling gloves, Mortar & pestle., Hypodermic Syringes, Holder & test tube, Plethysmometer (Ugo Basline SLR model-7140,Italy)

2.1 Medicinal plants (extracts)

Ethanol extract of *H.Benghalensis* were examined in two concentrations of 500mg/kg and 250mg/kg body weight of animal.

2.2. Control & Positive Control

2.2.1. Analgesic activity

1. Control – distilled water

2. Positive control – Diclofenac sodium (Beximco Pharma, Bangladesh)

Administered dose – 50mg/kg body weight animal

2.2.2. Anti-inflammatory activity

1. Control –Distilled water

2. Positive control – Diclofenac sodium

Administered dose – 50mg/kg body weight animal

2.3. Experimental animal

Swiss albino mice (male and female), weighing 20-30g bred in International Centre for Diarrheal Diseases and Research, Bangladesh(ICDDR,B) and grown in the Animal House of the Department of Pharmacy, North South University (NSU). Long Evans rats (male and female), weighing 100-170g of either sex, bred in NSU and ICDDR, B and grown in the animal house of the Department of Pharmacy NSU. All the animals were acclimatized one week prior to the experiments .The animals were housed under standard laboratory conditions (relative humidity 55-65%, room temperature 25.0± 2 ^{o}C, and 12 hours light dark cycle). The animals were fed with standard diet from ICDDR, B and had free access to filtered water [6, 7].

2.4. Plant Extraction method

2.4.1. Collection

The plant sample of *Hiptage Benghalensis* was collected from Ayurvedic Institution 'Back to Nature' on 18.06.2012 in the form of leaf shavings. The leaves of the plant were collected and washed with water several times.

2.4.2. Drying and grinding

The collected plant leaves were washed with water to rinse away undesirable materials and plant parts. The cleansed leaves were then partially dried by fan aeration and then fully dried in the oven at below 40°C for 2 days. The fully dried leaves were then grinded to a powdered form and stored in the refrigerator at +4°C for a few days.

2.4.3. Cold extraction (Ethanol extraction)

103gram of powered material were placed in a clean, flat bottomed glass container and soaked in 500 ml of 80%ethanol. The container was then sealed and subjected to occasional shaking and stirring for a period of 2 days. After 48 hours the homogenous mixture was filtered first by cotton material and twice through whatman filter paper to obtain a finer filtrate. The filtrate (Ethanol extract) obtained was evaporated by Rotary evaporator at 4 to 5 rpm and at 65ºc temperature. The separated filtrate was found to be a precipitate of dark green color and the gummy concentrate was designated as the crude ethanol extract of the leaves of *Hiptage Benghalensis*. It was then dried in the freeze drier and preserved at +4°C for two weeks.

2.5. Phytochemical Analysis

2.5.1. Study Design

Qualitative phytochemical tests for the identification of alkaloids, flavonoids, steroids, gum and carbohydrates, reducing sugar, saponins, tannin and terpenoids were carried out for the plant extract by the method described by Harborne and Sazada [*8,9*] .The freshly prepared extract of *Hiptage Benghalensis* was qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extract was performed using the following reagents and chemicals: Alkaloids with Wagner reagent, flavonoids with the use of concentrated HCl, tannins with 0.1% ferric chloride, and saponins with ability to produce suds. Gum was tested using Molish reagents and concentrated sulfuric acid, steroids with sulfuric acid, reducing sugar with the use ά-napthol and sulfuric acid and terpenoids with chloroform and conc. HCl.

2.6. Analgesic activity of Hiptage Benghalensis

2.6.1. Study design

Experimental animals were randomly selected and divided into four groups denoted as group-I, group-II, group-III, group-IV consisting of 6 mice in each group individual weighing was done to adjust individual doses. Here, distilled water was given to group-I, 50 mg/kg Diclofenac sodium was given to group II, and 250 mg/kg and 500mg/kg of the crude extract of *Hiptage Benghalensis* were administered to group III and group IV respectively.

2.6.2. Mice Screening

Young Swiss-albino mice aged 4-5 weeks, average weight 25-30 gram were used for this study. They were kept in standard environmental condition for one week in the animal house of the Department of Pharmacy, North south University, Bangladesh for adaptation after their purchase. The animals were provided with standard laboratory food and tap water *ad libitum* and maintained at natural day night cycle. After one week of adaptation and grooming, mice screening was performed prior to Hot plate test. In that experiment mice with significant response action (Licking, Shaking and Jumping) and response time (at the range of 0-20 seconds) were selected.

2.6.3. Hot plate test method

The Hot plate test was performed on the test subjects in a slightly modified version from the one described earlier [10]. Mice were divided into four groups consisting of six animals in each group. The mice of each group were placed in the Hot Plate apparatus maintained at a temperature of 55° ± 0.2°C for a maximum time of 20 second per exposure in order to obtain its response to electrical heat induced pain stimulus but at the same time to prevent blister formation and skin damage which might affect the result. Licking of the paws or jumping out of the beaker was taken as an indicator of the animal's response to heat-induced pain stimulus. The time for each mouse to lick its paws or jump out of the beaker was taken as reaction time (S). Before treatment, the reaction time was taken once. The mean of this one determination constituted initial reaction time before treatment of each group mice. Each of the test group were thereafter treated with Distilled Water (50mg/kg of body wt), Diclofenac Sodium (50 mg/kg of body wt) and ethanol extract at the doses of *H.Benghalensis* 250 mg/kg and 500 mg/kg body wt. orally. Reaction time was recorded as *latency period*, when the animals licks their hind and fore paws and jumped at 0, 30, 60,120,180 and 240 minutes after the treatment..

Percent analgesic score was calculated as:

(1)(PAS) = Tb-Ta/Tb × 100

Where, Tb= Reaction time (in second) before drug administration

Ta = Reaction time (in seconds) after drug administration

2.6.4. Acetic acid induced writhing test in mice

The analgesic activity of the samples was evaluated using acetic acid induced writhing method in mice. In this method, acetic acid is administered intra-peritonially to the experimental animals to create pain sensation. As a positive control, any standard NSAID drug can be used. In the present study as a positive control Diclofenac sodium was used to serve the purpose of standard NSAID. The ethanol extract of *H.Benghalensis* was administered orally in two different doses (250 and 500 mg/kg body weight) to the Swiss Albino mice after an overnight fast. Test samples and vehicle were administered orally 30 minutes prior to intra-peritoneal administration of 0.7% v/v acetic acid solution (0.1ml/10g) but Diclofenac sodium was administered 15 minutes prior to acetic acid injection. Animals were kept individually in glass beaker for observation. Each mouse of all groups were observed individually for counting the number of writhing they made in 15 minutes commencing just 5 minutes after the intra peritoneal administration of acetic acid solution. Full writhing was not always accomplished by the animal, because sometimes the animals started to give writhing but they did not complete it. This incomplete writhing was considered as half-writhing. Accordingly, two half-writhing were taken as one full writhing. The number of writhes in each treated group was compared to that of a control group while Diclofenac sodium (50 mg/kg) was used as a reference substance (positive control).

2.7. Anti-inflammatory Effect of *Hiptage Benghalensis*

2.7.1. Preparation of inflammatory agent

Preparation of inflammatory agent Carrageenan was used as inflammatory agent in this experiment. It was obtained from Jahangirnagar University. Carrageenan powder was suspended in 5 ml saline to make 0.1% suspension and kept in water bath for proper homogenization. The tube was kept in a beaker containing hot water (50±2°c) to prevent transformation into a jelly like compound.

2.7.2. Carrageenan-induced Rat Hind Paw Edema test

The ethanol extract of *Hiptage Benghalensis* on carrageenan induced inflammation in rat paw was investigated by following the method described previously.[11] Rats were randomly divided into four groups, each consisting of six animals, (weighing 150-200 gram) of which Group I was kept as control giving only water. Group II was given carrageenan as inflammatory agent. Group III and Group IV were given the test sample at the dose of 250 and 500 mg/kg body weight respectively. Half an hour after oral administration of the test materials, 0.1ml 0.1% carrageenan suspension was injected subcutaneously in left hind paw of each animal leading to the formation of edema *in situ* (localized inflammation). The volume of paw edema was measured at 1, 2, 3, 6, and 8 hours using water Plethysmometer after administration of carrageenan. The right hind paw served as a reference non inflamed paw for comparison. The average percent increase in paw volume with time was calculated and compared against the control group. Percent inhibition was calculated using the formula:

(2)% Inhibition of paw edema = [1- (Vt / Vc)] X 100

Where Vc and Vt represent average paw volume of control and treated animal respectively

2.8 Statistical analysis

All the results were expressed as Mean ± Standard deviation (SD). Data was analyzed using one-way ANOVA followed by Dunnett's t-test.The results obtained were compared with the vehicle control group. The P values P<0.05, P< 0.01 and P< 0.001 were considered as statistically significant. The confidence interval is 95%.All the statistical tests were carried out using SPSS statistical software.

3. RESULTS AND DISCUSSION

3.1. Phytochemical screening

Phytochemical screening of the ethanol extract of H.Benghalensis leaf and stem revealed the presence of various bioactive components such as tannins, flavonoids, saponins, gums, steroids, alkaloids, reducing sugar and terpenoids [12]. The result of phytochemical test has been summarized in the table below-

Table 1: Result of Phytochemical Screening of Plant Extract

Hiptage Benghalensis Extract	*Leaf*						
	Tannins	*Saponins*	*Flavinoids*	*Gums& Carbohydrates*	*Alkaloids*	*Reducing Sugars*	*Terpenoids*
80% ethanol	+++	++	++	+++	+++	+++	+++

Symbol (+) indicates presence of phytochemicals.

3.2. Analgesic activity

Table 2: Analgesic effect of the ethanol extract of *H.Benghalensis* using the hot –plate method. Statistical evaluation of the results shown in table:

Treatment Group	Dose	Latency Period(s)					
		0 min	**30min**	**1h**	**2h**	**3h**	**4h**
Control	------	10.70±.847	9.66±.937	8.00±.814	6.58±.641	5.52±.549	5.00±.443
Standard	50mg/kg	9.14± .524	11.02±1.00	12.60±.945	14.16±1.076***	15.96±.676***	12.48±.698***
H.Benghalensis	250mg/kg	7.68±.851	9.28±1.09	10.32±1.12**	11.28±1.07**	12.54±.912***	10.18±.747***
H.Benghalensis	500mg/kg	7.65±.312	9.22±.285	10.34±.273	11.72±.233**	12.68±.177***	10.19±.163***

Values in the results are expressed as mean ± SEM., Data was analyzed using one-way ANOVA followed by Dunnett's t-test.The results obtained were compared with the vehicle control group. The P values *P<0.05, **P< 0.01 and ***P< 0.001 were considered as statistically significant.

Table3: Percent inhibition of the standard and two different concentrations of the extract compared with their respective means at 0 hour

Treatment group	*Dose*	*% Inhibition*				
		30min	**1h**	**2h**	**3h**	**4h**
Standard	50mg/kg	20.56	37.00	54.90	74.61	36.54
H.Benghalensis	250mg/kg	20.83	34.37	46.87	63.28	32.55
H.Benghalensis	500mg/kg	20.52	35.16	53.20	67.75	33.20

3.2.1. Effect of plant extract on Hot-Plate test

Table 2 and 3 shows the analgesic effect of the ethanol extract of *H.Benghalensis* using the hot –plate method and percent inhibition of the standard and two different concentrations of the extract compared with their respective means at 0 hour. The ethanol extract of *H.Benghalensis* exhibited statistically significant ($p > 0.05$) analgesic effect in hot plate test of white albino mice. This was determined by analyzing data using one way ANOVA followed by Dunnet's post hoc test. However, the data shows that the dose dependent effect reached 67.75% at 180 minutes and 63.28% at the 180 minutes at the doses of 500 and 250 mg/kg-body weight respectively.

Table 4 :Result of Analgesic Activity of Hiptage Benghalensis in Acetic Acid Method
Statistical evaluation of the results shown in table:

Values are expressed as Mean±S.E.M. Differences between groups are determined by One-Way ANOVA followed by post hoc Dunnet test.

Treatment	**Dose**	***Total Writhing Counts***					***Mean± SEM***	***% Inhibition***
Control	-----	23	27	29	47	32	31.6000±4.11825	
Standard	50mg/kg	14	18	16	17	13	15.6000±.92736***	50.00%
H.Benghalensis	*250mg/kg*	19	19	21	21	18	21.4000±1.96469***	37.66%
H.Benghalensis	*500mg/kg*	16	18	7	11	18	14.0000±2.16795***	55.69%

*p<0.05 ,**p<0.01 and ***P< 0.001 compared to the control treated group.

3.2.2 Effect of plant extrat on Acetic Acid Writhing Test

Table 4 shows the effects of the extracts of *H.Benghalensis* on acetic acid induced writhing in mice. Both doses of the plant extract showed significant reduction ($p<0.05$) of writhing induced by the acetic acid after oral administration in a dose dependent manner. After oral administration of two different doses- 250 and 500 mg/kg body weight, the percent inhibition was 37.66% & 55.69% *respectively*.

3.3. Anti-inflammatory Activity

Table 5: **Anti-inflammatory effect of ethanol extract of *Hiptage Benghalensis on* carrageenan induced rat paw inflammation.**

Treatment Group	*Dose*	*Volume of Paw(ml)*					
		0 min	**1 h**	**2h**	**3 h**	**6 h**	**8 h**
Control	----	.71±.055	.88±.077	1.18±.007	1.46±.063	1.55±.066	1.62±.065
Standard	50mg/kg	.65±.039	.85±.058	.99±.036	1.24±.046	1.02±.028***	.79±.020***
H.Benghalensis	250mg/kg	.67±.057	.99±.101	1.19±.077	1.28±.054	1.09±.053***	.92±.032***
H.Benghalensis	500mg/kg	.68±.031	1.02±.081	1.21±.056	1.29±.131	1.08±.038***	.89±.027***

Values are expressed as Mean±S.E.M. Differences between groups are determined by One-Way ANOVA followed by post hoc Dunnet test. *p<0.05 ,**p<0.01 and ***P< 0.001 compared to the control treated group.

Table 6: Percent inhibition of the standard and two different concentrations of the extract compared with their respective means at 0 hour

Treatment	**Dose**	**%inhibition**				
		1 h	**2 h**	**3 h**	**6 h**	**8 h**
Standard	50mg/kg	29.97	51.68	89.91	56.27	20.49
H.Benghalensis	250mg/kg	48.61	77.08	91.07	63.54	36.61
H.Benghalensis	500mg/kg	48.74	77.19	88.88	57.89	29.53

3.3.1. Effect of plant extract on Carrageenan-induced Hind Paw Edema

Table 5 and 6 shows the anti-inflammatory effect of ethanol extract of *Hiptage Benghalensis on* carrageenan induced rat paw inflammation and percent inhibition of the standard and two different concentrations of the extract compared with their respective means at 0 hour**.** The ethanol extract of *H.Benghalensis* exhibited statistically significant ($p<0.05$) anti-inflammatory activity in Carrageenan-induced Hind Paw Edema of rat. This was determined by analyzing data using one way ANOVA followed by Dunnet's test. In control animals, the sub plantar injection of carrageenan produced a local edema that increased progressively to reach a maximal intensity four hours after the injection of the phlogistic agent. Ethanol extract of *H.Benghalensis* showed a significant dose depended reduction at both 250 and 500mg/kg body weight. However significant inhibition of edema was found to be 63.54% and 57.89% at six hour of study at a dose of 250 and 500mg/kg body weight respectively. Further significant inhibition was to be 36.61% and 29.53% at eight hour of study at a dose of 250 and 500mg/kg body weight respectively.

4. DISCUSSION

It's a long and tedious process to isolate pure, pharmacologically active constitutes from plants. Thus, it is necessary to have methods available which eliminate unnecessary separation procedures. Chemical screening is thus performed to allow localization and targeted isolation of new or useful constituents with potential activities. This procedure enables recognition of known metabolites in extracts or at the earliest stages of separation and is thus economically very important [13,14].

Preliminary qualitative phytochemical screening of *H.Benghalensis* leaf extract exhibited the presence of Tannins, saponins, flavonoids, carbohydrates and gums, reducing sugars, alkaloids, and terpenoids. Therefore it is assumed that these compounds may be responsible for the observed analgesic activity.

The presence of flavonoids represents the possibility of some biological activity of the extracts of *H.Benghalensis*. Such as it can modify allergens, viruses, and carcinogens indicating flavonoids have potential to be biological "response modifiers". It can also be used as an anti-allergic, anti-inflammatory [15], anti-microbial and anti-cancer activities. Flavonoids were reported to have a role in analgesic activity primarily by targeting prostaglandins [16, 17]. Flavonoids and other phenolic compounds of plant origin have been reported as antioxidants and as scavengers of free radicals. Antioxidants can also exert anti-inflammatory effects [18].

The presence of tannins represents the possibility of some biological activity of the extracts of H.Benghalensis. Such as antidiarrheal, hemostatic, antihemorrhoidal, anti-inflammatory, astringent, anti-infective. It can be used for immediate relief of sore throats, diarrhea, dysentery, hemorrhaging, fatigue, skin ulcers and as a cicatrizant on gangrenous wounds. It may have anti-viral effect which tannins have. It can also be used against poisons. There are also reports on the role of tannins in anti-nociceptive activity [19]. Besides, alkaloids are well known for their ability to inhibit pain perception [20].

As a result of adverse side effects, like gastric lesions, caused by NSAIDs and tolerance and dependence induced by opiates, the use of these drugs as anti-inflammatory and analgesic agents have not been successful in all the cases . Therefore, new anti-inflammatory and analgesic drugs lacking those effects are being searched all over the world as alternatives to NSAIDs and opiates. During this process, the investigation of the efficacy of plant-based drugs used in the traditional medicine have been paid great attention because they are cheap, have little side effects and according to WHO still about 80% of the world population rely mainly on plant-based drugs [21].

Effect of ethanol extract of *Hiptage Benghalensis* in hot plate method is shown in the table 2. It is one of the most common tests for evaluating the analgesic efficacy of drugs/compounds. The paws of mice and rats are very sensitive to heat at temperature which is not damaging to the skin. The responses are shaking, jumping, withdrawal of the paws and licking of the paws. The time until this response is prolonged after administration of centrally acting analgesics. *H.Benghalensis* extract at the dose of 250 and 500 mg/kg showed the significant ($P<0.05$) increase in latency time as compared to control. Positive control Diclofenac Na showed significant ($P<0.05$) analgesic activity at the dose of 50 mg/kg.The analgesic activity was expressed as mean increase in latency after drug administration ±SEM. *H.Benghalensis* exhibited potent analgesic activity at the dose levels of 250 and 500mg/kg. These extracts show analgesic activity at low dose of 250mg/kg even in first hour in test. These results indicate that ethanol extract of *H.Benghalensis* can produce significant analgesic effect.

Carrageenan induced paw edema is most widely use acute inflammatory model for studying anti-inflammatory activity and it includes two phases. First phase occurs within an hour of injection of phlogistic agent and is mediated through release of histamine serotonin and kinin. While the second phase which can be measured around 3 to 4 hours is related to release of prostaglandins . Carrageenan-induced edema involves the synthesis or release of mediators at the injured site. These mediators cause pain and fever [22]. Inhibitions of these mediators from reaching the injured site or from bringing out their pharmacological effects normally ameliorate the inflammation and other symptoms. In the present study, it has been shown that the ethanol extract of the *H.Benghalensis* possess a significant anti-edematogenic effect on paw edema induced by carrageenan. Slight inhibition of inflammation is observed in first phase and maximum in second phase, which is mainly due to release of prostaglandins. The possible anti-inflammatory effect may be due ro inhibition of cyclooxygenase enzyme which catalyzes the biosynthesis of prostaglandins and thromboxane from arachidonic acid. There are reports that flavonoids possess anti-inflammatory activity and some act as phospholipase inhibitors [23, 24, 25].Such inhibitors are able to decrease the inflammatory response to Carrageenan in rats [26, 27].

5. CONCLUSION

The present study indicated that the ethanol extract of *H.Benghalensis* may have potential use in medicine. In our study, the ethanol extract of the plant showed significant dose dependent inhibition of paw edema in rats and significant analgesic effect in mice. Thus, it is concluded that the ethanol extract of leaf of *Hiptage Benghalensis* produce significant anti-inflammatory and analgesic activities in dose defendant manner. Now our next aim is to isolate the leading compounds and to establish their chemical structure as well. Further studies should be undertaken to correlate the pharmacological activities with the chemical constituents of the leaf of *H.Benghalensis* and uncover specific mechanisms of action so that we may find a viable natural alternative to the traditional NSAIDs.

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COMPETING INTEREST

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

Author A Shehla.U.Hridi managed the literature searches, performed the experiment and statistical analysis, and wrote the first draft of the manuscript.
Author C MD.Fakhar Uddin Majumder assisted during the experiments and provided the logistic support.
Author B Nafisa Ferdous managed the literature searches, performed the experiment.
Author D Dr.JMA Hannan designed the study, wrote the protocol, and supervised the experiments.
All authors read and approved the final manuscript.

CONSENT

Not applicable

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care (NIH publication No. 8523,revised 1985) were followed, as well as specific national laws were applicable. All experiments have been examined an approved by the appropriate ethics committee of North South University.

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