Research paper

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

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Abstract:

Objective: *This research was focused on the qualitative and quantitative evaluation of anti-inflammatory and analgesic effects of Hiptage Benghalensis in laboratory animals and whether these effects were of any statistical significance. Phytochemical analysis of ethanol extract of Hiptage Benghalensis has indicated the presence of steroid, carbohydrate, flavonoid, alkaloid, tanin, phenol and, mangiferin and terpenoid-compounds (1).*

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

Result: *The experimental activities for the ethanol extract of Hiptage Benghalensis exhibited statistically significant ($p<0.05$) anti-inflammatory activity in Carrageenan induced Hind Paw Edema in Long Evans rat and analgesic activity by Hot Plate and acetic acid induced writhing method 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. Further studies should be undertaken to correlate the pharmacological activities with the chemical constituents of the leaf of Hiptage Benghalensis.*

Key words:

Analgesic, Anti-inflammatory, Carrageenan, Acetic Acid, Hiptage Benghalensis, Phytochemical.

I. Introduction

Hiptage Benghalensis (L) Kurz belongs to the family Malphigiaceae. It is a large, woody climber found in India , South East Asia and Philippines .This is a woody climbing shrub with clusters of pink to white and yellow fragrant flowers and 3-winged, helicopter-like fruits. Flowers look like a decorative accessory. The fragrance is very strong and pleasant, resembles fruity perfume. Leaves are narrow and drooping. The leaves and bark are hot, acrid, bitter, insecticidal, vulnerary , astringent, refrigerant, expectorant, cardio tonic, anti-inflammatory and useful in the treatment of biliousness, cough, burning sensation, thirst and inflammation, wounds, ulcers, and rheumatism; it also has the ability to treat skin diseases and leprosy. 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. So far no information is available for the analgesic and anti-inflammatory activity of the ethanol extract of so, the present study has been undertaken to evaluate the analgesic and anti-inflammatory activity of the ethanol extract of H.Benghalensis using hot plate method in Swiss albino mice and Carrageenan-induced Hind Paw Edema methods in long evans rat respectively.

2. Materials and Methods:

Hot Plate (Model – 35100, UGO BASILE, ITALY), Balance, Refrigerator, Beakers, Petri dishes & glass wrought, Safety rat handling gloves, Mortar & pestle., Hypodermic , Syringes, Holder & test tube, Hot water Bath , Plethysmometer, Acetic Acid , Carrageenan

2.1. Medicinal plants (extracts)

Extract 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

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± 20C, and 12 hours light dark cycle). The animals were fed with standard diet from ICDDR, B and had free access to filtered water (*2)*.

2.4. Plant Extraction method

2.4.1. Collection

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

2.4.2. Drying and grinding

The collected plant leaf was washed with water, separated from undesirable materials or plant parts, partially dried by fan aeration and then fully dried in the oven at below 40°C for 2 days. The fully dried leaves was then grinded to a powdered form and stored in there refrigerator at +4°C for a few days.

2.4.3. Cold extraction (Ethanol extraction)

103gm of powered material was taken in a clean, flat bottomed glass container and soaked in 500 ml of 80%ethanol, sealed and kept for a period of 2 days with occasional shaking and stirring. It was then 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 (Eyela n 1000, Tokyo Rikaki kai co.ltd, Rotary vacuum, Japan) 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** (*3,4*) .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 conc 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 (For both Hot-Plate and Writhing)

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 for group II, 250 mg/kg

for group III and 500mg/kg for group IV of the crude extract of *Hiptage Benghalensis*.

2.6.2. Mice Screening

Young Swiss-albino mice aged 4-5 weeks, average weight 25-30 gm. 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. Mice screening was performed before 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 employed for measurement of analgesic activity which was previously described by *Lanhers et al., (1992)* and modified by *Mahomed and Ojewole., (2004).* A comparison of Hot plate test was made between positive controls (Diclofenac Sodium), control and test sample given orally 30 minutes after hot plate induction. Positive analgesic activity was shown when sample animal gave longer number of stimuli than the control, or the sample. The temperature of the metal surface of the hot plate was maintained at 55 ± 0.2°C. Latency to a discomfort reaction (licking, shaking or jumping) was determined before and after drug administration. The cut-off time was fixed at 15s to avoid the damage to the animal paw. The latency was recorded at 0, 30, 60, 120, 180,240 min following oral administration of the agents. The prolongation of the sample

latency time compared with that of control was used for statistical comparison. Each mouse was placed in the beaker (on the hot plate) in order to obtain its response to electrical heat induced nociceptive pain stimulus. The time for each mouse to lick its paws or jump out of the beaker was taken (reaction time). Each mouse served as its own control (*5,6,*) before treatment, its reaction time was taken once. The mean of these values on determination constituted initial reaction time before treatment of the mouse. Each of the test mice were thereafter treated with either distilled water, diclofenac sodium (50mg/kg of body wt.) and ethanol extract at the doses of H.Benghalensis 250 mg/kg and 500 mg/kg body wt. orally. Thirty min after treatment, the reaction time of each group mice were again evaluated five times individually in one hour interval on this occasion. 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-peritoneally to the experimental animals to create pain sensation. As a positive control, any standard NSAID drug can be used. In the present study Diclofenac sodium was used to serve the purpose. The plant extract 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. Then the animals were placed on an observation table. 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*

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 hot water (50±2°c) containing beaker to prevent transformation into a jelly like compound.

2.7.1. 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 of Winter et al (1962) with minor modifications. Rats were randomly divided into four groups, each consisting of six animals, 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 (7,8*)* 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.

2.7.2. 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. P values <0.05 were considered as statistically significant.

3.Result

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 (1) 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 & Stem***						
	Tannins	***Saponins***	***Flavinoids***	***Gums& Carbohydrates***	***Alkaloids***	***Reducing Sugars***	***Terpenoids***
80% ethanol	*+++*	*++*	*++*	*+++*	*+++*	*+++*	*+++*

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

3.2. Anti-inflammatory Activity

Table 2: Anti-inflammatory effect of ethanol extract of *Hiptage Benghalensis on* carrageenan induced rat paw inflammation

Table 3: Percent inhibition of the standard and two different concentrations of the extract compared with their respective means at 0 hour

Treatment	%inhibition 1 hour	%inhibition 2 hour	%inhibition 3 hour	%inhibition 6 hour	%inhibition 8 hour
Standard	29.97	51.68	89.91	56.27	20.49
Hiptage (250mg/kg)	48.61	77.08	91.07	63.54	36.61
Hiptage (500mg/kg)	48.74	77.19	88.88	57.89	29.53

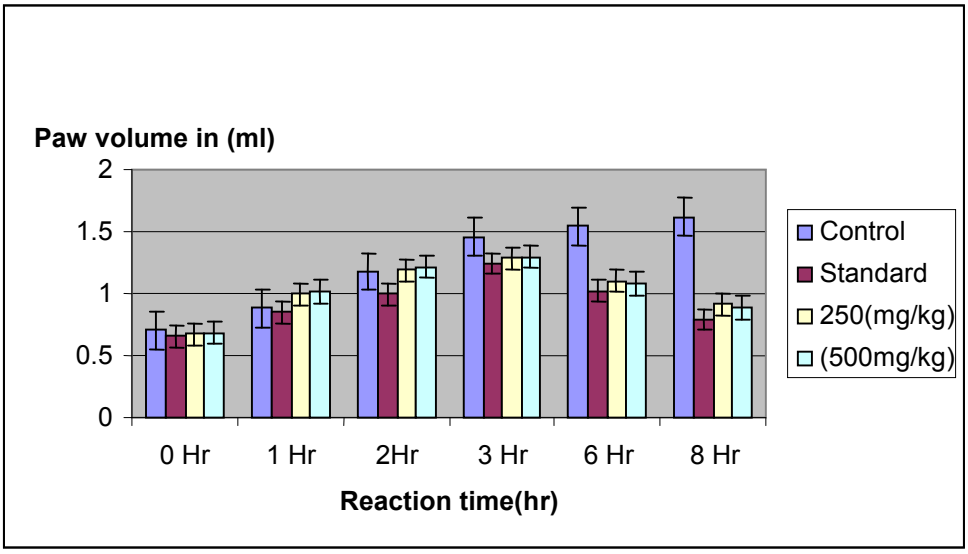


Figure 1: Anti-inflammatory activity of Hiptage by Paw edema method

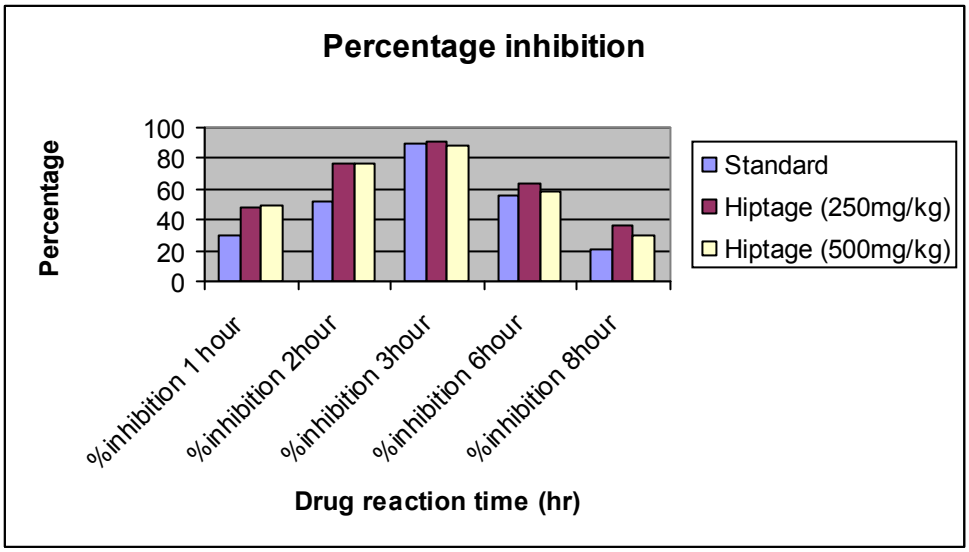


Figure 2: Percentage Inhibition of H.Benghalensis

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

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 Dunnett'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

Treatment	0 min	30 min	60 min	120 min	180 min	240 Hour
Control	10.70±.847	9.66±.937	8.00±.814	6.58±.641	5.52±.549	5.00±.443
Standard	9.14± .524	11.02±1.00	12.60±.945	14.16±1.076***	15.96±.676***	12.48±.698***
Drug 250 mg/kg	7.68±.851	9.28±1.09	10.32±1.12**	11.28±1.07**	12.54±.912***	10.18±.747***
Drug 500 mg/kg	7.65±.312	9.22±.285	10.34±.273	11.72±.233**	12.68±.177***	10.19±.163***

our 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.

3.3. Analgesic activity

Table 4: Analgesic effect of the ethanol extract of *H.Benghalensis* using the hot – plate method. Statistical evaluation of the results shown in table

Values in the results are expressed as mean ± SEM., [a] significantly different in comparison with control at P<0.05

Table 5: Percent inhibition of the standard and two different concentrations of the extract compared with their respective means at 0 hour

Treatment group	% Inhibition				
	½ Hour	1 Hour	2 Hours	3 Hours	4 Hours
Standard	20.56	37.00	54.90	74.61	36.54
Hiptage 250 mg/kg	20.83	34.37	46.87	63.28	32.55
Hiptage 500 mg/kg	20.52	35.16	53.20	67.75	33.20

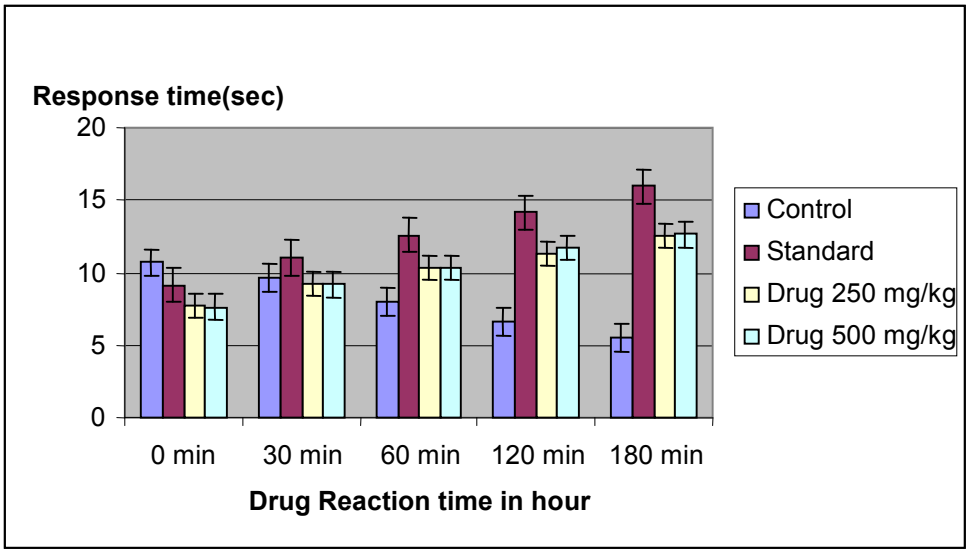
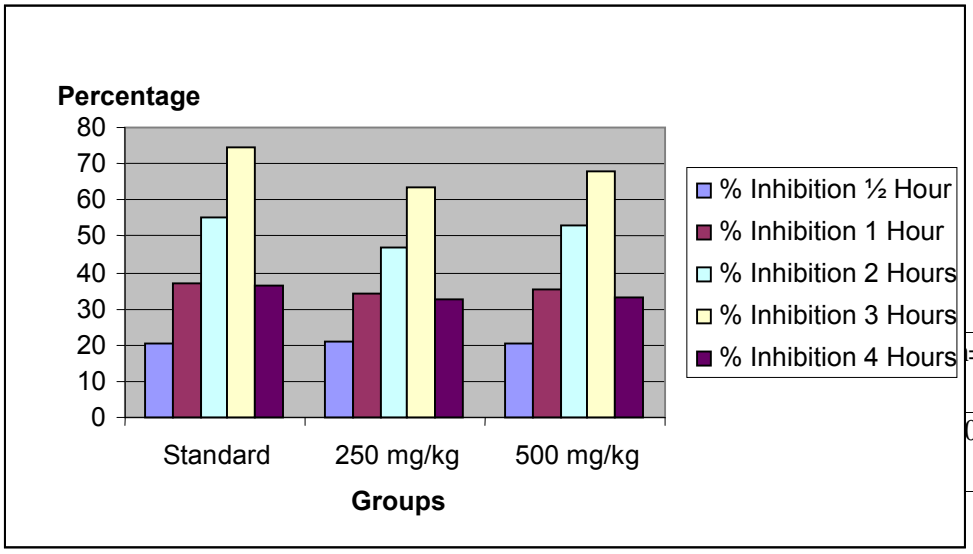


Figure 3: Analgesic activity of Hiptage by Hotplate method



± SEM	% Inhibition
00±4.11825	

Figure 4: % Inhibition of Hiptage

3.3.1. Effect of plant extract on Hot-Plate test

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 Dunnett'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.

3.3.2. Effect of Hiptage Benghalensis extract in acetic acid induced writhing

Table 6: Statistical evaluation of the results shown in table:

Standard	14	18	16	17	13	15.6000±.92736***	50.00%
Hiptage 250 mg/kg	19	19	21	21	18	21.4000±1.96469***	37.66%
Hiptage 500 mg/kg	16	18	7	11	18	14.0000±2.16795***	55.69%

Table 6 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*.

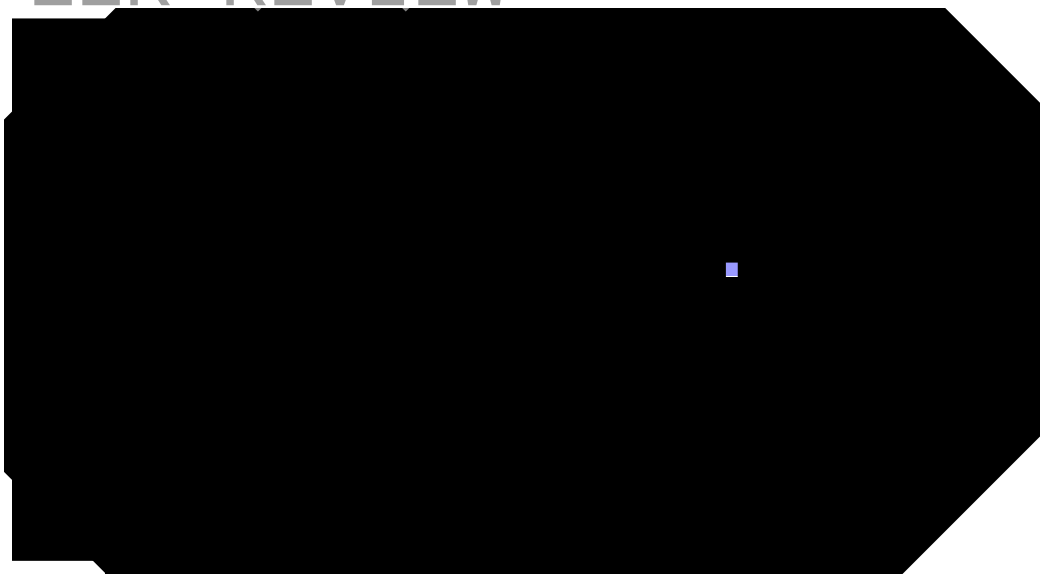


Figure 7: Analgesic activity by acetic acid method(IP)

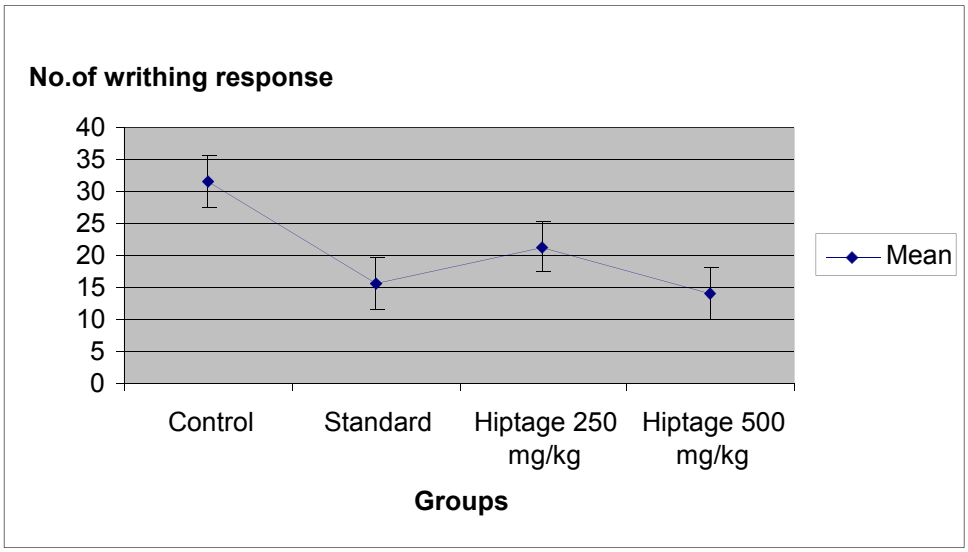


Figure 8: Analgesic activity by acetic acid method(IP)

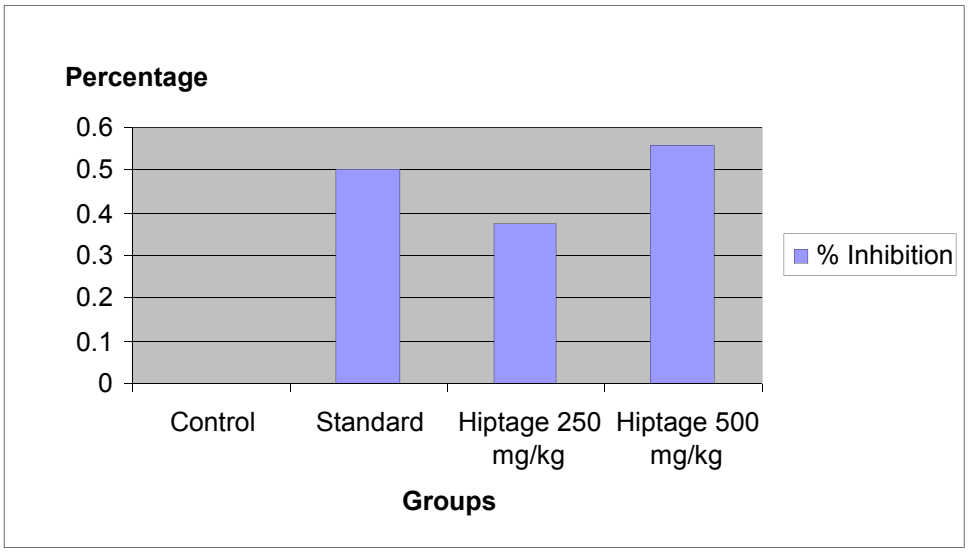


Figure 9: % Inhibition of Hiptage by acetic acid method(IP)

3.3.2. Acute toxicity

Oral administration of graded doses (250 & 500mg/kg) of the ethanol extract of H.Benghalensis to rats and mice did not produce any significant changes in behavior, breathing, cutaneous effects, sensory nervous system responses or gastrointestinal effects during the observation period. No mortality was recorded in any group after 24h of administering the extract to the animal.

4. Discussion

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(*9)*.

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.(10)Carrageenan-induced edema involves the synthesis or release of mediators at the injured site. These mediators cause pain and fever (11). 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. These are reports that flavonoids possess anti-inflammatory activity (12,13) and some act as phospholipase inhibitors (14).Such inhibitors are able to decrease the inflammatory response to Carrageenan in rats (15, 16)

Effect of ethanol extract of *Hiptage Benghalensis* in hot plate method is shown in the figures. It is one of the most common test 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 10 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.

The acetic acid-induced writhing is a sensitive method to evaluate peripherally acting analgesics. Ethanol extract of H.Benghalensis possess analgesic effects in the model of acetic acid-induced writhing test. Acetic acid induced writhing in mice finds much attention in the screening of analgesic drugs in acetic acid-induced abdominal writhing, the visceral pain model, and released arachidonic acid via cyclooxygenase and prostaglandin biosynthesis which played a role in the nociceptive mechanism. This model of response is thought to be mediated by peritoneal mast cells acid sensing ion channels and the prostaglandin pathway. In other words, the acetic acid induced writhing has been associated with increased level of PGE2 and PGF2α in peritoneal fluids as well as lipoxygenase products (17).The increase in prostaglandin levels within the peritoneal cavity then enhances inflammatory pain by increasing capillary permeability. Results of the present studies show that ethanol extract of H.Benghalensis produced significant analgesic effect which may be due to the inhibition of the synthesis of the arachidonic acid metabolite.

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 and significant analgesic effect. 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.Thus, it is concluded that the ethanol extract of fruit of Hiptage Benghalensis produce significant anti-inflammatory and analgesic activities in dose dependent manner.

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7. Conflict of Interest

Authors have no Conflict of Interest

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