Study of Bioactivities of *Holarrhena pubescence* Growing in Bangladesh

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ABSTRACT: The stem bark of *Holarrhena pubescence* was tested for anti-diabetic, analgesic, anti-inflammatory and antidiarrheal activities. After 1, 4 and 12 weeks of treatment protocol in alloxan-induced diabetic rats, it showed very negligible reduction of blood glucose level. In 12 weeks treatment protocol *H. pubescence* demonstrated that, the extract at 300 and 600 mg/kg bw can reduce blood glucose level by 3 and 4 mmol/l, respectively. Bark extract possessed strong analgesic, anti-diarrheal and mild anti-inflammatory activities. A dose of 600-mg/kg showed 69.50% ($p<0.05$) of writhing inhibition compared to indomethacin (73.76%) for acetic acid-induced analgesic method. In formalin-induced anti-nociception, the extract at 300 and 600 mg/kg bw showed dose dependent anti-nociception (67.42% and 69.007%, respectively, $p < 0.05$). The anti-diarrheal study demonstrated significant anti-diarrheal potential in a dose dependent manner as demonstrated by reduction of defecation by 64.92% and 71.43%, for 300- and 600-mg/kg bw, respectively.

Key words: *Holarrhena pubescence*, Anti-diabetic, Analgesic, Anti-inflammatory, Anti-diarrheal.

INTRODUCTION

Holarrhena pubescence (Buch.-ham.) Wall. (syn- *Holarrhena antidysenterica* (Roxb. ex Fleming) Wall.) commonly known as kurchi, is a flowering plant of Apocynaceae family of Indian subcontinent and also indigenous to Bangladesh.[1] Its stem bark has been well reported for antibacterial, antidiarrheal, anti-malarial, muscle relaxant, CNS depressant activities.[2-6] Seeds of this plant has potentials for free radical scavenging, anti-diabetic, hypoglycemic, antidiuretic, anti-hyperlipi-dimic and *in vivo* and *in vitro* anti-urolithic activities.[7-12] Leaves of H. *pubescence* are reported for anti-anthelminthic, anti-inflammatory, analgesic and antioxidants activities.[13-15] Seed and bark contain various important alkaloids. For example, pubadysone [11a-hydroxy-18,20-oxido-3-oxo-pregna-1,4,17(20)-triene], puboestrene [3-acetoxy-17-oxo-1,3,5 (10)-estratri ene] and pubamide [3,18-dioxo-11a-hydroxycona-1,4-diene] have been isolated from the bark of *H. pubescence*.[16] Bark is enriched with steroidal alkaloids such as conessine, isoconessimine, conessimin, conarrhimin and conimin. These alkaloids could be potential candidates for further development of new drugs against AD.[17] So we designed our research to find out the ant-diabetic, anti-diarrheal, analgesic and anti-inflammatory activities of stem bark of H. *pubescence* which are not reported previously. We also used different methods of finding these pharmacological activities.

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METHODS

Chemicals and drugs. Alloxan was purchased from Sigma Chemical Company, USA. Indomethacin and loperamide were procured from Square Pharmaceuticals Ltd. Formalin and acetic acid (Mark - Germany) were used to induce pain. Carrageenan was purchased from Sigma Chemical Co. (USA). All

other chemicals used in the experiments were generous gifts from Eskeyef Pharmaceutical Company Ltd., Bangladesh. The chemicals were of analytical grade.

Animals. The test rats were collected from the International Centre for Diarrheal Disease Research, Bangladesh (icddr,b). The experiments were performed by using Long-Evans male rats weighing about 200 - 220 gram, aged between 2 -2.5 months. Feeding of animals was done with normal pellet, along with drinking water and they were maintained at natural day and night cycle.

Experimental design. In performing anti-diabetic study, after one week of acclimation, the test animals were divided into five major groups. Long Evan male rats (90) were divided into three different time frames to perform 1 week, 4 weeks and 8 weeks treatment protocol. For 1 week protocol, 5 groups had been assigned with total 30 test rats and groups were assigned as normal, control, glibenclamide, and MEHP 300-mg/kg bw, 600-mg/kg bw. For 4 weeks and 12 weeks treatment protocol, same grouping had been followed. Besides this, after acclimation for 7 days, Long Evan rats (24) were randomly divided into four groups (normal, standard and two MEHP doses) to analyze acetic acid induced analgesic test. Each group had 6 rats as sample. The same protocol was followed for formalin-induced analgesic, carrageenan induced anti-inflammatory and castor oil induced anti-diarrheal test.

Authentication of plant. The specimen of *H. pubescence (*Buch.-Ham.) Wall. was submitted to the Bangladesh National Herbarium (BNH), Dhaka, Bangladesh where it was given the accession number: DACB-48725.

Preparation of plant extract. After collecting the plant bark, it was cleaned with water and dried at room temperature (24-26°C) for one week. The completely dried bark was cut into smaller piece and ground to powder, sieved and 400 gram of sieved powder was taken into a conical flask followed by soaking it with 2000 ml of 95% methanol. After that, the mixture was coarse filtered by a cotton plug followed by Whitman filter paper number 1. Finally, the filtrate was rotary evaporated using Bibby RE-200, Sterilin Ltd., UK at 5-6 rpm and optimum temperature to get a dark brown coloured concentrate that was designated as the crude methanol extract of *H. pubescenc*.

Phytochemical screening. The methanol extract of bark of *H. pubescence* was qualitatively analysed for identifying the presence of various compounds like tannins, flavonoid, anthraquinone, glycosides terpenoids, alkaloids and phenol.

Assessment of anti-diabetic activity

Induction of experimental diabetes. The induction of diabetes was performed by allowing single intraperitoneal injection of alloxan monohydrate at a dose of 120 mg/kg body weight. Alloxan was dissolved in citrate buffer at pH 4.5 and injected immediately within few minutes to avoid degradation. After 72 hours, from the retro-orbital plexus, blood was collected and blood glucose level was determined by using auto analyzer (Microlab 2000) from all surviving rats.

Preparation of test samples. Diabetic rats received treatment with i.p injection of glibenclamide (1.2 mg/70kg bw) as standard. Oral administration of methanolic extract of MEHP bark was conducted in a dose of 300- and 600-mg/kg bw for 1-, 4- and 12-weeks.

Assessment of analgesic Activity

Acetic acid-induced writhing method. Anti-nociception `study was performed according to the protocol described by Fontenele *et al.*[12]

Nociception was induced by injection (i.p.) of 0.1 ml/10 g acetic acid solution (10 ml/kg). Test animals were grouped into four containing 6 rats each, where negative control and positive control groups were administered with distilled water and indomethacin (10 mg/kg, i.p.) consecutively 30 minutes before acetic acid. The rats were pretreated with methanolic extract of *H. pubescence* stem bark at 300- and 600-mg/kg i.p. 30 minutes before acetic acid. Five minutes after the i.p. injection of acetic

acid, the number of abdominal constriction (writhing responses) was counted up to next 10 minutes.

Formalin-induced writhing method. Test protocol described by Dubuisson and Dennis was followed with slight modification. Here5% formalin in distilled water was prepared as pain inducing agent. Rats were grouped as negative control (distilled water), positive control (Indomethacin) and two extract groups (300 and 600 mg/kgbw) containing 6 rats each. Each group was administered with their respected treatments before formalin injection in the dorsal surface of left hind paw. Writhing response was observed in two phases like 0-5 minutes (early phase) and 15-35 minutes (late phase). Results are expressed as percentage inhibition.

Assessment of anti-inflammatory activity

Carrageenan promoted rat paw inflammatory method. Anti-inflammatory potential was measured by carrageenan induced edema method. Preweighted rats randomly divided into four groups carrying 6 rats each were delivered with their respected treatment with distilled water, indomethacin (10 mg/kg), 300-mg/kg bw and 600-mg/kg bw of MEHP. After 30 minutes of treatment, carrageenan (1%) was injected into the rat paw to induce inflammation. Generation of inflammation (edema) was measured using plethysmometer at 0, 30, 60,120, 180 and 240 minutes of carrageenan injection. Data is presented as mean ±SEM.

Assessment of anti-diarrheal activity

Castor oil induced anti-diarrheal test. Rats were selected by screening of their diarrheal response with 0.5 ml of castor oil. Rats were taken in individual cages, the floors of which were lined with blotting paper and every hour, the floor lining was changed. Diarrhea was induced by oral administration of 0.5 ml castor oil to each rat, 30 minutes before the above treatments. Every hour, total weight of fecal output, total weight of wet feces, total number of fecal output, and number of wet feces were recorded. Percent (%) inhibition of diarrhea was calculated as follows:

(%) inhibition of diarrhea = (mean no. of wet defecation/mean wet defecation of control)×100

Statistical analysis, Graph Pad Prism (version 4.0) computer program (Graph pad Software San Diego, CA, USA) was used and the results are expressed as mean ± SEM. A one-way analysis of variance (ANOVA), followed by Dunnett's post-hoc test or students paired or unpaired *t*-test were used in this study where needed.

RESULTS AND DISCUSSION

Preliminary phytochemical analysis. The result of qualitative chemical analysis of *H. pubescence* (MEHP) stem bark contained mainly alkaloids, glycosides, terpenoids, flavonoids, phenolics, tannins, anthraquinones etc. On the other hand carbohydrates, saponins, reducing sugars, proteins, vitamin C were not reported.

Blood glucose level. After administration of alloxan inexperimental rat, blood glucose level was significantly increased from (18 mmol/l) when compared with normal rats (7.12 ± .024 mmol/l) (Figure 1A). After treatment for one week with standard drug glibenclamide, MEHP 300 and 600 mg/kg bw respectively, glibenclamide, as a potential anti-diabetic drug, reduced blood glucose level to 7. 56 ±.16 mmol/l but MEHP failed to decrease blood glucose level.

On the other hand, in case of 4 weeks and 12 weeks treatment protocol, glibenclamide successfully reduced blood glucose level as 7.1 ± 0.178 mmol/l and 6.1± .145 mmol/l, which is very significant but in case of 4 weeks treatment protocol, the methanolic bark extract of MEHP only showed minor reduction of blood glucose level and the values were 17.56 ± 0.122 and 17.33 ± 0.180 mmol/l for 300 and 600 mg /kg bw. For 12 weeks treatment protocol, the blood glucose level were reduced by only 2 mmol and 3 mmol/kg bw which are not significant (Figures 1B and 1C).

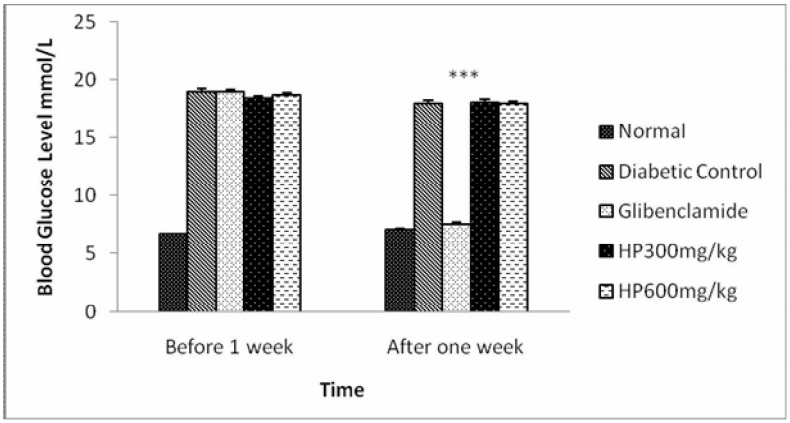

Figure 1A. Blood glucose level before and after one week ($^{***}p< 0.01$ and n = 6) treatment.

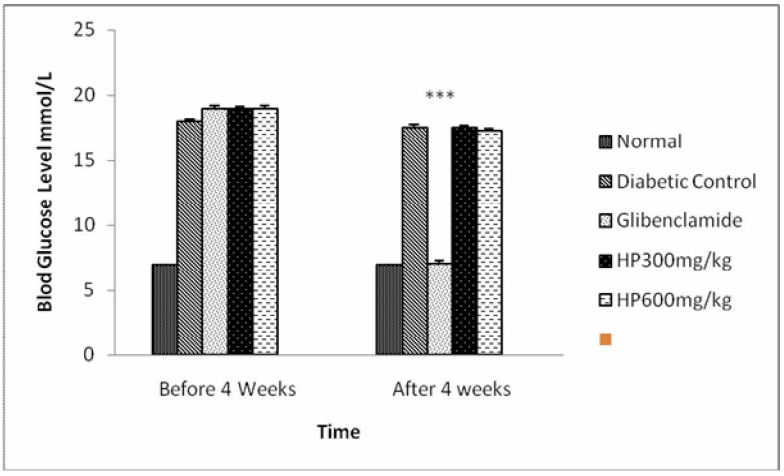

Figure 1B. Blood glucose level before and after four weeks ($^{***}p< 0.01$ and n=6) treatment

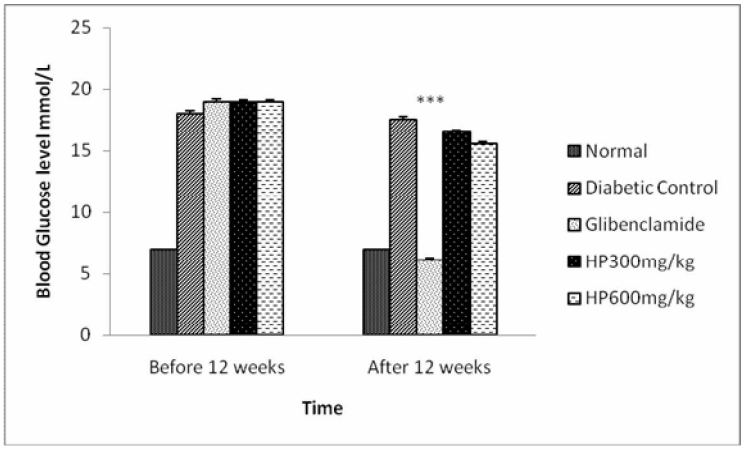

Figure 1C.Blood glucose level before and after twelve (12) weeks($^{***}P< 0.01$ and n=6) treatment

Acetic acid-induced writhing test. In acetic acid-induced method, MEHP showed inhibition of writhing response in dose dependent mannerwhen compared with distilled water (Figure 2). The MEHP at 600-mg/kg bwshowed strongly significant inhibition of writhing response 69.5% inhibition whereas standard indomethacin revealed 73.76 % of inhibition.

Formalin-induced writhing test. As shown in figure 3, writhing responses were counted in two phases for formalin-induced analgesic protocol. In late phase, 300 and 600 mg/kg bw of MEHP demonstrated significant percentage inhibition 67.42 and 69.007%, respectively. In addition, in early phase, only the higher dose of MEHP gave significant writhing inhibition (61.57%).

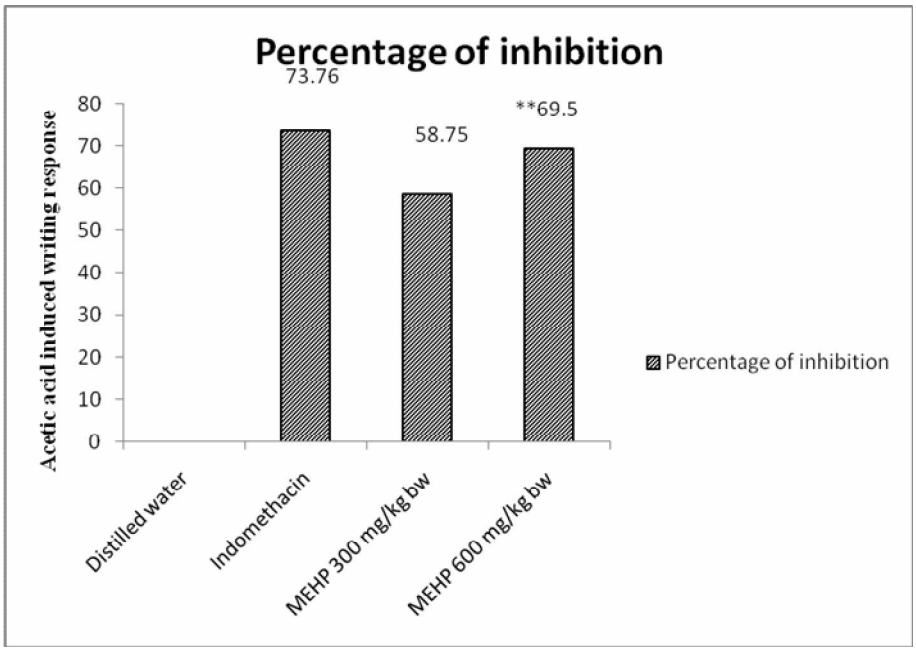

Figure 2. % Inhibition of acetic-acid induced writhing response of *H. pubescence* in rat shown by MEHP ($^{**}p < 0.01$ and n = 6)

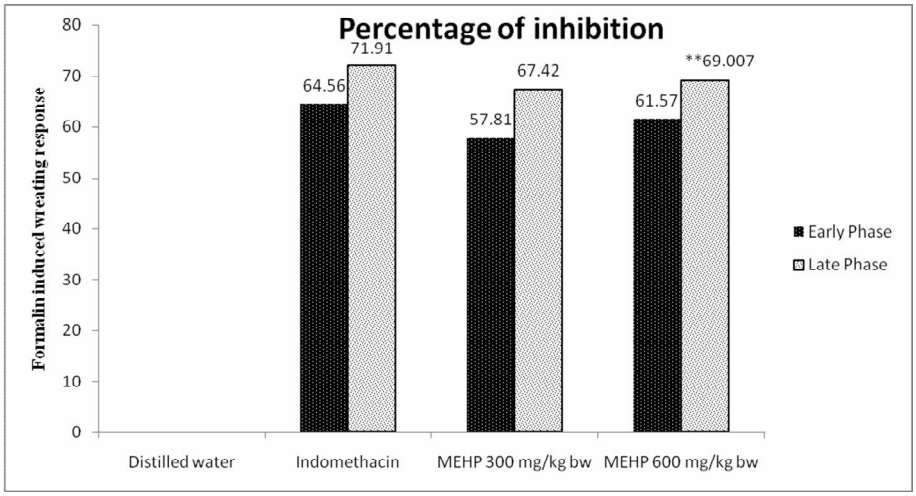

Figure 3. % Inhibition of formalin-induced writhing response in rat by *H. pubescence* extract ($^{**}p < 0.01$ and n=6)

Anti-inflammatory study. In Table 1, anti-inflammatory study of the MEHP extract reveals that higher dose showed slightly significant anti-inflammatory potentials compared to the distilled water at 30 minutes and 60 minutes after carrageenan administration.

Anti-diarrheal potential assessment. The MEHP demonstrated significant anti-diarrheal potential in dose dependent manner. Figure 4 represented the percentage inhibition of fecation for MEHP where higher dose inhibited 71.43% and lower dose inhibited 64.92%.

Table1.Data presenting paw thickness in carrageenan-induced anti-inflammatory study of MEHP.

Groups	Distilled water	Indomethacin 10 mg/kgbw	MEHP 300 mg/kgbw	MEHP 600 mg/kgbw
Time interval				
0 minute	0.94 ± 0.01	0.90 ± 0.06	0.93 ± 0.07	0.92 ± 0.04
30 minutes	1.13 ± 0.03	0.98 ± 0.04	1.07 ± 0.05	1.02 ± 0.06
60 minutes	1.27 ± 0.04	1.06 ± 0.05	1.19 ± 0.05	1.09 ± 0.06
120 minutes	1.53 ± 0.05	1.21 ± 0.07*	1.43 ± 0.06	1.36 ± 0.05*
180 minutes	1.64 ± 0.06	1.37 ± 0.06**	1.59 ± 0.07	1.53 ± 0.09**

Values expressed as mean ± S.E.M (*n*=6). (*) indicates statistically significant using one way analysis of variance, followed by Dunnett's multiple comparison test (*p< 0.05 and **p< 0. 01)

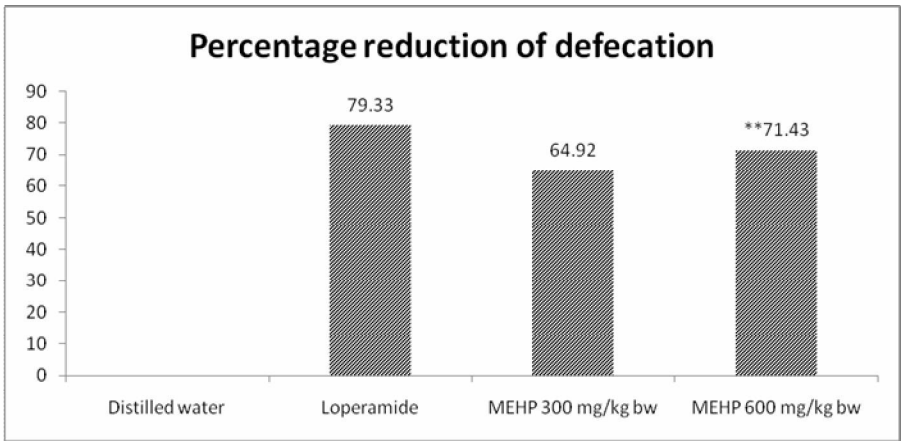

Figure 4. % Defecation for castor oil induced anti-diarrheal method (**p < 0. 01 and n = 6)

Our study showed that glibenclamide produced significant decrease in blood glucose level in alloxan-induced diabetic rats whereas MEHP failed to exert any significant effect in the reduction of blood glucose level.[18] With the examination of analgesic activity, it can be summarized that standard indomethacin showed significant pain relieving potential that can be comparable with our tested plant. The MEHP samples demonstrated significant analgesic potential. It can also be assumed with their traditional use. Acetic acid specifically induce inflammatory pain. This test method is basically used to evaluate peripheral pain relieving potential. Early phase of formalin test indicates neurogenic pain and late phase represents pains which are controllable by NSAIDs. It revealed strongly significant analgesic potential at late phase. The MEHP showed strongly significant analgesic potential, whereas in inflammation it showed non-significant effect as anti-inflammatory drugs.[19,20] Previousstudies showed that presence of phytochemicals like terpenoids, flavonoids, phenolics and tannins are responsible for

anti-nociception and anti-inflammatory potential. Anti-diarrheal study by castor oil demonstrated significant anti-diarrheal potential of MEHP in a dose dependent manner. It has been already established that phytochemicals present in the plant have anti-diarrheal potential and it supports previous studies.[5-11]

CONCLUSION

The methanolic extract *H. pubescence* of stem bark was found exibit significant analgesic and anti-diarrheal activities by decreasing writhing responses and the intestinal fluid accumulation in rats. But anti-inflammatory and anti-diabetic activities are not well reported. These can be possibly correlated with various phytoconstituents of the *H. pubescence*. However, further pharmacological studies are required to explore the exact mechanism of actions of the extract.

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