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IN-VITRO ANTI-INFLAMMATORY, ANTI-OXIDANT AND *IN-VIVO* ANALGESIC, ANTI-DIARRHEAL ACTIVITIES OF FRACTIONAL LEAF EXTRACTS OF *HIBISCUS SURATTENSIS*

Sayema Sultana[1], Abdullah Al Faruq[1], Nahid-Al-Rashid[1], Dr. Talat Nasim[2] and Md. Qamrul Ahsan[1]*

[1]Department of Pharmacy, Faculty of Science and Engineering, Southern University Bangladesh.
[2]Lecturer in Therapeutics, School of Pharmacy and Medical Sciences, University of Bradford, Bradford, UK.

***Corresponding Author: Md. Qamrul Ahsan**

Department of Pharmacy, Faculty of Science and Engineering, Southern University Bangladesh.

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ABSTRACT

Objective: To determine the effect of fractional extracts (ethanol, chloroform and n-hexane) of *Hibiscus surattensis* in treating inflammation, oxidant, pain and diarrhea using *in vitro* and *in vivo* experimental models. **Methods:** The activities of different fractional plant extracts were tested for protein denaturation of egg albumin. Additionally, inhibition of DPPH free radical, acetic acid-induced writhing and castor oil-induced diarrheal models were applied for evaluation of anti-inflammatory, anti-oxidant, analgesic and anti-diarrheal activities respectively. **Results:** All tested extracts demonstrated a significant ($^{*}P< 0.05$, $^{**}P< 0.005$) mild to moderate activity of inhibition of protein denaturation compared with the positive control group of acetyl salicylic acid. Extracts also showed significant ($^{*}P< 0.05$) moderate inhibition of DPPH free radical scavenging activity compared with the standard drug ascorbic acid at the similar doses. In analgesic test, these extracts significantly ($^{*}P<0.05$) reduced the number of writhing and increased the mean latent period of defecation in castor oil-induced diarrheal model mice at the dose of 500 mg/kg body weight. **Conclusions:** These results suggest that the crude leaf extracts possesses significant anti-inflammatory (mild to moderate), anti-oxidant (moderate), analgesic and anti-diarrheal activities. These observations offer the opportunity for the identification of more active chemical entities, which may support the clinical and experimental uses of this plant.

KEY WORDS: Anti-inflammatory; anti-oxidant; analgesic; anti-diarrheal and *Hibiscus surattensis*.

INTRODUCTION

The *Hibiscus* has various species belonging to family Malvaceae*,* many of them used medicinally and was comprises of about 275 species in the tropics and sub-tropics and most *Hibiscus* species have a remarkable color pattern with the base of corolla forming a deep-colored heart.[1]

Hibiscus surattensis (*H. surattensis*) is a medicinal plant belongs to Malvaceae family. These plants are available in the region of Chittagong and Chittagong Hill Tracts in fallow lands and scrub forests which are locally known as Sowa Amela (Tanchangya). It is a prostrate or climbing annual plant. All parts, including the weak stems and leaf stalks, covered with small downward-pointing soft prickles and hairs. Pairs of oval leafy stipules beside the stalks are characteristic. Leaves are usually reddish and flowers with very showy orange-yellow, over 7 cm across, petals dark redmaroon at the centre. The 6 or more outer sepals divide into a narrow erect lobe and a broader oval lobe. The 5 inner sepals lengthen to 2.5 cm in fruit, tip pointed, covered with hairs.[2] Various medicinal properties of *Hibiscus* have been observed. For example, in West Africa, *H. surattensis* extract is utilized with other species in malaria treatment, with the leaves ingested as a decoction.[3] In Nigeria, *H. surattensis* flowers are used for the treatment of hypertension.[4] The stems and leaves of *H. surattensis* are used as a lotion to treat venereal disease and ureteritis in India and the eastern regions of South Africa.[5] Anti-inflammatory, antifungal, antipyretic, and antihelminthic activities were identified from stems and root extracts of *H. taiwanensis*.[6] Flowers of *H. tiliaceus* are widely used for birth control and for treating skin infections.[7] *H. rosa-sinensis* extracts exhibit anticancer, antioxidant and antibacterial activities.[8,9] Sorrel (*H. sabdariffa*), a medicinal herb commonly uses to make drink and pickle, is used as a folk medicine for the treatment of hypertension, liver diseases, and fever.[10-13] *H. sabdariffa* has been reported to possess antihypertensive, antioxidant, anti-cancer, anticlastrogenic, hypolipidaemic, hepatoprotective, anti-stress, antispasmodic, diuretic and antidiarrheal activities.[14] The decoction of the plant seeds is given to augment or induce lactation in poor letdown and maternal mortality.[15] Previous studies have been

indicated that flowers and leaves of *H. rosa-sinensis* are found to possess anti-infectious, antimicrobial, antioxidant, anti-inflammatory, anti-diarrheic and antipyretic activity.[16-19] Antibacterial and antioxidant activities were found from other *Hibiscus* species namely *platanifolius, Cannabinus, mutabilis, esculentus* and *tiliaceus.*[20-22] Despite the extensive studies of other *Hibiscus species*, anti-inflammatory, analgesic and antidiarrhoeal activities of *H. surattensis* have not been reported. Hence, there is a need to determine the anti-inflammatory, analgesic and antidiarrhoeal activities of *H. surattensis* extracts.

In this study, we have demonstrated that the crude leaf extracts of *H. surattensis* possesses significant anti-inflammatory (mild to moderate), anti-oxidant (moderate), analgesic and anti-diarrheal activities. These observations offer the opportunity for the identification and isolation of more active chemical entities, which may support the clinical and experimental applications for various disorders.

MATERIALS AND METHODS

Selection and Identification

The plant named as *H. surattensis* (Family: Malvaceae) was selected based on its medicinal uses. The traditional practitioners called as "kabiraj" and the tribal people of hill tracts were the main source of reliable information about the traditional uses of this plant. Taxonomical identification of this plant was carried out by the experts of Bangladesh Forest Research Institute Herbarium (BFRIH), Chittagong. The herbarium sheet was prepared following the standard procedure and specification suggested by the expert of the institute. The sheet was signed by the taxonomist and preserved in pharmacognosy laboratory, Department of Pharmacy, Southern University Bangladesh.

Collection and Garbling

Fresh leaves were collected from the hill tracts of Sitakunda Eco Park, Chittagong, in the month of June and July 2016 which was the period when the plant grows the most. The extraneous, undesired substances from the plant material were removed at two stages. At first the rotten leaves, stems etc were removed by hands immediately after collection. The soil was removed by sieving through a net aided by a flow of air from an electric fan before the plant materials were dried.

Drying and Grinding

The leaves were then subjected for shade dry at temperature not exceeding 50°C. Next, these leaves were grinded into coarse powder with the help of a grinder. The powder were stored in airtight containers and kept in a cool, dark and dry place until extraction was commenced.

Extraction and Fractionation of Plant Materials

Solvent-solvent partitioning was done using the protocol design by Kupchan and modified by Wagenen *et al.*[23] For hot extraction, 150 gm of plant powder was subjected in a Soxhlet Apparatus with 800 mL of ethanol (96%) continuously for 48 hrs and the obtained extract was collected, filtered and made to evaporate the solvent below 50°C temperature and reduced pressure. After evaporation of the solvent, a gummy concentrate was obtained which was designated as hot ethanol crude extract. From ethanol crude extract about 10g extract was dissolved in double distilled water (DDW) and then fractionated using fractionating column with n-hexane and subsequently with chloroform. Fractionation was done using 50 mL solvent each time until 150 mL n-hexane and chloroform were used and each time after vigorous shaking the mixture was allowed to stand. Solvent layers were separated and decanted. The remaining extract was used as fraction of ethanol. The obtained extract was collected, filtered and made to evaporate the solvent below 50°C. After evaporation of the solvent, it rendered into a gummy concentrate. The gummy concentrate was weighed and stored 4°C in clean air tight closure containers.[24]

Chemicals

All chemicals and solvents used in this study were of analytical grade and purchased from Merck, Germany. Standard drugs such as loperamide, diclofenac sodium and acetyl salicylic acid were obtained from Square Pharmaceuticals.

Experimental Animals

Swiss albino mice of both sexes (male and female), 6-7 weeks of age, weighing between 25-30 g, were collected from the Bangladesh Council of Scientific and Industrial Research (BCSIR) Chittagong for *in vivo* experiment. All the mice were maintained under standard laboratory conditions of temperature: (25.0 ±1.0 °C), relative humidity: 55-65% and 12 hrs light/12hrs dark cycle and water *ad libitum*. All protocols for animal experiment were followed by Institutional Animal Ethics Committee and appropriate measures were taken to minimize the pain or discomfort of animals.[25] The animals were fasted overnight but allowed fresh water *ad libitum.*

Preliminary Phytochemical Screenings

The fractionated extracts were performed to various tests (Table 1) for determining the chemical nature of the phyto constituents.[26]

Antioxidant Activity

The stable DPPH free-radical scavenging activity was determined using the modified method described by Chang *et al.*[27] 0.1mL of ethanol extract of *Hibiscus surattensis* (EEHS), n-hexane extract of *Hibiscus surattensis* (NEHS) and chloroform extract of *Hibiscus surattensis* (CEHS), at various concentrations (20, 40, 60, 80 and 100 μg/mL) was added up to 3mL of 0.004% methanol solution of DPPH. All the reaction tubes were kept in dark except control tubes for 30 minutes. After 30 minutes, absorbance of the resulting solution was measured against a blank at 517 nm.The percentage

DPPH radical scavenging activities (%SCV) were calculated (Table 2) by comparing the results of the test with the control (not treated with extract) using following formula:

% SCV = [(A0 - A1)/A0] × 100

Where, SCV = Radical scavenging activity,

Ao = Absorbance of the control and

A_1 = Absorbance of the test (extracts / standard)

IN VITRO ANTI-INFLAMMATORY ACTIVITY

The method was described by Habibur Rahman *et al.*[28] The reaction mixture (5 mL) consisted of 0.2 mL of egg albumin solution (from fresh hen's egg), 2.8 mL of phosphate-buffered solution (PBS, pH 6.4) and 2 mL of varying concentrations (100, 250, 500 µg/mL) of drug. A similar volume of double-distilled water served as the control. Next, the mixtures were incubated at 37 ± 2°C in a BOD incubator for 15 minutes and then heated at 70°C for five minutes. After cooling, their absorbance was measured at 660 nm by using the vehicle as a blank. Diclofenac sodium in the concentrations of 100, 250 and 500 µg/mL was used as the reference drug and treated similarly for the determination of absorbance. The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\% \text{ inhibition} = 100 \times [V_t / V_C - 1]$$

Where, V_t = absorbance of the test sample; Vc = absorbance of control.

IN VIVO ANALGESIC ACTIVITY

Acetic acid-induced writhing test

This test was done using the method described by Collier *et al.*[29] Muscle contractions were induced in mice by intra peritoneal injection of 0.7% solution of acetic acid (10 mL/kg). For test groups, 500mg/kg of leaves extracts of EEHS, NEHS and CEHS were selected. For standard (25mg/kg), 0.025 gm of Diclofenac-Na (*CLOFENAC*, 50mg/Tab) was taken and a suspension of 10 mL was made and for control group, 50 mg of Tween-80 was added with double distilled water (DDW) to 5 mL. For preparation of 0.7% acetic acid solution, 0.7 mL glacial acetic acid was mixed with DDW to 100 mL.

IN VIVO ANTIDIARRHEAL ACTIVITY

The method, described by Shoba and Thomas, 2001 was followed for this study.[30] The animals were screened initially by giving 0.4 mL of castor oil and only those showing diarrhea were selected for the final experiment. The animals were then, divided into control, positive control and three test groups containing three mice in each group. Control group received vehicle (1% Tween-80 in water) at a dose of 10 mL/kg body weight orally. The positive control group received loperamide at the dose of 3 mg/kg orally; test groups received the EEHS, NEHS and CEHS at the dose of 500 mg/kg body weight orally. Each animal was placed in an individual cage, the floor of which was lined with blotting paper. The floor lining was changed every hour. Diarrhea was induced by oral administration of 0.4 mL castor oil to each mouse, 30 minutes after the above treatments. During the observation period (4hrs), the total latency periods (first diarrheal stool after the administration of castor oil) and the number of diarrheic feces excreted by the animals were recorded. A numerical score based on stool consistency was assigned (normal stool =1 and watery stool = 2) and frequency defecation was recorded.

Statistical Analysis

The data were presented as mean standard error mean (±SEM)). The statistical data were analyzed by one way SPSS windows software version 20 (ANOVA) followed by Dunnett's t-test for multiple comparisons. For the comparison between two groups, Student's t-test was employed. The significant difference was considered at $P<0.05$ and $P<0.005$.

RESULTS

Phytochemical Screening

We first determined the chemical constituents present at different extracts. It was observed that the EEHS contained alkaloids, steroids, tannins, reducing sugar, gums whereas CEHS was found to have alkaloid, steroids, reducing sugars, gums and tannins. On the other hand, NEHS had alkaloids, reducing sugars, steroids and gums (Table 1). All three fractioned extracts contained alkaloids, gums, reducing sugars and steroids.

Table 1: Chemical constituents present in different extracts.

Chemical Compounds		Consequences		
(Examination)	Name of the test/reagent	EEHS	CEHS	NEHS
Alkaloids	Mayer's test	+	+	+
	Dragendorff's test	+	+	+
	Wagner's test	+	+	+
	Hager's test	+	+	+
	Tannic acid test	+	+	+
Steroids	Salkowski test	+	+	+
	Libermann-burchared test	+	+	+
Tannins	Ferric chloride test	-	-	-
	Potassium dichromate test	+	+	-
	Keller-Killiani test	+	+	-
Reducing sugars	Fehling's test	+	+	+
	Benedict's test	+	+	+
Gums	Molisch's test	+	+	+
EEHS=Ethanol extract of *H. surattensis*; NEHS= n-hexane extract of *H. surattensis*; CEHS= Chloroform extract of *H. surattensis*.				

IN VITRO ANTI-INFLAMMATORY ACTIVITY

The *in vitro* anti-inflammatory effect of *Hibiscus surattensis* was evaluated against protein denaturation of egg albumin. The percent inhibition of protein denaturation of EEHS was moderate 46.65% ($P < 0.005$) at a concentration 500 µg/mL compared with those of NEHS and CEHS extracts. The standard drug of acetyl salicylic acid showed maximum inhibition of 89.13% ($P < 0.005$) at the same concentration. Results are summarized in Table 2.

Table 2: Effect of different extract of *H. surattensis* on protein denaturation activity.

Treatment groups	Concentration (µg/mL)	Mean Absorbance (Mean ± SD)	% MIPD
Control (Ethanol)	---------	0.408	0
Positive Control (ASA)	500	0.0443±0.0005**	89.13
	250	0.0656±0.0011**	83.91
	125	0.0850±0.0062*	79.17
EEHS	500	0.2176±0.0058**	46.65
	250	0.3086±0.0011*	24.35
	125	0.3520±0.0062**	13.73
NEHS	500	0.3170±0.0052*	22.3
	250	0.3536±0.0192*	13.32
	125	0.3746±0.0071*	8.17
CEHS	500	0.3326±0.0110*	23.27
	250	0.3586±0.0032**	19.2
	125	0.3956±0.0075**	14.05

ASA= Acetylsalicylic acid; % MIPD= Percent mean inhibition of protein denaturation; EEHS=Ethanol extract of *H. surattensis*; NEHS= n-hexane extract of *H. surattensis*; CEHS= Chloroform extract of *H. surattensis;* n=3, each group; SD = Standard deviation, *$P< 0.05$, **$P< 0.005$ significant compared to control.

ANTIOXIDANT ACTIVITY

We next investigated the anti-oxidant activities of the extracts by determining free radical scavenging activities. We found that all three extracts tested showed moderate anti-oxidant activities in a dose-dependent manner. Minimum inhibition was observed at 20 µg/mL whilst the maximum inhibition was found at100 µg/mL. NEHS exhibited 47.55% inhibition of DPPH free radical at 100µg/mL. EEHS and CEHS extracts elicited inhibition of 45.64% and 40.47% at100 µg/mL respectively. The percent inhibition DPPH activity of ascorbic acid showed higher activity compared with all fractionated test extracts. Results are summarized in Table 3.

Table 3: DPPH free radical scavenging activity of various extracts of *H. surattensis* leaves.

Concentration (µg/ml)	% inhibition of DPPH by ASC	% inhibition of DPPH by EEHS	% inhibition of DPPH by NEHS	% inhibition of DPPH by CEHS
20	38.92	17.17	18.75	18.37
40	50.45	24.39	26.8	25.39
60	66.06	30.71	34.35	33.52
80	73.77	37.17	41.82	40.01
100	89.46	45.64	47.55	40.47

DPPH=1, 1-Diphenyl-1,2- picrylhydrazine ; EEHS=Ethanol extract of *H. surattensis*; NEHS= n-hexane extract of *H. surattensis*; CEHS= Chloroform extract of *H. surattensis.*

ANALGESIC ACTIVITY

In *in-vivo* analgesic activity test, the EEHS, NEHS and CEHS significantly reduced the number of writhing. EEHS, NEHS and CEHS showed significant writhing prolongation in analgesic test at the dose of 500mg/kg body weight and the activities were showed 24.11% ($P<0.005$), 25.53%, ($P<0.05$) and 17.73% ($P<0.05$) respectively. The standard drug diclofenac-sodium exerted 63.83% ($P<0.005$) activity at the dose of 25 mg/kg body weight (Table 4).

Table 4: Effect of different extracts *H. surattensis* on acetic acid induced writhing in mice.

Treatment groups	Dose	Number writhes	% Protection
Control	1% Tween-80	94±3.61	------
Diclofenac-Na	25mg/kg	34±2.64*	63.83
EEHS	500mg/Kg	71.33±1.53**	24.11
NEHS	500mg/Kg	70.46±4.01*	25.53
CEHS	500mg/Kg	77±4.04*	17.73
Values are expressed as mean ± Standard deviation (SD) of three in each group; *$P< 0.05$, **$P< 0.005$ significant compared to control.			

IN VIVO ANTIDIARRHEAL ACTIVITY

Anti-diarrheal activity of the extracts was determined in castor oil induced diarrheal mice. EEHS and NEHS extracts significantly prolonged the time in minutes for castor oil induced diarrhea and increased the percent latency period by 15.24% ($P < 0.005$) and 16.9% ($P < 0.005$) respectively at the dose of 500 mg/kg body weight whereas very little effect was observed by CEHS at the same dose. The standard drug loperamide at the dose of 3mg/kg showed significantly ($P < 0.005$) increased (76.81%) latency period as shown in Table 5.

Table 5: Effect of the different extracts of *H. surattensis* on the latent period (minutes) of castor oil induced diarrheal in mice.

Treatment group	Dose	Mean latent period	Mean no. of stools	% increase of latency period
Control	1% Tween-80	39.33±3.61	0.67±1.51	------
Loperamide	3mg/kg	168.33±3.05*	4.33±1.52**	76.81
EEHS	500mg/kg	46.03±1.53*	5.66±1.52**	15.24
NEHS	500mg/kg	47.33±2.08*	6.33±1.52**	16.9
CEHS	500mg/kg	41.33±2.51*	10±1.0*	4.8
EEHS=Ethanol extract of *H. surattensis*; NEHS= n-hexane extract of *H. surattensis*; CEHS= Chloroform extract of *H. surattensis;* Values are expressed as mean ± SD of three in each group; *$P< 0.05$, **$P< 0.005$ significant compared to control.				

DISCUSSION

This study demonstrates that the leaf extracts of *H. surattensis* possesses significant anti-inflammatory (mild to moderate), anti-oxidant (moderate), analgesic and anti-diarrheal activities.

The inflammation is caused by denaturation of proteins. Mizushima Y and Kobayashi M showed that different concentrations of anti-inflammatory drugs such as aspirin, celecoxib, and ibuprofen induce protein denaturation.[31] The percent inhibition of protein denaturation activities of the EEHS, NEHS and CEHS demonstrated mild to moderate anti-inflammatory activity at various concentrations tested. The presence of alkaloids, flavonoids, tannins and phenols in the extracts may inhibit prostaglandin synthetase which has been shown for its anti-inflammatory effects.[32]

The reduced level of DPPH represents as an indicator of potential antioxidant activity.[33]

Our EEHS, NEHS and CEHS extracts exerted significant activities compared with standard drug of ascorbic acid at the same concentration (20µg/mL to 100µg/mL). However, the highest antioxidant activity was obtained from ascorbic acid compared with the fractional extracts.

Our EEHS, NEHS and CEHS extracts showed significant (*$P< 0.05$, **$P< 0.005$) analgesic activity. Acetic acid induces an increased level of PGE2 and PGF2 in the peritoneal fluid, which are responsible induction of pain.[34-35] Various analgesic drugs like ibuprofen, diclonefac sodium and aspirin have been reported that inhibit acid induced writhing by inhibition of prostaglandin synthesis.[36] We speculate that the anti-analgesic activities of EEHS, NEHS and CEHS extracts might be mediated through the activation of PGE2 and PGF2. Further studies are required to confirm this speculation.

In the castor oil induced diarrhea model, EEHS, NEHS and CEHS produced a significant (*$P< 0.05$, **$P< 0.005$) antidiarrheal activity compared with standard drug of loperamide. This could be due to active metabolite of ricinoleic acid which is liberated by the action of lipases in the upper part of the small intestine.[37] Ricinoleic acid mediates its action by binding to EP3 prostanoid receptors on smooth muscle cells and facilitates the accumulation of fluid in the intestine by inhibiting absorption and enhancing secretion of fluid and electrolytes.[38]

In summary, we have demonstrated that the fractioned leaf extracts of *H. surattensis* showed moderate *in-vitro* anti-inflammatory, antioxidant, and *in-vivo* analgesic and antidiarrheal activities. Further investigation of mechanism of action of pharmacological effects and identification of the active compounds are needed to

assess clinical and experimental applications of active ingredients of *H. surattensis* in various human disorders.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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