Rudimentary Pharmacological Inspection of the Ethanolic Extract of Grewia hirsute Vhal

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Authors’ contributions

This work was carried out in collaboration among all authors. Authors MB and MSR made significant contributions to conception design and conduction of research. Authors MB and MD performed all the experiments. Authors MSR and MB made data collection, statistical analysis, graphical representation and interpretation. The article was written by Authors MSR, MB, MZ, MD. Critical revision of the article was done by authors MAA, MRA, MAAM, AI, RB, SA, MRM and WA. MMB and MAA contributed equally. Authors MSR and MD contributed equally and shared the correspondence. All authors read and approved the final content of the manuscript. Finally, MSR submitted the article.

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ABSTRACT

Background: Screening of plant kingdom for various pharmacologic activities with an aim of discovering new active constituents is a continuous process and a part of systemic analysis.

Purpose: The purpose of this study was to investigate hypoglycemic, hypolipidemic, anti-ulcer, and thrombolytic effects of the ethanolic extract of Grewia hirsuta Vahl. (EEGH) in different in vivo and in vitro paradigms in both Swiss Albino mice and Long Evans rats.

Materials and Methods: After an oral acute toxicity study, adult male mice and rats according to the testing procedure were treated with EEGH (200, 400, 2000 and 4000 mg/kg, p.o.) and (1000 mg/70kg B.W. p.o Metformin, 10 mg/70kg, B.W. p.o Simvastatin, and 30 mg/70kg, B.W. p.o Omeprazole), and subjected to three in vivo tests and one in vitro test according to various established testing protocol.

Results: After testing glucose tolerance test, for hypoglycemic activities, 400 mg/kg EEGH both in 6 weeks and 12 weeks exhibited moderate anti-diabetic effect (8.89 ± 0.09 and 8.12±0.28 mmol/L respectively). Additionally, combination therapy of both 400 mg/kg EEGH + Metformin revealed a substantial fall of sugar level from 16.78± 0.1 to 6.4 ± 0.04 mmol/L, confirming a sign of moderate potentiation. No lipid-lowering effect was observed in the timed-treatment procedure. In the anti-ulcer effect, n-butanol showed a comparable ulcer inhibition index (90.56%) compared to the standard Omeprazole inhibition index of 91%. Additionally, ethyl acetate fraction showed 62% and 66% clot lysis capacity by 200 and 400 mg/kg EEGH, whereas standard Streptokinase’s capacity was 80%. The EEGH showed notable anti-ulcer activities and moderate anti-diabetic and thrombolytic activities.

Conclusion: Results extend the current understanding of the impacts of Grewia hirsuta Vahl. on biological parameters and indicate that this plant might be promising for the management of diabetes mellitus, anticoagulant, and gastric ulcer disorders prominently.

Keywords: Grewia hirsuta vahl; gastric ulcer; blood clot lysis; hyperglycemia; lipid profile.

1. INTRODUCTION

Grewia hirsuta Vahl. commonly known as Nagbala (Sanskrit) and Kukurbicha (Bengali) under the family Malvaceae [1], is an undershrub, erect and fulvous tree [2]. It has 30-90 cm high branches (slender), 5-11cm leaves (oblong linear-lanceolate, stellate, small hairy above), flowers white turning yellow, fruit 2-4 cm lobed (shining brown, hairy) [3]. It is reported as the flowering plant of the Asian region and indigenous to Bangladesh, Cambodia, Southeast India, Myanmar, Laos, Malaya, Sri Lanka, Nepal, Vietnam, Thailand, and the Peninsula of Malaysia. It has several synonyms: Grewia obliqua, Grewia roxburghii, Grewia trichodes, Grewia tomentosa, Grewia Pilosa. It is a small tree having coarsely and gray-brown hairy branchlets. It blossoms in June-July with white flowers having narrowly ovate petals, and drupe is 2-lobed. It has extensive use in Ayurvedic medicine and this Nagbala is named Ayurvedic lingo. Ayurvedic preparation is used to cure the wound, cough, heart disease, dyspepsia, fever, dysentery, and diarrrhea [4].

The plant Grewia hirsuta Vahl. has therapeutic effects proved by its traditional uses like the analgesic, anti-ulcer, antidiarrhoeal, anti-rheumatoid agent, and abortifacient agent around the world [5,6]. These plants contain an alkaloid, flavonoid, tannin, reducing sugar, carbohydrate, and steroid, reported by this author in an earlier publication [1]. Wagholikar et al. (2017) reported the presence of flavonoid, glycoside, and carbohydrate in moderate and protein, alkaloid, the tannin in minor amounts using two doses (Kwath and Churna) of G. Hirsuta Vahl root [2]. GC-MS study of the G. Hirsuta Vahl by Rajan MD et al. revealed the presence of the basic compound like aldehyde, ketone, aliphatic compound, tannin, curcumene, resin, sesquiterpene, tetra-terpene, sesquiterpene alcohol, fatty acid, unsaturated fatty acid, myristic acid, salt of tannin, sesquiterpene oxide, amino acids, palmic acid, peptide, amino compound, linoleic acid, oleic acid, gingerol, alkaline [5]. Oral toxicity test had been done before inspection of four different pharmacological activities. The compound (4Z, 12Z)-cyclopentadeceno-4, 12-dienone isolated from G. Hirsuta Vahl. proved its efficacy as an anti-diabetic agent in both in-vitro and docking studies [6,7].

In 2000, WHO stated an approximate estimation that 171 million peoples worldwide have diabetes, and in percentage, it is observed that
2.8% population are suffering from diabetes currently [8]. WHO also reported that 366 million patients worldwide would have diabetes, increasing the total estimated 171 million people by 2030 [9]. Diabetes mellitus in overall physiological aspects is a chronic escalating metabolic disorder typified by impeded insulin sensitivity, sluggish insulin secretion, and gradual failure of β-cells for secreting insulin [10]. Diabetes is responsible for the progressive increase of glucose in the blood and the production of free radicals. Hyperglycemia causes impairment of cholesterol influx and efflux balance. These lead to intracellular stockpiling of lipid in vascular cells. This high level of glucose and lipid are responsible for the formation of atherosclerosis [11]. Nathan et al., 2005 and Tuomilehto, 2003 claimed that hyperglycemia might be responsible for dysfunction associated with the liver and kidney [12,13]. SGPT and SGOT are the two prime liver enzymes for monitoring inflammation in hepatocytes. SGPT level is closely related to fatty liver in non-alcoholic fatty liver disease (NAFLD). Obesity is now considered the vital risk factor of NAFLD, which is also believed to be the promoter of the pathogenesis of type-2 diabetes [14]. Adiponectin released from the adipose tissue is also thought to have an impact on NAFLD. Because the low concentration of adiponectin in NAFLD represents an increased SGPT and SGOT [15]. Begum et al. proved that hypoglycemic agents with lipid-lowering agents decrease serum SGPT and SGOT Levels [11]. So new medicine is an option to treat metabolic disorder-related complications.

Ulceration is an inflammatory disease, and inflammation is subjected both in the inner and outer layer of skin and in mucus membrane, which is exacerbated by the overuse of drugs, irregular food habits, stress, and many other reasons [16]. Recurrent usage of NSAIDs to treat different inflammatory conditions may lead to bleeding on the GI mucosa with progressing ulceration [17]. Peptic ulcers represent the ulceration of the gastrointestinal tract, specifically the duodenum and stomach. Helicobacter pylori infection is thought to be the reason for peptic ulcer with other previously described causes. Studies present that 70% of gastric ulcers and mostly all duodenal ulcers result from H. Pylori infection. Sometimes peptic ulcers may extend into the mucosal layer and the stomach and duodenum’s smooth muscle layer. Treatment of peptic ulcers is programmed to either enhance the mucosal defense system or counteract different ulceration factors [18]. Due to toxicity associated with the available marketed potent anti-ulcer drugs, researchers are focusing on the need searching new medication as an alternative [19].

A thrombus (formation of a blood clot) is the coagulated mass of blood. It is developed in the systemic circulation for collapsing homeostasis in a process known as thrombosis [20,21]. Portal hypertension can be explained as thrombosis of a portal vein caused by thrombus formation in the vein, leading to the portal vein’s constriction [22]. Thrombus formation in the blood vessel leads to create a blockage which ultimately interrupts blood flow into the myocardioyte or brain [23], presenting serious consequences like atherothrombotic diseases such as myocardial infarction, cerebral clot formation, stroke, escalated pressure of blood, deep brain thrombosis, heart problems, and occlusion of the peripheral artery. Venous thrombosis is considered the second-highest attributing factor for the death of cancer patients [24]. This blockage may progress into ischemia, presenting hypoxia in the cell, causing necrosis. Thrombolytic agents remodel the blockage of the blood vessel to enhance the regular blood flow [25]. In the modern era, thromboembolism is associated with morbidity and fatality among the population, which can be successfully treated with various thrombolytic medicating agents like alteplase, urokinase, anistreplase, herbal blood anti-coagulation preparation, Streptokinase, tissue plasminogen activator as well as various folk medicine depending on the area, and habitat [26–28]. Despite available thrombolytic agents, various side effects (higher dose, poor fibrin selectivity, bleeding) associated with these medications have made the drug scientists ardent to establish a newer alternative of present thrombolytic agent with maximum therapeutic effects [25]. Streptokinase and urokinase are responsible for the risk of bleeding [29], extreme anaphylactic reaction, and shortage of specificity. Therefore, treatment with Streptokinase is narrowly sought for immunogenicity and other minor thrombo-embolic events [30]. Herbal products are the safest among the various treatment modules, as their origin is "natural" [31]. For time demand and urgency of patients, the exploration and formulation of herbal preparation from numerous plant and animal sources as antiplatelet [32,33], anticoagulant [34,35], antithrombotic [36], and thrombolytic activity have become the prime concern. Epidemiologic studies have provided evidence.
that foods with experimentally proved antithrombotic effect could reduce thrombosis risk [37].

This research work was designed as a continuation of previous work [38], and only a little research has been done on this *Grewia Hirsuta* Vahl. plant. After screening various literature based on the nature of phytoconstituents present in *Grewia Hirsuta* Vahl. and locally extensively used as folklore medicine (Kabiraji Medicine); it was planned to assess Bangladesh species’ preliminary therapeutic potential. For this purpose, by conducting four in-vivo tests presenting acute toxicity test, hypoglycemic potential and liver enzyme function test, anti-ulcer test, as well as one in-vitro test, namely thrombolytic test, were planned to explore the therapeutic potential of the plant concerned.

### 2. MATERIALS AND METHODS

#### 2.1 Plant Processing and Extraction

The whole plant *Grewia Hirsuta Vahl.* was collected from Asulia, Savar, Bangladesh, in the middle of February 2019. It was washed with water for removing dirt and shade dried with adequate airflow for ten days. Careful measurement was taken to prevent fungal attacks within leaves. The specimen was preserved in the National Herbarium of Bangladesh after identification by a botanist from Dept. of Botany, the University of Dhaka, and a voucher specimen (SPD/DU: 2345/2019) was deposited there. The dried plant was powdered and sieved. The powdered plant (1000 gm) of the sieved plant was soaked in 2000 ml of ethanol into two amber glass bottles (500gm in each bottle). Occasional shaking was done for 12days manually. The mixture was filtered firstly with cotton fabric following by Whitman filter paper. The filtrate was finally evaporated by using a rotary evaporator (Bibby RE-200, Sterilin Ltd., UK) at a temperature of 68°C with suitable rpm (5-6 rpm) to obtain crude ethanol extract (21.576 g). Wine color gummy concentrated ethanolic extract of *Grewia Hirsuta* Vhal. (EEGH) was then fractionated as described by Tunh et al. [38], and successive fractional extractions of n-hexane, petroleum ether, chloroform, n-butanol, ethyl acetate, and water was done to yield soluble fraction. All yields were (4.134g n-hexane, 2.567g petroleum ether, 3.621g chloroform, 2.546g n-butanol, 3.781g ethyl acetate 4.231g water) preserved for use in various in-vitro and in-vivo studies.

#### 2.2 Tested Animals

A total number of 257 test animals (200 Long Evans Rats + 15 Swiss Albino Mice) were procured from the animal house, icddr,b. Both Swiss Albino mice (age 4-6 weeks; weight 100-140 gm) and Long Evans rats (age 4-6 weeks; weight 200 to 220 g) were purchased to assess acute toxicity test, oral glucose tolerance, anti-diabetic activities, liver enzyme test, and anti-ulcer test. Grouping of animals was done randomly according to each test protocol. To prevent coprophagy, animals were appropriately caged. Both mice and rats were provided distilled water and standard pellet purchased from icddr,b for their diet. Animals were kept at ambient temperature and maintained at dark/light cycles. Acclimatization for the necessary time was confirmed before each test. In handling and sacrificing animals, ethics had been strictly maintained according to the rule illustrated [39–42]. For grouping in various test protocols, 12 mice and 12 rats were used in acute toxicity tests, 12 rats were used in OGT tests. A total of 70 rats were for anti-diabetic grouping, a similar number was also used for blood lipid tests, and 42 rats were used for anti-ulcer tests for EEGH. Another 35 rats were used in 5 groups for the fractional extraction of Ethyl Acetate, n-hexane, PET, CCL4, and n-butanol. Twenty healthy volunteers studying under 18 to 23 were selected from the Department of Pharmacy, East West University, Dhaka, Bangladesh, to collect blood to assess thrombolytic activities.

#### 2.3 Chemicals & Drugs

For oral glucose tolerance tests and anti-diabetic activities, Glucose, Metformin, Simvastatin, and anti-ulcer activity, Omeprazole was collected from Eskeyef Pharmaceutical Limited, Dhaka, Bangladesh. For liver enzyme concentration assessment SGPT, SGOT kits, and induction of diabetes in rat model Alloxan were brought from Sigma-Aldrich, USA. For thrombolytic activity, a lyophilized streptokinase vial (2,000 000 IU) was procured from Sanofi-aventis Bangladesh Ltd. All other chemicals used in this research work were analytical grade purchased from a certified local vendor.

#### 2.4 Acute Toxicity Study

Before commencement of any type of pharmacological inspection studies of acute oral
toxicity was done. For this purpose, four different doses (200, 400, 2000, and 4000 mg/kg) of aqueous extract of *Grewia Hirsuta Vahl* were administered only once in experimental mice and rats. According to OECD (Organization for Economic Development 2001) guideline, the 'Ups and Down' method was selected after grouping mice and rats. Six animals are used in each group. After administering of dose, behavioral changes were closely monitored with a time interval every 20 minutes for the first 3 hours, every 40 minutes for the next 6 hours, and daily for 48 hours. Behavioral changes include changes in skin hair, eyes, saliva, mucous membrane, diarrhea, lethargy, respiration, circulation, sleep, CNS and motor activities, etc. Observations were followed by the previously studied procedure [43].

2.5 Assessment of Anti-diabetic and Hypo-lipidemic Activities

2.5.1 Oral glucose tolerance test

A total of 12 Long Even rats were grouped into two groups to assess oral glucose tolerance test for one week. Two groups were the glucose control group, and Metformin treated group. Metformin was used as a standard drug, and doses were calculated from the prescribed human dose that is 1000 mg/70 kg body weight per day. Following previously studied protocol [44] with slight modification. Glucose was fed orally in both groups using a gastric feeding tube at a dose of 2 g/kg. Dosing was done once only. Blood glucose level was tested after 0, 45, 90, and 135-minute intervals. Metformin dose is 1 g /70 kg body weight per oral per day. Following this dose, Metformin fed to metformin control group last 1 hour of glucose administration.

2.5.2 Anti-diabetic test

2.5.2.1 Animals grouping and induction of Alloxan

Long Evans (70) rats were grouped based on two separate time frames, six weeks treatment protocol and 12 weeks treatment protocol.

In 6 weeks, treatment protocol rats are divided into five groups (7 rats in each group); normal control, alloxan control, metformin control, 200mg/kg per p.o of EEGH, and 400mg/kg per p.o. of EEGH. In 12 weeks, treatment protocol rats were classed into six groups (7 rats in each group); standard control, alloxan control, metformin control, 200mg/kg per p.o of EEGH and 400mg/kg per p.o. of EEGH, and combined 400 mg/kg + metformin 1 g/70 kg body weight per oral. In normal saline, alloxan monohydrate was injected intra-peritoneal route in rats that were fasted for 12 hrs. All rats in the control group, metformin control group, and extract control groups were injected at a dose of 120mg/kg body weight in successive five days. All animals are kept seven days in resting condition. On the 8th day, blood glucose was determined with a glucometer (One Touch Horizon, USA). From the 8th day after induction of diabetes, treatment in both protocols was started. The institutional review board approved the protocol. Blood glucose level was assessed after finishing 6 weeks and 12 weeks treatment protocol.

2.5.3 Hypolipidemic test

The same official procedures were used in the case of assessing lipid-lowering activities by Simvastatin. Simvastatin administered (i.p) at a dose of 10 mg/70kg BW/day. The level of TC, TG, LDL, and HDL was assessed following the same time frame and protocol, and levels were checked by a diagnostic kit manufactured by Sigma Aldrich, USA.

2.5.4 Preparation of dosage of active drugs and test sample

Metformin dissolved in normal saline and administered at a dose of 1 g/70kg b.w. per oral/day, 200 and 400 mg/kg b.w. p.o. EEGH dose was used. For combination therapy, the 1 g/70kg b.w. dose of Metformin and 400 mg/kg b.w. p.o. /day dose of *G. Hirsuta Vahl* were used. Prior administration of Simvastatin (10 mg/70 kg BW/day) was also dissolved in normal saline.

2.5.5 Collection of blood serum and liver

After completing the 6 and 12- weeks protocol, rats were anesthetized by chloroform to collect an average 5 ml blood and blood mix with heparin to maintain its liquidity. Collected blood was then centrifuged at 4000 rpm to 15 minutes to collect serum.

2.5.6 Liver enzyme (SGPT and SGOT) Levels

To perform this, test the International Federation of Clinical Chemistry (IFCC) standard was
followed [45]. SGPT and SGOT diagnostic kit manufactured by Sigma-Aldrich, USA, was used in assessing serum enzyme level.

2.6 Anti-ulcer Activities

To assess ethanol-induced anti-ulcerant properties of the G. Hirsuta vahl. the method explained by Baglikova et al. and Ganguly et al. was followed with some moderate modification [46]. The Long Evan rats weighing 200 ± 20 gm were kept at fasting for 24 hours in a separate cage to prevent coprophagy and allowed to drink water for two hours before the experiment commenced. The experimental animals were divided into six groups, each consisting of 7 rats (Table 1). Gastric ulcers were induced with freshly prepared ethanol-acid (24 ml/kg of 0.5 M HCl in 70% ethanol) in rats of Group II, III, IV, V, and VI after thirty minutes of pre-treatment with sample in respected groups. All these rats were sacrificed by cervical dislocation procedure after 90 min of ulcer induction, and their stomachs were immediately excised. Each stomach was opened in long curvature and washed

With distilled water Group I and Group II can serve as comparing the model to assess the events of ulceration. The gastric mucosa of each rat was examined for ulcers by a magnifying lens, and scoring of ulcers for ethanol and its five different fractions as EtOAc, n-hexane, PET, CCl₄, and n-butanol (Table 2). The mean ulcer score for each animal was expressed as ulcer index. For the precision of the result, the study was repeated thrice. Ulcer Score done as: Normal = 0, Red Coloration = 0.5, Spot ulcer = 1, Hemorrhagic Streak = 1.5, Ulcer = 2, Perforation = 3.

Ulcer index = (Ulcer area/ Total Stomach) *100

The percentage of ulcer protection was determined as follows: % ulcer protection = ((Control mean ulcer index-test mean ulcer index)/Control mean ulcer index) *100

For Fractional extraction similar protocol was conducted with its five different fractions; EtOAc, n-hexane, PET, CCl₄, and n-butanol.

2.7 Test for Thrombolytic Activities

2.7.1 Collection of streptokinase and specimen

After purchasing a commercially available lyophilized streptokinase vial (2 000 000 IU from Sanofi-Aventis Bangladesh Ltd), 5 mL of sterile distilled water was added within it by syringe and mixed properly. This suspension was considered the stock solution from which 100 µl (32,000 I.U) was utilized for in-vitro clot lysis. A total of 10 healthy male volunteers having no history of oral contraceptive or anticoagulant therapy for the last 30 days were selected conveniently. Five milliliters of venous blood per volunteer was drawn. To enhance the formation of the clot, this 5 ml of blood was distributed to each ten previously weighed sterile Eppendorf tube (0.5 mL/tube) and incubated at 37 °C for 45 min. After the formation of the clot, serum was completely removed from the tubes without disturbing the clot formed. Weight of the clot was determined by weighing the Eppendorf tube with clot using the formula: Clot weight = Weight of tube containing clot - Weight of tube alone. The experiment was run twice for two different doses.

2.7.2 Preparation of herbal extract, positive and negative control

100 µL of the EEGH with various concentrations (2, 4, 6, 8, 10, 12, 14, and 16 mg/mL respectively) was added to the tubes (0.5mL/tube) and labeled properly. As a negative non-thrombolytic control, 100 µL of sterilized distilled water and positive control 100 µL Streptokinase were used.

2.7.3 Clot lysis and statistical analysis

Clot lysis was done by a previously reported study [47]. All the positive control, negative control, and plant extract tubes were incubated again at 37 °C for 90 min. After incubation, the excreted fluids were removed from the tubes, and the tubes were also weighed to observe the difference in weight after clot disruption. The difference obtained in weight was calculated, and the result was expressed as the percentage of clot lysis following equation.

Clot lysis (%) = (weight of released clot/clot weight) × 100

The procedure was repeated ten times with the blood collected from ten different volunteers. Clot lysis is presented as a percentage (%). Data are expressed as mean±SEM, and weight differences were tested by paired t-test.

2.8 Test for Reducing Power Activities

Different extracts of EEGH were subjected for testing reducing power activities. Ascorbic acid
(1mL) was used as the reference standard. A method describes by Lee, K [48] employed in this test. According to the method, different extracts and standards were mixed with 3mL 0.2 M potassium buffer and 3 mL of potassium ferricyanide in various test tubes numbered. Then heat for 20 minutes at 50°C and additionally 10% of 3 mL trichloroacetic acid solution was added in the test tube and subjected for centrifuged at 3200 rpm for 12 minutes. Finally, 3 mL of the supernatant solution was taken from the mixer and mixed with 0.1% 0.5 mL ferric FeCl₃ solution and 3 mL distiller water. Absorbance was taken at 700 nm.

3. RESULTS

3.1 Effect of Plant Extract on Acute Toxicity Studies

Up and Down methods have been accomplished to depicts the acute toxicity of the aqueous and ethanolic extract of EEGH at different doses (200, 400, 2000, and 4000 mg/kg) in both mice and rats caused no death. Throughout short- and long-term close monitoring, no lethal effects were observed. No toxic effect was reported for up to 14 days. Moreover, movement, eating habits, posture, fur brightness, and skin color were normal. Therefore, the ethanolic and aqueous extract seemed safe, and oral median lethal dose (LD₅₀) in both mice and rats necessarily greater than 4000 mg/kg.

3.2 Effect of Metformin in Oral Glucose Tolerance Test (OGT)

Fasting blood glucose level was determined by Metformin, a drug that reduced blood sugar level in the different time intervals in hyperglycemic rats over 135 minutes (Fig. 1). Time interval was 0, 45, 90 and 135 minutes. Reduced blood glucose level was observed 45 minutes after an oral metformin dose of 1000mg/70 BW per day. In 45 minutes, the blood glucose level was 7.15 ± 0.104 mmol/L, and a successive reduction was followed at a time of 135 minutes, and the blood glucose level was average 6.10 ± 0.011 mmol/L. It was noted that after 45 minutes’ blood glucose level in the glucose control group was 11.03 ± 0.221 mmol/L. Thus, Metformin could be used as a promising control to observe the efficacy of the test samples.

| Groups | Treatment |
|--------|-----------|
| Group I | 6 ml/kg/day distilled H₂O, p.o.; (Normal control, NC) |
| Group II | 6 ml/kg/day H₂O + 24 ml/kg of 0.5 M HCl in 70% EtOH |
| Group III | 200 mg/kg/day EEGH + 24 ml/kg of 0.5 M HCl in 70% EtOH |
| Group IV | 400 mg/kg/day EEGH + 24 ml/kg of 0.5 M HCl in 70% EtOH |
| Group V | 400 mg/kg/day EEGH+ Omeprazole, 30 mg/kg/day + 24 ml/kg of 0.5 M HCl in 70% EtOH |

Table 1. Grouping of experimental animals for screening anti-ulcer activities

| Groups | Treatment |
|--------|-----------|
| Group I (Control) | 6 ml/kg/day distilled H₂O, p.o.; (Normal control, NC) |
| Group II (Omeprazole) | Omeprazole, 30 mg/kg/day + 24 ml/kg of 0.5 M HCl in 70% EtOH |
| Group III(Ethanol) | 400 mg/kg/day EEGH+ Omeprazole, 30 mg/kg/day + 24 ml/kg of 0.5 M HCl in 70% EtOH |
| Group IV (Ethyl Acetate) | 400 mg/kg/day Ethyl Acetate extract+ Omeprazole, 30 mg/kg/day + 24 ml/kg of 0.5 M HCl in 70% EtOH |
| Group V (n-hexane) | 400 mg/kg/day n-hexane extract + Omeprazole, 30 mg/kg/day + 24 ml/kg of 0.5 M HCl in 70% EtOH |
| Group VI (PET) | 400 mg/kg/day PET extract+ Omeprazole, 30 mg/kg/day + 24 ml/kg of 0.5 M HCl in 70% EtOH |
| Group VII (CCL₄) | 400 mg/kg/day CCL₄ extract+ Omeprazole, 30 mg/kg/day + 24 ml/kg of 0.5 M HCl in 70% EtOH |
| Group VIII (n-butanol) | 400 mg/kg/day n-butanol extract+ Omeprazole, 30 mg/kg/day + 24 ml/kg of 0.5 M HCl in 70% EtOH |

Table 2. Grouping of experimental animals for screening anti-ulcer activities for ethanol and its five different fractions as EtOAc, n-hexane, PET, CCL₄ and n-butanol
3.3 Effect of EEGH in Blood Glucose Level in 6 Weeks

After induction of diabetes, various rat groups were treated with Metformin (1000 mg/70 kg per day per oral) and 200 and 400 mg/kg of EEGH for 6 weeks (Fig. 2). Both normal and alloxan-controlled groups were monitored simultaneously. Persistent treatment with aforesaid dose for 6 weeks' period lower blood glucose level from 17.45 ± 0.31 to 6.50 ± 0.051 mmol/L by Metformin, 16.41 ± 0.34 to 10.4 ± 0.13 mmol/L by 200 mg/kg EEGH and from 16.20 ± 0.34 to 8.89 ± 0.09 mmol/L by 400 mg/kg EEGH.

![Graph showing blood glucose levels](image)

Fig. 1. Oral glucose tolerance test. Data were presented as mean ± SEM; n=7 in each group. Unpaired t-test was done where is p-value range from *p<0.05 and *** p<0.01

![Graph showing blood glucose levels](image)

Fig. 2. Blood glucose level reduction before and after six weeks. Data were presented as mean ± SEM; n=7 in each group, *p<0.05. An unpaired t-test was done, and p-value range from *p<0.05 and *** p<0.01
3.4 Effect of EEGH in Blood Glucose Level in 12 Weeks

The above study viewed the potential glucose-lowering effects of EEGH; we aimed to analyze the combined effects with Metformin. Regular treatment with both Metformin and combined dose (400 mg/kg EEGH + Metformin) for a prolonged period of 12 weeks it was revealed that both doses were significantly reduced blood glucose level from 16.56 ± 0.34 to 6.20 ± 0.05 mmol/L and 16.78 ± 0.1 to 6.4 ± 0.04 mmol/L respectively (Fig. 3). And both 200mg/kg EEGH and 400 mg/kg EEGH displayed moderate sugar lowering effect, (200mg/kg: 17.0 ± 0.34 to 9.87 ± 0.21 mmol/L and 400 mg/kg: 16.20 ± 0.34 to 8.12 ± 0.28 mmol/L). The drug Metformin and EEGH do not affect each other’s functions and might be used combined as a potential anti-diabetic therapy.

3.5 Evaluation of the Impacts of the EEGH on the Biochemical Parameters (TG, TC, LDL & HDL) in 6 Weeks and 12 Weeks Treatment

Plasma lipids are related to diabetes and cardiovascular diseases (CVD), prompting the authors to monitor these biochemical parameters in abnormal conditions compared with the standard drug simvastatin. Table 3 illustrated lipid level reducing Simvastatin’s capacity, EEGH (200 and 400 mg/kg), and combined of both (drug and extract) after successive 6 and 12 weeks treatment separately. After 6 weeks treatment it was observed that Simvastatin, 200 mg/kg EEGH, 400 mg/ kg EEGH alleviated TC by 29%, 4% and 5%, TG by 42%, 11%, and 10%, LDL by 30%, 3% and 7%. In 12 weeks treatment procedure, combined dosages (400mg/kg EEGH + Simvastatin) regimen was introduced and found that Simvastatin, combined dose, 200mg/kg EEGH and 400 mg/kg EEGH turn down TC level by 42%, 43%, 11% and 12%, TG level by 38%, 40%, 5% and 7%, LDL level by 31%, 33%, 2% and 7% respectively. On the other hand, HDL level was surged both in 6 and 12 weeks of treatment. In 6 weeks Simvastatin, 200 mg/kg EEGH and 400 mg/kg EEGH escalate HDL by 52%, 10%, and 18% consequently. In 12 weeks after treatment with an extended period with the inclusion of combined dose displayed that Simvastatin, combined dose (400 mg/kg + Simvastatin), 200 mg/kg, and 400 mg/kg EEGH also elevated HDL level by 50%, 51%, 6%, and 13% respectively.

![Fig. 3. Blood glucose level reduction before and after 12 weeks. Data were presented as mean ± SEM; n=7 in each group, *p<0.05. An unpaired t-test was done p-value range from *p<0.05 and *** p<0.01](image-url)
Table 3. Assessment of the biochemical parameters (mg/dL) after 6 and 12 weeks

| Parameters      | Experimental animal groups                           | Normal     | Diabetic   | Simvastatin | 200 mg/kg EEGH | 400 mg/kg EEGH | 400 mg/kg EEGH+Simvastatin |
|-----------------|------------------------------------------------------|------------|------------|-------------|----------------|----------------|---------------------------|
| TC in 6 weeks   |                                                      | 170.25±3.2 | 230.54±1.2 | 164±1.22    | 221.67±5.2     | 219.78±3.9     | -                         |
| TC in 12 Weeks  |                                                      | 161.54±1.9 | 246.34±3.5 | 143±2.21*   | 219.19±1.8     | 217.23±1.1     | 140.23±3.4*               |
| TG in 6 Weeks   |                                                      | 129.34±1.3 | 201.22±3.3 | 125.2±1.1   | 198.27±2.6     | 190.23±5.2     | -                         |
| TG in 12 weeks  |                                                      | 130.56±3.1 | 198.23±2.2 | 123.6±3.2*  | 188.34±2.2     | 185.66±4.2     | 120.87±1.5*              |
| LDL in 6 weeks  |                                                      | 131.56±2.1 | 190.34±1.1 | 133.2±1.9   | 185.23±1.4     | 176.45±2.9     | -                         |
| LDL in 12 weeks |                                                      | 131.45±1.9 | 189.98±1.5 | 130.98±1.6  | 163.44±2.5     | 175.98±2.1     | 128.78±3.1*              |
| HDL in 6 Weeks  |                                                      | 60.45±3.32 | 32.67±4.32 | 64.75±2.8*  | 34.25±2.8      | 32.89±3.65     | -                         |
| HDL in 12 Weeks |                                                      | 58.34±1.98 | 32.67±4.32 | 64.75±2.8*  | 34.25±2.8      | 32.89±3.65     | 65.56±3.11*              |

Total Cholesterol: TC; TG: Triglyceride; LDL: Low-density lipoprotein and HDL: High-density lipoprotein. Data were presented as mean ± SEM; n=7 in each group, *p<0.05 represents the statistically significant values. An unpaired t-test was done.

Table 4. Evaluation the effects of the EEGH on the liver enzymes SGPT and SGOT after 6 and 12 weeks treatment protocol

| Liver enzymes (U/L) | Experimental animal groups                           | Normal   | Diabetic   | Simvastatin | 200 mg/kg EEGH | 400 mg/kg EEGH | Simvastatin + 400 mg/kg EEGH |
|---------------------|------------------------------------------------------|----------|------------|-------------|----------------|----------------|-----------------------------|
| SGPT in 6 weeks     |                                                      | 24.3±1.2 | 82.3±5.1   | 22.77±3.1   | 77.67±2.2      | 75.23±3.2      | -                           |
| SGPT in 12 weeks    |                                                      | 24.1±0.81| 81.55±3.2  | 19.12±2.1*  | 75.23±4.1      | 74.77±1.8      | 19.45±3.1*                 |
| SGOT in 6 weeks     |                                                      | 32.3±4.2 | 77.3±4.2  | 38.2±2.4    | 70.12±1.9      | 63.34±3.1      | -                           |
| SGOT in 12 weeks    |                                                      | 31.78±3.1| 78.87±1.8 | 35.10±1.1*  | 69.99±2.5      | 73.12±2.4      | 32.23±3.3*                |

Data were presented as mean ± SEM; n=7 in each group, *p<0.05 represents the statistically significant values. An unpaired t-test was done.
3.6 Assessment of the Effect of EEGH on the Liver Enzymes SGPT and SGOT

SGPT and SGOT are the common liver marker enzymes that provide information about liver damage or injury. Generally, these enzymes reside within the liver cells and spill into the blood when cells are damaged. The elevated levels of SGPT and SGOT during hypoglycemic condition was compensated by the combined dose regimen significantly (P<0.05). Table 4 instantiated effect of Simvastatin, 200 mg/kg EEGH, 400 mg/kg EEGH, and Combined dose (Simvastatin and EEGH) on liver enzymes. In 6 weeks, SGPT and SGOT level depleted 72% and 51% by Simvastatin, 5% and 9% by 200 mg/kg EEGH, and 9% and 18% by 400 mg/kg EEGH. In 12 weeks, combined dose and Simvastatin reduced liver enzyme concentration of SGPT and SGOT level by 70%, 77%, and 59%, 55% compared to the diabetic control group.

3.7 Effect of EEGH Extract on the Ulcer

To assess the mucosal cell's damage of gastric lining, the effect of crude extract of EEGH (400 mg/kg) and its five different fractions; EtOAc, n-hexane, PET, CCl4, and n-butanol was introduced. Rats were sacrificed after 60 minutes of ulcer production. Stomachs were open along the greater curvature and rinsed carefully with distilled water and the 0.9% saline to remove clots—each inner mucosal lining placed in a glass slide to the observed lesion. The lesion was magnified with BASUNE 10X handheld Magnifier USA for rudimentary observation. Intimate observation revealed that hemorrhagic lesions, redness, and subchronic gastritis were frequently noted. Damage of mucosal lining due to permeability, cell damage, and cell necrosis was markedly reported. Table 5 displayed that the control group stimulated ulcer index 6.78 ± 1.12 without any inhibition. Omeprazole (Standard) and n-butanol showed nearly comparable ulcer index inhibition percentage (91 and 90.56%) followed by PET 89% and CCl4 88% n-hexane showed (72%) lower inhibition percentage among all (Table 5).

3.8 Effect of EEGH Extract on Thrombolytic Activities

Following the procedure of incubation at 37°C for 90 minutes observation period, 100μl Streptokinase exhibited above 80% clot lysis, while water as a negative control showed only 4.5% lysis capacity (Fig. 4). The mean difference of clot lysis between standard and negative control was <0.0008 after measuring the p-value and considered extremely significant. After treating with both doses 200 mg/kg and 400 mg/kg EEGH, it showed that ethyl acetate displayed 62% and 66% clot lysis capacity. Both pet ether and n-butanol showed nearly 43% clot lysis properties on an average after ingestion of both doses. All other fraction offers moderate thrombolytic activities and ranges from 30% to 46%.

4. DISCUSSIONS

Numerous phytoconstituents acted as a vital source of finished pharmaceutical dosages form and are intensely used as folk medicine in the various localized area throughout the world. Till to date, the plant kingdom is considered a promising source of drugs, and worldwide more than 30% of finished dosages form prepared from plant sources [49,50]. In screening plant identification of safety, the level is significant to fix a dose during rudimentary pharmacological evaluation procedure [51] the acute toxicity study of Grewia hirsuta Vh. through oral route in both mice and rats using four different doses 200, 400, 2000, and 4000mg/kg revealed no sign of toxicity. According to OECD guidelines, substances having LD50> 5000 mg/kg considered

| Groups   | Ulcer index | % of inhibition |
|----------|-------------|-----------------|
| Control  | 6.78±1.12   | -               |
| Omeprazole    | 0.72±0.012 | 91.23%          |
| Ethanol       | 0.73±0.023 | 90.34%          |
| Ethyl acetate | 0.87±0.056 | 88.34%          |
| n-hexane      | 0.64±0.78  | 72.48%          |
| PET           | 0.31±0.56  | 89.23%          |
| CCl4          | 0.54±0.89  | 88.34%          |
| n-butanol     | 0.26±0.23  | 90.56%          |

Table 5. Effects of EEGH extracts on various parameters in ethanol-induced ulcer
Fig. 4. Clot lysis percentage by seven different extracts of EEGH done by paired t-test analysis. Data was represented by average ± SEM and *p* < 0.05 was considered significant.

as category five, and this postulation was well supported by Globally Harmonized Classification System (GHS) [52]. This study suggested that LD50>4000 mg/kg is safe. This finding is supported by the previously studied genus of *Grewia asiatica* [53–56].

From the OGT test, it was observed that Metformin is well tolerated and has the capacity to reduced blood glucose level over two hours; blood glucose level was 6.50±0.22mmol/L. Metformin is considered a well-tolerated drug and can reduce blood glucose levels, supporting previous studies [57–59]. No experimental rats showed any glucose intolerance in this study.

In assessing the effect of EEGH in blood glucose level after a period of six weeks, it was unfolded that treatment with Metformin and 200 and 400 mg/kg EEGH dose, Metformin lowered sugar level in the blood, and both extracts truncate glucose level moderately. The mechanistic procedure is yet to be known and needs further cell-level clarification [60,61]. On the other hand, in 12 weeks’ treatment proceeding, both 400 mg/kg and the combination of 400 mg/kg EEGH+ Metformin dose regimen revealed the satisfactory reduction of blood sugar level. The combination dose was designed to find out the additive or synergistic effect; on the contrary, 200 and 400 mg/kg EEGH was selected to find out the individual hypoglycemic effect of EEGH. These two timed- treatment protocols for screening out the hypoglycemic effect of *Grewia hirsuta* Vhal. they have revealed comparable findings with the genus of grewia [62–67].

In determining the lipid-lowering credentials of *Grewia hirsuta* Vhal. extract of ethanol was used both in 6- and 12-weeks plan of action. It was spotted that only Simvastatin lowers the TC, TG, and LDL level, but plant extract was failed to show a notable effect. Simvastatin also surged HDL level, and even combination dose was unable to reveal any synergistic or additive effect in anti-diabetic and anti-lipidemic effects [46,61,68]. From the above findings, this study suggests screening active constituents responsible for anti-diabetic potentials and confirming that lipid-lowering capacity is absent in this plant. Various active constituents of Grewia genus, including grewialin, optivanin, alkaloids, flavones, lignans, triterpenoids, etc., are reported to be present, and thorough screening could help to find out active constituents responsible for anti-diabetic potentials [69–72].

Liver marker enzymes SGPT and SGOT level was also checked out by EEGH in both 6- and 12-week testing protocols. No test has been done previously on assessing the SGPT and SGOT level either using the plant *Grewia hirsuta* Vhal. or any genus of grewia. Extensive study results declared that this EEGH has a notable lack of capacity to lower elevated SGOP and SGOT levels in alloxan-induced diabetic rats. No similar literature was found to support these findings.

In testing gastric mucosa cell lining protection, ethanol is used as a gastric cell lining damaging agent. Two steps had assessed Anti-ulcer credentials of *Grewia hirsuta* Vhal. Firstly, ethanol extract of *Grewia hirsuta* Vhal. was used to find out the anti-ulcer credentials at a dose of 200, 400 mg/kg/day along with a combination (400 mg/kg/day EEGH+ Omeprazole) (Table 1).
The individual dose 400 mg/kg/day EEGH exhibited a remarkable ulcer inhibition capacity of 90.34%. Secondly, subsequent fractional extraction of EtOAc, n-hexane, PET, CCl₄ and n-butanol were assessed to further confirmed anti-ulcer potentials among six different extracts (Ethanol, EtOAc, n-hexane, PET, CCl₄ and n-butanol) of Grewia hirsuta Vhal., n-butanol and Ethanol exhibited nearly similar percentage of inhibition as standard Omeprazole (90.56%, 90.34%, and 91.23% respectively). The third highest inhibition was reported by PET, which was 89.23%. All different fractions showed an average above 70% inhibition (n-hexane 72.48%, PET 89.23%, CCl₄ 88.34%), which claims that EEGH extract has notable anti-ulcer activities. This study is like another genus of Grewia Hirsuta, namely Grewia asiatica L. [73–75].

Streptokinase was used as the reference standard for assessing thrombolytic activities and showed 80% clot lysis capacity. Extensive testing with different fractions of EEGH in both doses 200 and 400 mg/kg revealed that 200 and 400 mg/kg EEGH extract in ethyl acetate showed significant (62% and 66%) clot lysis capacity followed by n-butanol. All fractions show moderate thrombolytic activities. No single report was found after an extensive literature search on testing thrombolytic activities of Grewia hirsuta Vhal. But identified in another genus of Grewia bicolor [70,76].

5. CONCLUSION

Extract in ethanol of G. hirsuta Vhal. manifested potent anti-ulcer activities but moderate anti-diabetic and thrombolytic potentials. On the other hand, this plant has no lipid-lowering capacity. For all claimed activities, cell-level mechanisms need to be understandable with different research approaches. To consider this plant as a potent source of therapeutic activities, screening of active constituents is mandatory.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

ETHICS APPROVAL

The Biomedical Research Center approved all experimental procedures, University of Dhaka, Bangladesh (Ref. no. BMRC/EC/2018-19/331).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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