RESEARCH ARTICLE

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Antihyperglycemic, Neuropharmacological, Cytotoxic, Anticoagulant, and Anti-inflammatory Pharmacological Evaluations of *Vernonia elaeagnifolia* DC Leaves Secondary Bioactive Metabolites

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ABSTRACT

The current investigation was carried out to confirm the antihyperglycemic, cytotoxic, neuropharmacological, anticoagulant, and anti-inflammatory effects of the ethanolic extract of *Vernonia elaeagnifolia* leaves in different experimental models. In the oral glucose tolerance test, the plant extract demonstrated significant ($p<0.05$) antihyperglycemic activity by reducing the elevated blood glucose level in mice at both doses (250 mg/kg and 500 mg/kg) when compared to the control groups. The crude ethanolic leaf extract, however, didn't afford to block the α-glucosidase enzyme up to 0.5 mg/mL. The experimental mice showed a noticeable ($p<0.001$) decrease in locomotor and exploratory activities in the hole cross and open field tests, as well as head dipping in the hole-board test model in the neuropharmacological activity assay at both doses (250 mg/kg and 500 mg/kg). The extracts also showed cytotoxic activity in a brine shrimp lethality bioassay with an LC_{50} of 91.929 µg/mL and showed significant concentration-dependent anticoagulant activity ($p<0.001$). Moreover, the extract (10–500 mg/kg) caused a significant ($P<0.01 – 0.001$) dose-dependent reduction of inflammation induced in rats by using two different models: formalin-induced paw edema and egg-albumin-induced paw edema. From the above results, it is clear that the crude extract of *V. elaeagnifolia* DC leaves has significant pharmacological potential in different *in vitro* and *in vivo* study models. So, it could be a source of a substance for isolating lead that could be used to treat several diseases.

Keywords: Anticoagulant, Antihyperglycemic, Anti-inflammatory activities, Cytotoxic, Neuropharmacological, *Vernonia elaeagnifolia*.

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I. Introduction

Since the birth of civilization, humans have used ethnopharmacological characteristics of plants or extracts of them to treat a variety of ailments, and even it has thrived alongside it. Plants are a novel source of new medication since they have evolved over millions of years by showing unique, structurally diverse secondary active metabolites to endure hostile conditions [1]. According to the World Health Organization (WHO), around 80% of the world's population

still uses plant-based traditional medicines as the first line of treatment for ailments [2]. Moreover, *V. elaeagnifolia* DC is an Asteraceae family (also recognized as Toran vel, Curtain creeper) climbing shrub and creeper that contains secondary bioactive materials such as tannins, proteins, flavonoids, phenols, alkaloids, and saponins. Traditionally, *V. elaeagnifolia* DC is commonly used to reduce phlegm and cough, sprain, inflammation, ulceration of the nose and stomach, joint pain, upper respiratory infections, and skin diseases [3]-[6]. In Bangladesh, this plant is used in folk medicine and the leaves are used as leech repellent by the locals. In addition, the plants are commonly found flourishing in homes and gardens, particularly as ornamental plants on fences [7]. From the published data, research has confirmed that *V. elaeagnifolia* DC exhibits anti-hyperlipidemic, anti-microbial, antioxidant, and anti-fungal properties, but no research on antihyperglycemic, neuropharmacological, cytotoxicity, anticoagulant or anti-inflammatory properties has been done. Hence, the present study is intended to investigate the aforementioned activities of ELVE.

II. Materials and Methods

A. Plant Extract Preparation

Vernonia elaeagnifolia DC. leaves were collected during the day from healthy host plants in Boyra, Khulna division, Bangladesh in May 2015. Experts at the Bangladesh National Herbarium in Dhaka recognized the plant components and a voucher specimen (DACB 43461) was placed for future reference. The leaves were washed and dried in the shade after being collected. After that, the shade-dried leaves were pulverized into a coarse powder with the help of a suitable grinder, (Capacitor start motor, Wuhu motor factory, China). Then, powdered material (approximately 250 g) was soaked for a week at room temperature in 95% ethanol, with intermittent shaking and stirring followed by filtering through Whatman filter paper (Whatman, United Kingdom), and solvent evaporation at 50°C using vacuum distillation equipment (Heidolph Collegiate, LABOROTA 4000, Germany). On a dry weight basis, the extraction yield was around 3.6% w/w. The final dried extract was kept at 4°C until it was used in the experiment [8].

B. Experimental Animals

For *in-vivo* pharmacological studies, young healthy Swiss-albino mice aged 4-5 weeks (20-25 gm) and rats aged 3-4 months (100-110 gm) were purchased from the Animal Resources Branch of the Centre for Diarrheal Disease Research, Bangladesh (ICCDR, B). The animals were housed in the animal house of the Pharmacy Department, Khulna University, under pathogen-free circumstances and conventional laboratory conditions (relative humidity 55-65%, room temperature (23.0 ± 2.0°C), and a 12-hour light-dark cycle). In addition, ICCDR, B formulated diet, and filtered water was fed to the animals. For the lethality bioassay, *Artemia salina* leaches (Brine shrimp eggs) was acquired from an aquarium market in Katabon, Dhaka. The Swiss Academy of Medical Sciences and the Swiss Academy of Sciences issued the “Ethical Principles and Guidelines for Scientific Experiments on Animals (1995)” as a guideline for treating animals during the experiment time.

C. Chemicals and Standards

α-Glucosidase (Type I, lyophilized powder from *Saccharomyces cerevisiae,* ≥10 units/mg protein, EC 3.2.1.20, product code- G5003-100UN), p-nitrophenyl α-D-glucoside (Product Code- N1377), dimethyl sulfoxide (product code- 472301), Tween® 80 (Product Code- P1754), formalin (product code- HT501128) were purchased from Sigma-Aldrich Chemie GmbH (Germany). Glibenclamide (antihyperglycemic), diazepam (CNS depressant), and diclofenac sodium (NSAID) were bought from Square Pharmaceuticals Limited. Acarbose (antihyperglycemic) and warfarin sodium (anticoagulant or blood thinner) was generous gift from Pacific Pharmaceuticals Ltd and Incepta Pharmaceuticals Ltd., respectively. Vincristine sulfate (antineoplastic or cytotoxic) was provided by Beacon Pharmaceuticals Limited. All other analytical-grade reagents were purchased from Zakia Enterprise (Dhaka, Bangladesh). Purified water was prepared using Lan Shan RO Water Purifier, Model: LSRO-575-G (Taiwan) in the working lab.

D. Acute Toxicity Test

The acute toxicity test was performed according to the guidelines published by the Organization for Economic Cooperation and Development (OECD 401) to evaluate the safe dose(s) to be utilized in the following in vivo test models [9]. The experimental animals were chosen at random and fasted for 16 hours overnight. For mice and rats, they were placed into five groups, each consisting of five mice and rats. ELVE was administered to each mouse and rat in each group at doses of 500, 1,000, 1,600, and 2,000 mg/kg body weight while the animals in the control group were given distilled water. In addition, a sterile feeding needle was used to provide all dosages orally. For the first two hours, each animal was carefully monitored to note lethality and any physical signs of toxicity. After that, they were then monitored at 6-hour intervals for 24 hours, and then every 24 hours for the next 14 days.

E. Evaluation of Antihyperglycemic Activity

1) In-vitro α-glucosidase inhibition test

The inhibition of the α-glucosidase enzyme was colorimetrically quantified by measuring the glucose release from the Paranitrophenyl-glucopyranoside(*p*NPG) substrate, using the approach proposed by Shuyuan Liu *et al.* [10]. At 37°C, 10 µL of crude extracts dissolved in DMSO at various concentrations (from 0.156 to 4.0 mg/mL) were incubated in a 96-well plate with 20 µL of enzyme solution (1 unit/mL) and 112 µL of potassium phosphate buffer (PBS, pH 6.8). After 15 minutes of incubation, the reaction was started by adding 20 µL of *p*NPG to each well and incubating the mixture for the next 15 minutes at 37°C. Then, the reaction was eventually stopped by adding 80 µL of Na_2CO_3 solution and measuring the final absorbance at 405 nm with a microplate reader (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer). Acarbose was used as a standard at varying concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL). The crude extract's α-glucosidase inhibitory activity was reported as the IC_{50} based on the % inhibition calculated using the equation below:

$$\% \text{ Inhibition} = 1 - \left(\frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{sample blank}}}{\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}}}\right) \times 100$$

where Abs $_{\text{sample}}$ was the absorbance of the mixture of PBS, *α*-glucosidase enzyme, sample (crude extract/Acarbose), and *p*NPG; Abs $_{\text{sample blank}}$ was the absorbance of the mixture of PBS, sample (crude extract/Acarbose), and *p*NPG; Abs $_{\text{control}}$ was the absorbance of the mixture of PBS, α-glucosidase enzyme and *p*NPG; Abs $_{\text{blank}}$ was the absorbance of the mixture of PBS and *p*NPG.

2) In-vivo Oral Glucose Tolerance Test (OGTT)

Oral glucose tolerance tests in the mouse model were evaluated using slight modifications of the Djilani, Toudert, and Djilani reported method [11]. A total of 25 mice were divided into five groups randomly, with five mice in each group, and fasted overnight. Group 1 received 2% Tween-80 in water at a dose of 10 mL/kg body weight; Group 2 received reference medicine glibenclamide at a dose of 10 mg/kg body weight; and Groups 3 to 5, the treatment groups, received ELVE at doses of 100 mg, 250 mg, and 500 mg per kg body weight, respectively. Before providing control, standard, and test samples, the blood glucose levels of all mice in each group were monitored with a glucometer (EZ Smart-168, Tyson Bioresearch Inc., Taiwan) at the fasting stage. With the use of a sterile feeding needle, a blood sample was collected by punching the tail tips. Moreover, to prevent infection and irritation, the exposed portion of the tail was covered with povidone-iodine ointment after the blood was collected. Control, standard, and test samples were given to each group of mice 30 minutes before they were given glucose orally using a feeding needle. After glucose administration, blood glucose levels were tested 30 minutes, 90 minutes, and 150 minutes afterward. A clean environment and strong ethics were maintained throughout the experiment.

F. Evaluation of Neuropharmacological Activity Test

1) Open field test

The open-field test model was evaluated using slight modifications proposed by Shilpi *et al.* to conduct the neuropharmacological activity of crude extract [12]. In this experiment, 25 experimental laboratory mice (25-30 g) were randomly selected and put into five groups, each with five animals. Group-1, referred to as the control group, was given 1% tween 80 in water at a dose of 10 mL, Group-II, referred to as the standard group, was given diazepam at a dose of 1 mg, and Group-III and group-IV referred to as the test groups, were given ELVE at doses of 250 mg and 500 mg, respectively. A sterile feeding needle was used to administer the control, reference medication, and extract orally. After receiving their treatments, each mouse in each group was placed in one of the corners of square grids filled with black and white colors in sequential order (100 cm × 100 cm × 40 cm). During the whole study period, the number of squares visited by each mouse was recorded for 3 minutes at intervals of 0, 30, 60, 90, 120, and 180 minutes. The entire experiment took place in a completely silent environment.

2) Hole cross test

The hole cross-test was evaluated using slight modifications of Uddin *et al.* [13]. For the test, a wooden box (30 cm × 20 cm × 14 cm) was utilized, which was divided into two chambers by a wall with a 3 cm diameter hole at a height of 7.5 cm from the bottom. A total of 25 mice were divided into five groups at random and given the same treatment as in the previous experiment. After the control, standard, and test samples had been treated, each mouse was carefully placed on the darker side of the wooden box, and spontaneous movement through the hole was counted for 3 minutes at 0, 30, 60, 90, 120, and 180 minutes. During the experiment, a sound-attenuated environment was tightly maintained.

3) Hole board test

The experiment was carried out with minor modifications to the procedure reported by File, and Wardill [14]. In this experiment, a hardwood board (40 cm × 40 cm) with 16 evenly dispersed 3 cm diameter and 2.2 cm depth holes were used. Twenty-five mice were divided into different groups and given the treatment. Each group's mouse was placed on the board's edge after receiving its treatment. The number of heads dipping into the holes was counted for three minutes at 0, 30, 60, 90, 120, and 180 minutes during the observation period. Throughout the testing, a sound-attenuated environment was strictly maintained

G. Brine Shrimp Lethality Bioassay

The cytotoxic activity of ELVE was studied by adopting brine shrimp lethality bioassay as illustrated by Meyer *et al.* [15]. Artemia salina eggs were hatched in simulated seawater with appropriate aeration and matured to nauplii for up to 48 hours at room temperature (25-30°C). The test solution was made by dissolving ELVE in simulated seawater using the cosolvent Dimethyl Sulfoxide (DMSO), then serial dilution to create 10 mL solutions ranging from 5 µg/mL to 320 µg/mL in each test tube. The standard, vincristine sulphate, was serially diluted in each test tube to generate a 10 mL solution ranging from 0.312 µg/mL to 5 µg/mL concentration. DMSO in 10 mL simulated seawater was used as a control. Under the illumination, each test tube containing 10 live brine shrimp nauplii in each group was left uncovered. Using a 10X Handheld Magnifying Glass, the number of alive shrimps was counted and recorded after 24 hours. To avoid statistical error, the experiment was repeated three times and the results were confirmed. The percentage mortality rate of brine shrimp nauplii at each concentration was estimated using the following equation:

$$\% \text{ Mortality} = \frac{C_a - S_a}{C_a} \times 100$$

where C_a = Avg. no. of alive shrimp in the control test tube and Sa = Avg. no. of alive shrimp in the test sample or standard test tube.

H. Evaluation of Anticoagulant Activity

The crude extract's anticoagulant activity was determined using the prothrombin time (PT) test method described by Anisuzzman *et al.* [16]. With the use of a disposable polypropylene sterile syringe, blood was taken from the vein of each healthy volunteer's right arm and stored in centrifuge tubes coated with 3.8% trisodium citrate to prevent blood coagulation. To collect pure plasma, blood samples were spun at 3,000 rpm for 15 minutes with the use of a centrifugal

machine. The platelet-enriched plasma was carefully removed from suspended blood particles and kept at 40°C until experiments were conducted. Each test tube included 100 µL ELVE at various concentrations ranging from 43.75 mg/mL to 350 mg/mL, 200 µL platelet plasma from each volunteer, and 300 µL $CaCl_2$ at a concentration of 25 mmol, all of which were thoroughly mixed. Instead of crude extracts, 100 µL of 0.9 % saline and 100 µL warfarin were used in two test tubes for the control and positive controls, respectively (standard). All test tubes were incubated at 37°C to enhance plasma clotting, which was visually seen by tilting the test tube often, and the clotting time was recorded with the help of a stopwatch.

I. Evaluation of In-vivo Anti-inflammatory Activity

1) Formalin-induced paw edema model

The in-vivo inflammatory efficacy of ELVE crude extract was evaluated in rats' paw edema caused by injecting 100 µL of 2 % formalin into the sub-planter area of the rats' right hind paw according to the method described by Sadeghi *et al.* [17]. Twenty-five experimental rats (160-170 g) were screened at random and divided into four groups (5 rats per group): control, standard, and two test groups. The extract (250 mg/kg and 500 mg/kg, p.o.) and diclofenac sodium (10 mg/kg, p.o.) were administered 30 minutes before the formalin challenge to the test and control groups, respectively. A digital caliper was used to measure the thickness of the paw 30, 90, 150, and 210 minutes after formalin injection. The percentage (%) of edema inhibition is computed using the following formula:

$$\%\ \text{Inhibition of edema} = \frac{T_o - T}{T_o} \times 100$$

where T_o is the thickness of the paw of control group rats; T is the thickness of paw edema of test groups/standard group rats.

2) Egg-albumin-induced paw edema model

To test the anti-inflammatory potentiality of ELVE, paw edema was generated by injecting egg albumin on the sub-plantar surface of the right-hand paw of rats according to the method described by Rungqu *et al.* [18]. Twenty-five rats (160-170 g) were divided into groups as described above, and each baseline right hind paw diameter was measured with a digital caliper. The control group received 10 mL/kg of tween 80 (10%) as a vehicle; the standard group received 10 mg/kg of diclofenac sodium as a reference medicine; and the two test groups received 250 and 500 mg/kg of crude extract, respectively. Using a sterile feeding needle, all dosages were administered orally. After 30 minutes, each rat was given 100 µL ml of 50% (v/v) fresh raw egg albumin to produce edema. A digital caliper was used to measure paw sizes (thickness) at 30, 90, 150, and 210 minutes following albumin injection. The results were expressed as a percentage (%) of edema inhibition, which was determined using the following formula:

$$\%\ \text{Inhibition of edema} = 1 - \frac{\text{The thickness of paw edema of the test or positive control group}}{\text{The thickness of the paw of the control group}}$$

J. Statistical Analysis

Statistical analysis was carried out using Microsoft Excel and GraphPad Prism version 8.0 (GraphPad Software Inc., San Diego, CA). The mean and standard error of the mean was used to express the experimental results. For statistical comparison, Bonferroni's test (Two-way ANOVA) was used. Data were considered statistically significant due to *p< 0.05 in all experiments. The median lethal value (LC_{50}) in the brine shrimp lethality test was determined using Probit analysis software (Ldp line software, USA).

III. RESULT

A. Acute Toxicity

Neither death nor any side effects were observed in animals for an entire period of 14 days following oral administration of different doses of extract (500, 1,000 1,600, and 2,000 mg/kg body weight). Hence, these results indicate that the ethanolic leave extract of the plant was not toxic up to an oral dose of 2,000 mg/kg body weight.

B. Evaluation of Antihyperglycemic Activity

1) In-vitro α-glucosidase Inhibition

Fig. 1 summarizes the in-vitro α-glucosidase inhibitory activity of ELVE and standard. The crude extracts had no discernible inhibition of the α-glucosidase enzyme up to 0.5 mg/mL, whereas Acarbose, a reference drug, showed α -glucosidase inhibitory activity with an IC_{50} of 0.38 mg/mL.

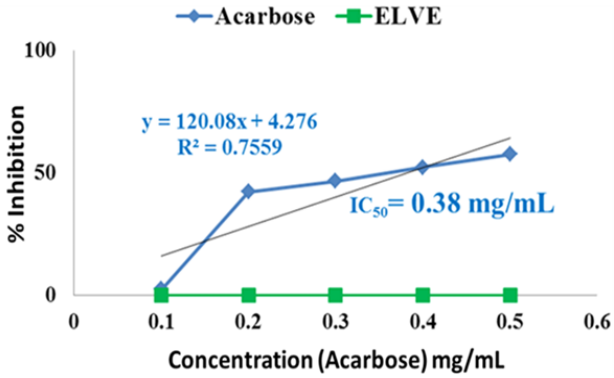

Fig. 1. α-glucosidase inhibitory assay Decrease of blood glucose concentration after 30 minutes of glucose feeding and continues for up to 150 minutes.

2) In Vivo Oral Glucose Tolerance Test (OGTT)

The glucose tolerance efficacies of the ELVE in glucose-loaded mice were presented in Table I. ELVE, at the dose of 250 and 500 mg/kg body weight, significantly (*p<0.05 and ***p<0.001) manifested the reduction of fasting blood glucose concentration at 30 min after feeding glucose (3 g/kg) in comparison to the control and continued for up to 150 minutes. However, the lowest dose (100 mg/kg body weight) of ELVE did not show a significant effect in lowering blood glucose levels.

C. Evaluation of Neuropharmacological Activity Test

1) Open field test

The crude leaf extract at 250 mg and 500 mg per kg body weight doses reduced the number of squares traveled by mice in a dose-dependent and significant manner (Fig. 2).

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TABLE I: EFFECTS OF ELVE ON GLUCOSE-LOADED MICE (MEAN ± SEM) (N=5)

Group	Fasting state (mmol/L)	30 min (mmol/L)	90 min (mmol/L)	150 min (mmol/L)
Control	5.70 ± 0.24	13.26 ± 0.67	9.74 ± 0.35	7.16 ± 0.24
Standard	5.96 ± 0.12	7.38 ± 0.19***	4.88 ± 0.21***	3.18 ± 0.17***
100 mg/kg ELVE	5.82 ± 0.12	13.02 ± 0.55	9.08 ± 0.24	7.06 ± 0.093
250 mg/kg ELVE	5.92 ± 0.13	11.08 ± 0.43***	6.92 ± 0.38***	5.96 ± 0.37*
500 mg/kg ELVE	5.76 ± 0.14	9.72 ± 0.32***	5.90 ± 0.48***	5.1 ± 0.35***

*p <0.05 vs. control, ***p <0.001 vs. control.

The reduction was manifested at 30 minutes and persisted up to 90 minutes for both test groups, with the standard group receiving diazepam (1 mg/kg body) in comparison to the control group. At 90 minutes, it was obvious that both the reference drug and ELVE had the greatest effect.

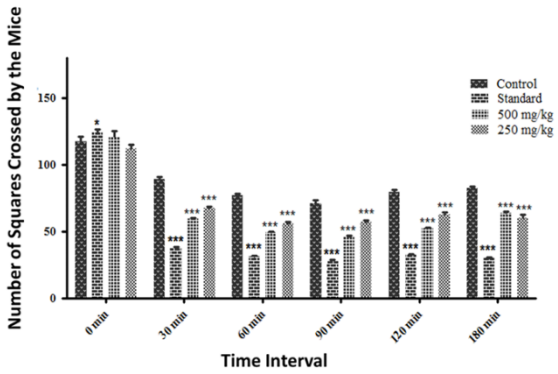

Fig. 2. Neuropharmacological effect of ELVE on the open field test model. *p<0.05 vs. control, ***p<0.001 vs. control. Values are expressed as the mean ± standard error of the mean (n = 5).

2) *Hole cross test*

The results (Fig. 3) showed that at the dose of 500 mg/kg body weight, the locomotor activity of mice decreased significantly (*p<0.05, **p<0.01, and ***p<0.001) from the second observation period (30 min) to the fifth observation period (120 min). However, when compared to the control group, half of the highest doses did not affect the mice's locomotion.

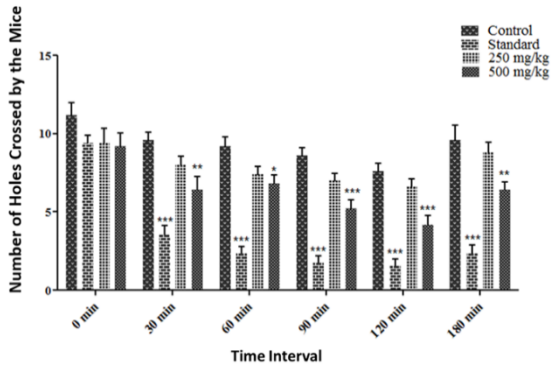

Fig. 3. Neuropharmacological effect of ELVE on hole cross test. *p<0.05 vs. control, ***p<0.001 vs. control. Values are expressed as the mean ± standard error of the mean (n = 5).

3) *Hole board test*

The crude extract showed significant potentiality in terms of head dipping at the dose of 500 mg/kg body weight at 60 minutes (*p<0.05) and persisted up to 180 minutes with significant effect (***p<0.001), whereas, at the lowest dose (250 mg/kg body weight), the crude showed significant effect from 5th to 6th time point (*p<0.01 and ***p<0.001) in comparison with control.

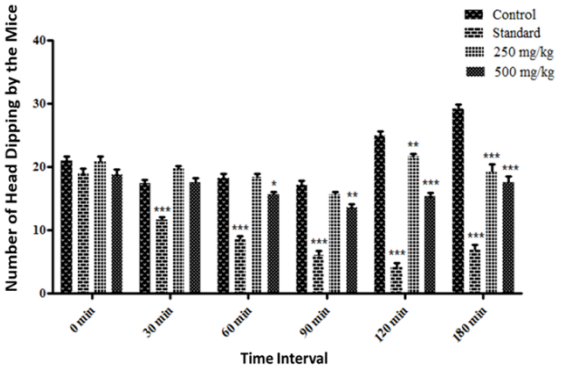

Fig. 4. Neuropharmacological effect of ELVE on hole board test. *p<0.05 vs. control, ***p<0.001 vs. control. Values are expressed as the mean ± standard error of the mean. (n = 5).

D. *Brine Shrimp Lethality Bioassay*

The crude ethanolic extract and the standard, vincristine sulfate, both demonstrated brine shrimp lethality in a dose-dependent manner and demonstrated a linear correlation (approx.) between sample concentration (ELVE and standard) and % response (mortality). The median lethal concentration (LC_{50}) of leaf extracts and vincristine sulfate was calculated to be 91.929 g/mL (Fig. 5) and 0.415 g/mL, respectively, in this assay (Fig. 6) by using Probit analysis software.

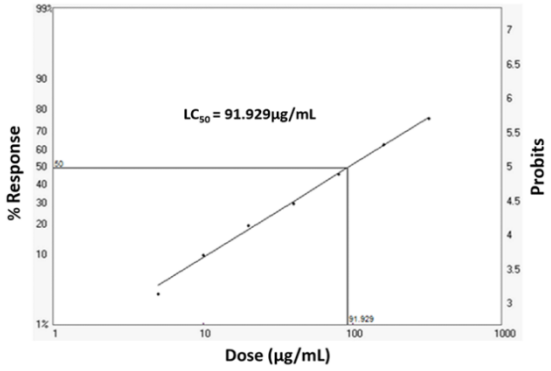

Fig. 5. Median Lethal concentration of ELVE for Brine Shrimp.

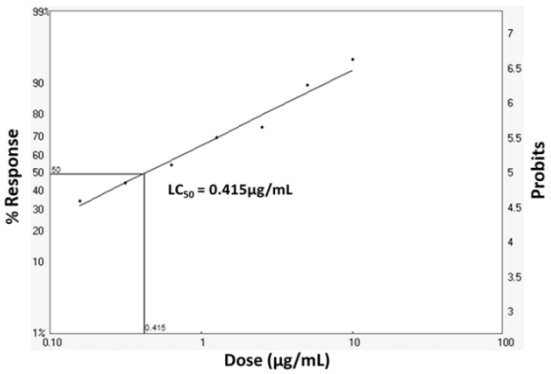

Fig. 6. Median Lethal concentration of vincristine sulphate for Brine Shrimp.

E. Anticoagulant Activity Test

In the in-vitro anticoagulant activity test, crude extract at different concentrations significantly increased (*p<0.05, and ***p<0.001) PT of normal human plasma compared to baseline clotting time (1.16 to 2 minutes). The crude extract had the greatest effect at 350 mg/mL, prolonging plasma clotting time (40.16 ± 1.72 minutes), comparable to warfarin at 0.1 mg/mL, which had a clotting time of 62.86 minutes.

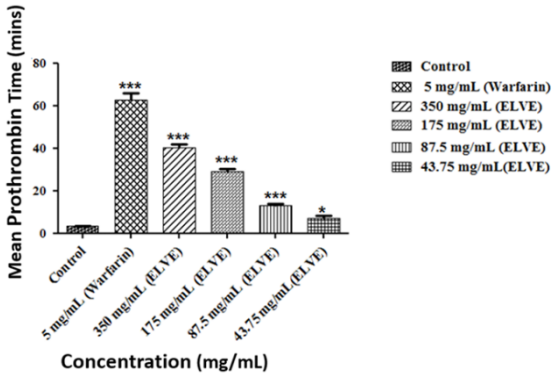

Fig. 7. The in-vitro effect of ELVE on clotting time of plasma in prothrombin time test. *p<0.05 vs. control, ***p<0.001 vs. control. Values are expressed as the mean ± standard error of the mean (n = 5).

F. Evaluation of In-vivo Anti-inflammatory Activity

1) Formalin-induced paw edema model

According to the results shown in Table II, formalin-induced significant inflammation in the hind paws of the control rats, but the crude extract at both doses (250 mg/kg and 500 mg/kg of body weight) inhibited significantly (***p<0.001) the development of formalin-induced paw edema. The highest dose of the leaf extract reduced paw edema by 71.17 % at the 4th hour of the experiment, which was comparable to the standard drug, Diclofenac sodium, which inhibited paw edema by 82.92 % at a dose of 10 mg/kg of body weight.

1) Egg-albumin-induced paw edema model

The results (Table III) showed that ELVE at 250 mg/kg and 500 mg/kg body weight caused a significant (p<0.01-0.001) and dose-dependent anti-inflammatory activity in rats by reducing egg albumin-induced edema in comparison to the thickness of pedal edema in the control group rats. The crude extract at 500 mg/kg body weight showed prominent inhibition on the 4th hour by 66.67%, which was comparable to the standard drug, Diclofenac sodium, which showed inhibition at 77.77 % at that time point.

IV. Discussion

Biological effects are dependent on secondary active metabolites found in medicinal plants, which may fluctuate in different parts of the plant in terms of the extent or/and functional groups, although leaves are thought to be the hub of maximal potential sources of bioactive substances. [19]. As a result, the current study was conducted on the leaves of *Vernonia elaeagnifolia* DC, which possessed plenty of secondary bioactive chemicals [3]. Before conducting the pharmacological study, the safe or toxic dose of crude extract should be investigated. In an acute toxicity test, ethanolic extract up to an oral dose of 2,000 mg/kg body weight showed no allergic manifestations or mortality over a 14-day observation period. As a result, it assured that the ELVE had a broad therapeutic window.

The anti-diabetic activity of the test extract was assessed using the α-glucosidase inhibitory assay and the oral glucose tolerance test. In a diabetic patient, α-glucosidase catalyzes the breakdown of the terminal (1-4)-linked *α*-glucose residues successively from the non-reducing ends of polysaccharides to release a single α-glucose molecule in the small intestine, resulting in significant glucose absorption and an exaggerated rise in blood sugar within a short period following a meal (postprandial hyperglycemia). The in-vitro α–glucosidase inhibitory activity is measured by measuring the *α*–glucosidase-mediated yellow-colored para-nitrophenol synthesis from the hydrolysis of the *α*–D-glucopyranoside portion of *p*NPG (Substrate) at 405 nm in the presence of sodium carbonate, a terminator augmented yellow color [20].

TABLE II: Effect of ELVE on Formalin-induced Paw Edema Model in Rats (mean ± SEM), (n=5)

Treatment	Dose (mg/kg)	Change of paw edema in mm (% inhibition)			
		1st h	2nd h	3rd h	4th h
Control	-	2.86 ± 0.02	2.98 ± 0.02	2.88 ± 0.02	2.81 ± 0.02
Diclofenac sodium	10	2.14 ± 0.02*** (25.17%)	1.11 ± 0.02*** (62.75%)	0.86 ± 0.02*** (70.13%)	0.48 ± 0.03*** (82.92%)
ELVE	250	2.72 ± 0.02*** (4.89%)	2.63 ± 0.02*** (11.74%)	1.71 ± 0.01*** (40.62%)	1.47 ± 0.02*** (47.69%)
ELVE	500	2.59 ± 0.02*** (9.44%)	2.35 ± 0.02*** (21.14%)	1.15 ± 0.01*** (60.07%)	0.81 ± 0.01*** (71.17%)

***p <0.001, when compared with control.

TABLE III: Effect of ELVE on Egg Albumin-Induced Paw Edema Model in Rats (mean ± SEM) (n=5)

Treatment	Dose (mg/kg)	Change of paw edema in mm (% inhibition)			
		1st h	2nd h	3rd h	4th h
Control	-	2.91 ± 0.03	3.05 ± 0.03	3.10 ± 0.03	3.06 ± 0.03
Diclofenac sodium	10	2.26 ± 0.03*** (22.34%)	1.29 ± 0.03*** (57.71%)	0.95 ± 0.03*** (69.35%)	0.68 ± 0.03*** (77.77%)
ELVE	250	2.75 ± 0.03** (5.50%)	2.60 ± 0.03*** (14.75%)	1.90 ± 0.03*** (38.70%)	1.72 ± 0.03*** (43.79%)
ELVE	500	2.55 ± 0.02*** (12.37%)	2.29 ± 0.03*** (24.91%)	1.31 ± 0.03*** (57.74%)	1.02 ± 0.03*** (66.67%)

**p <0.01 and ***p <0.001, when compared with control.

In this assay, ELVE demonstrated no inhibition of the α -glucosidase enzyme at concentrations of up to 0.5 mg/mL. In the other study, an oral glucose tolerance test was used to assess the body's ability to utilise glucose, which is a convenient and common way for proving the antihyperglycemic action of a test sample. The results (Table I) demonstrated that ELVE at 250 mg/kg and 500 mg/kg body weight exhibited statistically significant (*p<0.05 and ***p<0.001) and dose-dependent antihyperglycemic activity by lowering blood glucose levels in glucose-loaded nondiabetic mice. The lowest dose (100 mg/kg body weight), on the other hand, had no statistically significant effect; rather, glucose levels were about identical to control animals. In general, oral hypoglycemic agents maintain blood glucose levels through one or more of the following mechanisms: (i) retarding the postprandial glucose level by the inhibition of intestinal α–glucosidase, (ii) intestinal glucose release-blocking by inhibiting sodium-linked glucose transporters (SGLTs) located in the mucosal of the small intestine, (iii) extrapancreatic and pancreatic secretions [22]. Because the crude extract had no action in in-vitro alpha-glucosidase inhibition activity, the crude extract may exhibit antihyperglycemic activity via one of the two aforementioned pathways or a combination of them. The presence of alkaloids, flavonoids, and tannins in *Vernonia elaeagnifolia* DC leaves may play a key role in reducing hyperglycemia in glucose-loaded mice [23], and [24]. Since this was a preliminary study, further bio-guided separation of the responsible compound(s) from the crude extract and thereof molecular analysis should be carried out to demonstrate the exact underlying molecular mechanism(s).

The neuropharmacological activity of ELVE was carried out by adopting three distinct in-vivo test models, namely Open Field Test model, the Hole Cross Test model, and the Hole Board Test model. The action of crude extract on CNS could be evaluated by observing its effect on the locomotors' activity of the experimental mice. Locomotor activity refers to the excitability of the CNS and the decrease of this activity by depression of the central nervous system is considered as a sedative effect [25]-[27]. At the highest dose in the Open Field Test and Hole Cross Test models, a statistically significant (***p<0.001) reduction in locomotor activity was observed (Fig. 2 and Fig. 3), compared to the control group. When the lowest dose (250 mg/kg body weight) was used, there was a significant (***p<0.001) reduction in locomotor activity in the Open Field test model, but only a mild activity was shown in the Hole Cross test model when compared to the control group mice. In both test models, the sedative reference drug diazepam behaves similarly to a crude extract but to a greater extent at a dose of 10 mL/kg body weight. As a result, leaf extract may have CNS depressant properties. Head dipping (exploratory behavior) of mice is observed in the Hole broad test model, which is directly related to their emotional state [28]. The agent with depressant activity will gradually reduce head dipping, and vice versa [29], and [30]. In this experiment, the ELVE significantly (***p<0.001) and time-dependently reduced the number of head dipping of mice at both doses (250 mg/kg and 500 mg/kg) compared to the control (Fig. 4). A crude extract may exert depressant activity via one or both of the following mechanisms: (i) positive allosteric modulation of gamma-aminobutyric acid (GABA) receptors (ii) an increase in the amount of GABA [31], and [32]. GABA, the main inhibitory neurotransmitter, serves a variety of functions, including maintaining normal brain function and neuronal activity, processing information, synchronizing neuronal networks, and mediating various cognitive diseases [33]. As a result, the extract may enhance GABA-mediated hyperpolarization of postsynaptic neurons or/and will directly activate GABA receptors. Plant extracts endowed with flavonoids and alkaloids have been shown to have a CNS depressant effect by either attaching to the benzodiazepine site of the GABAergic complex system or directly or indirectly modulating GABA receptors [26]-[27] and [34]-[36]. Furthermore, the leaf extract contains tannin, which has been shown to have nonspecific CNS depression activity [37]. As a result, the presence of alkaloids, flavonoids, and tannins, among other phytoconstituents, in *Vernonia elaeagnifolia* leaf extract could be responsible for its CNS depressant activity [3].

The brine shrimp lethality bioassay is a cost-effective, time-efficient, and risk-free method for determining the cytotoxicity of a crude extract [38]-[39]. The Brine shrimp lethality bioassay is not a specific pharmacological activity testing protocol; rather, it aids in the establishment of a strong correlation between cytotoxicity and anticancer properties [40]. Enzyme inhibition, ion channel interference, or cytotoxicity could be the underlying mechanisms for nauplii lethality [16], and [41]. The crude extract and the standard drug, vincristine sulphate, were found to have concentration-dependent mortality of brine shrimp nauplii (Fig. 5 and Fig. 6). Because of its LC_{50} value of 91.929 g/mL less than 250 g/mL, the extract could be classified as toxic and a potential source of a variety of phytoconstituents, including antimicrobial agents, anticancer agents, antimalarial drugs, and insecticides [42], and [43]. The abundance of phytochemical groups (alkaloids, flavonoids, and tannins) in the plant extract may be responsible for Brine shrimp cytotoxicity [3], and [44].

The extract's in-vitro anticoagulant activity is measured by measuring the time it takes a blood sample to clot, also known as prothrombin time. Prolongation of Prothrombin time is used to assess an agent's anticoagulant activity because it indicates the blockage of the extrinsic clotting factor pathway, which includes factors V, VII, and X, all of which are generally activated by vitamin K [45]. In the current study, crude extracts showed a significant (*p<0.05, and ***p<0.001) and concentration-dependent increase in prothrombin time when compared to the control (Fig. 7). Because Coumarin can inhibit the activation of the above-mentioned vitamin K-dependent clotting factors, the anticoagulant effect of the crude extract could be attributed to the presence of Coumarin in plant extract [45], and [46]. Furthermore, the plant extract is said to be rich in flavonoid and alkaloid compounds that exhibit anticoagulant activity by forming a complex with clotting factors to inhibit the conversion of prothrombin to thrombin and, as a result, prevent the conversion of soluble fibrinogen to insoluble fibrin clot [47], and [48].

Inflammation is defined as a complex biological response of vascular tissues to potentially harmful stimuli (pathogens, damaged cells, or irritants). It is characterized by redness, swelling, joint pain, joint stiffness, loss of joint function, heat,

and weakness. Inflammation is currently treated with nonsteroidal anti-inflammatory drugs. The anti-inflammatory study was conducted using two models: formalin-induced paw edema and egg albumin-induced paw edema. The prevention of the extent of edema induced by formaldehyde is one of the most suitable test procedures for evaluating the chronic anti-inflammation potentiality of crude extract because it closely resembles human arthritis [49]. Formalin-induced pain and peripheral tissue inflammation via neurogenic component response and tissue-mediated response. The neurogenic response is mediated by substance P and bradykinin, while the later tissue-mediated response is mediated by histamine, 5-HT, PGs, and bradykinin [50]. The egg albumin-induced paw edema model, on the other hand, is thought to be a useful tool for studying a therapeutic agent's anti-inflammatory potential [51]. Edema prompted by egg albumin can occur in three stages: the initial phase (the first 90 minutes) is characterized by the release of histamine and serotonin, the second phase (90–150 minutes) is marked by bradykinin release, and the third phase (after 180 minutes) is flagged by prostaglandin release [52]. As shown in Table II, oral administration of ELVE inhibits formalin-induced paw edema development in rats in a dose-dependent manner and exerted a significant (***$p<0.001$) anti-inflammatory effect throughout the observation period, with maximum percentage inhibition of 47.69% and 71.17% on the fourth hour at doses of 250 and 500 mg/kg body weight, respectively. In comparison to the control, ELVE at both doses caused a significant (**$p<0.01$ - ***$p<0.001$) dose-dependent and time-dependent anti-inflammatory activity by preventing the volume of egg albumin-induced edema in rats (Table III). On the fourth hour, at doses of 250 and 500 mg/kg body weight, respectively, maximum inhibition of 43.79% and 66.67% were observed. The crude extract's anti-inflammatory activity in reducing paw edema could be characterized by inhibiting the release of chemical mediators such as histamine, bradykinin, serotonin, and prostaglandin, among others. It has been reported that the plant extract is enriched with alkaloids, flavonoids, tannins, and coumaric acid, all of which have anti-inflammatory properties [3], and [45]. Flavonoids reduce inflammation by blocking molecules such as COX, cytokines, TNF- α, and matrix metalloproteinases [50]. Tannins have anti-inflammatory effects by modulating the release of inflammatory cytokines and inhibiting the production of nitric oxide (NO) and prostaglandins [53]. Alkaloids and Coumarin demonstrate anti-inflammatory properties by neutralizing chemical mediators such as histamine, serotonin, and others [54], and [55]. Based on the anticoagulant and anti-inflammatory effects of crude extract, it can be assumed that the crude extract contains anti-inflammatory phytoconstituent(s) that inhibit platelet cyclooxygenase, thereby impairing thromboxane A2-dependent platelet aggregation, similar to aspirin [56].

V. Conclusion

The current study is based on a variety of methodological approaches that indicate the ethanolic extract of *Vernonia elaeagnifolia* leaves is fortified with anti-diabetic, neuropharmacological, cytotoxic anticoagulant, and anti-inflammatory modulating compounds. As a result, a further metabolome-based bio-guided phytochemical investigation should be carried out to determine whether the observed pharmacological activities are due to the presence of well-known phytochemicals or to the presence of a new chemical entity.

Abbreviations

ELVE: Ethanolic leaves extract of *Vernonia elaeagnifolia*; OGTT: Oral glucose tolerance test; WHO: World health organization; ICCDR, B: Centre for diarrheal disease research, Bangladesh; CNS: Central nervous system; NSAID: Non-steroidal anti-inflammatory drug; OECD: Organization for economic cooperation and development; *p*NPG: Paranitrophenyl-glucopyranoside; PBS: Potassium phosphate buffer; DMSO: Dimethyl sulfoxide; SEM: Standard error of the mean; ANOVA: Analysis of variance; LC50: median lethal concentration; COX: Cyclooxygenase.

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Conflict of Interest

The authors state that they do not have any conflicts of interest.

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