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Research Article

Investigation of Analgesic, Anti-Inflammatory, Hypoglycaemic, Neuropharmacological and Cytotoxic Properties of *Clerodendrum viscosum* (Leaves)

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Abstract

Clerodendrum viscosum (CV) has been used traditionally to treat medical problems like asthma, ulcer, inflammation, pyrexia, diabetes, malaria, skin diseases, snakebite and tumor by folk practitioners. The present study evaluated the analgesic, antiinflammatory, neuropharmacological and cytotoxic properties *C. viscosum* (leaves) in rodents. Swiss albino mice of either sex weighing 25-30 gm and SD rats (150-180 mg) were divided into control (DW), standard (model specific) as well as test groups (n=6). Analgesic potential was evaluated using acetic acid-induced writhing and formalin induced paw-licking test. Anti-inflammatory properties were evaluated by xylene and croton oil induced ear edema test. Glucose tolerance was evaluated by OGTT in normal rats. Pentobarbital induced sleeping time test was applied to assess neuropharmacological activity. Also, Brine shrimp lethality bioassay method was employed for cytotoxicity evaluation. The alcoholic extracts showed significant antinociceptive activity in acetic acid test ($p<0.01$) and formalin test ($p<0.05$) at the dose of 1000mg/kg bw. The crude extract reduced inflammation significantly ($p<0.01$) in both xylene and croton oil induced ear edema test. At the dose of 1000mg/kg it increased glucose tolerance significantly ($p<0.05$) in normal rats. CV extract significantly ($p<0.01$) increased sleeping time indicating CNS depressant effect. The extract exhibited a potent cytotoxicity against brine shrimp (LC_{50}=316.23µg/ml). *C. viscosum* leaves showed analgesic, anti-inflammatory, hypoglycemic and CNS depressant effect against experimentally induced model mice. It also possessed cytotoxic properties and further studies are required to evaluate these effects and the potential of the plant.

Keywords: Analgesic; Anti-inflammatory; Antidiabetic; *C. viscosum;* Pentobarbital

Introduction

Pain is an unpleasant sensory and emotional experience associated with tissue damage [1]. Inflammation is a primary physiologic defense mechanism against infection, burn, toxic chemicals, allergens or other noxious stimuli [2]. Analgesic and anti-inflammatory agents usually relieve pain and inflammation and the existing therapeutic agents against pain and inflammation have serious adverse effects like drowsiness, nausea, gastrointestinal bleeding and ulceration [3,4]. These limitations of synthetic drugs encouraged searching for new agents and medicinal plants as well as their bioactive compounds have been documented to have advantage over such toxicity in treating pain and inflammation [5]. There are approximately 5000 plant species in Bangladesh of which about 1000 are thought to possess medicinal properties and are used in the traditional systems of medicine that serves as the primary healthcare for most of the people of Bangladesh. So, research in medicinal plants is a vital sector for the discovery of promising drugs in Bangladesh [6].

Clerodendrum viscosum (abbreviated as CV) belongs to the family of Lamiaceae, commonly known as Bhat in Bengali, is a perennial woody shrub or undershrub of about 2-4 feet in height [7-9]. The plant is found as a weed grown along the roadside and wasteland, which is available in the tropical regions of Asia including India, Myanmar, Pakistan, Thailand, Srilanka and Bangladesh. The plant contains saponins, flavonoids, alkaloids and glycosides [8,10,11]. Clerodin and Hentriacontane have been isolated from its flowers. *C. viscosum* is one of the most well-known natural health remedies in traditional practices in Bangladesh and is used for antiseptic and expectorant. Also used in ethno-medicine in the treatment of scorpion sting, tumors, leprosy and skin diseases [10,11]. The plant has been known as tonic, antipyretic and anthelmintic. The leaf and root have been widely used in asthma, tumors, dandruff, pyrexia, ascaricide, convulsion, diabetes, gravel, malaria, scabies, sore, spasm, scorpion sting, snakebite and tumor [12]. The present study investigated the analgesic, antiinflammatory, neuropharmacological and cytotoxic properties of *C. viscosum* (leaves).

Materials and Methods

Plant collection and Extraction

Clerodendrum viscosum (CV) leaves were collected, identified and authenticated from the department of Botany, Jahangrnagar University, Savar, Dhaka, Bangladesh. The collected materials were thoroughly washed in water, dried and pulverized. Then powder was extracted in soxhlet apparatus with ethanol, dried and finally a solid

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mass was obtained and preserved for analysis.

Experimental animals

For the experiment, Swiss albino mice of either sex, 6-7 weeks of age, weighing between 25-30 g, were collected from the animal research lab in the Department of Pharmacy, Jahangirnagar University, Savar, Dhaka. Animals were maintained under standard environmental conditions (temperature: 27.0±1.0°, relative humidity: 55-65 % and 12h light/12h dark cycle) and had free access to feed and water *ad libitum*. All protocols for animal experiment were approved by the institutional animal ethical committee.

Analgesic activity evaluation

Acetic acid induced writhing test: Four groups of six mice each were pretreated with the normal saline (10ml/kg), diclofenac-Na (100mg/kg) and *C. viscosum* (500mg/kg and 1000mg/kg) respectively. Forty-five minutes later, each mouse was injected with 0.7% acetic acid. The number of writhing response was recorded for each animal during a subsequent 5 min period after 15 min of the I.P. administration of acetic acid [13]. The percentage inhibition was calculated using the formula:

$$\%\ inhibition = \frac{(Mean\ number\ of\ wriths\ by\ control - Mean\ number\ of\ wriths\ by\ treated\ group)}{(Mean\ number\ of\ wriths\ by\ control\ group)} \times 100$$

Formalin-induced Paw licking test: Mice were divided into 4 groups of 6 animals each. Group 1 (control) received normal saline, group 2 (STD) received diclofenac-Na (100mg/kg). Groups 3 and 4 received the test extract 500mg/kg and 1000mg/kg p.o respectively. After 1 hour of drug administration, 2.7% formalin was injected into the dorsal surface of the left hind paw. The time spent for licking on the injected paw was recorded. Animals were observed for the 5 min post formalin (acute phase) and for 5min starting at 20th min post formalin (delayed phase) [14].

Anti-inflammatory action evaluation

Xylene induced ear edema test: Mice were divided into 4 groups of 6 animals each. Group 1, the control group received normal saline, p.o., group 2, the standard group received diclofenac-Na (100 mg/kg). Groups 3 and 4 received the test extract (500mg/kg and 1000mg/kg). One hour later, each animal received 20µl of xylene on the anterior and posterior surfaces of the right ear lobe. The left ear was considered as control. Both ears were excised one hour after xylene application and circular sections were taken and weighed. The percentage of ear edema was calculated as inflammation based on the weight of left ear without xylene [15].

Croton oil induced ear edema test: Mice were divided into 4 groups of 6 animals each. Group 1, the control group received normal saline, p.o., group 2, the standard group received diclofenac-Na (100mg/kg). Groups 3 and 4 received the CV extract (500mg/kg and 1000mg/kg). One hour later, each animal received 15µl of croton oil on the posterior surfaces of the right ear lobe and 15µl acetone on the inner surface of left ear lobe. Both ears were excised one hour after croton oil application and circular sections were taken, using a cork borer with a diameter of 3mm, and weighed [16].

Test for Hypoglycemic effect

Overnight fasted normal rats weighing 150-180 gm of either sex were divided into 4 groups of six animals each. Group-I served as control group (treated with water), group-II served as standard group (glibenclamide 10mg/kg), Group-III and IV served as ethanolic extract of *C. viscosum* 500mg/kg and 1000mg/kg respectively. Thirty minutes after administration, glucose solution was administered orally in every group. Blood sample was drawn from the tail vein by severing the tail tip and blood glucose level was measured by glucometer (Accu Check, USA) at 0, 60 and 120 minutes of treatment with glucose [17].

Neuropharmacological activity evaluation

Pentobarbital induced sleeping time test: Briefly, the animals were given (IP) a single dose of the vehicles, diazepam (2.5mg/kg), or the extracts (500 & 1000mg/kg dose). After 30 min, pentobarbital (45mg/kg, IP) was injected to induce sleep. The mice were considered asleep if stayed immobile and lost its righting reflex when positioned on its back. The time interval between pentobarbital injection and onset of sleep was recorded as sleep latency. Then total sleeping time was recorded. The animal was judged to be awake if it could return to upright position [18].

Test for cytotoxicity

Brine shrimp lethality bioassay: The eggs of Brine Shrimp were hatched at around 37°C with constant oxygen supply for two days. For the experiment, 20mg of extract was dissolved in 1ml of DMSO and adjusted up to 20ml by 3.8% NaCl. Then the solutions of varying concentrations (1600-12.5µg/ml) were obtained by serial dilution technique. 10 nauplii were exposed to different concentrations and each concentration was prepared as duplicate. The test tubes were kept at room temperature for about 24 hours and percent of mortality of napulli was counted. The median lethal concentration (LC_{50}) was determined as the measure of toxicity of the plant extract [19].

Statistical analysis

Microsoft Office Excel (2007) was used as a statistical tool for inhibition assay. Statistical analysis for animal experiments was carried out by one-way ANOVA following Dunnet's post hoc test using SPSS 16.0 for windows. Data were presented as Mean±SEM. The results obtained were compared with the control group. $P<0.05$, $p<0.01$ and $p<0.001$ were considered to be statistically significant, highly significant and very highly significant respectively.

Result

In the acetic acid-induced abdominal writhing which is the visceral pain model [20], the result presented in Figure 1, showed that the alcoholic extract of *C. viscosum* at 500mg/kg reduced the number of writhing insignificantly whereas at 1000mg/kg it reduced the number of writhing response highly significantly ($P<0.01$) when compared to the control group. The antinociceptive power was 36.32% and 59.70% respectively indicating that the extract has potent analgesic effect at 1000mg/kg, which was slightly lower but comparable to the reference drug (diclofenac-Na, 100mg/kg).

Formalin induced paw licking test indicated that, the extract of *C. viscosum* caused a dose-dependent decrease in licking time. At both first five as well as second five minutes, the effect was significant ($p<0.05$) at dose 1000mg/kg as compared to the control group treated with vehicle only. At early phase, the extract at 500mg and 1000mg/kg dose, inhibited algesia by 26.76% and 53.04% respectively. This inhibitory effect at late phase was 80.50% and 86.17% 500mg and 1000mg/kg doses respectively as compared to the control group

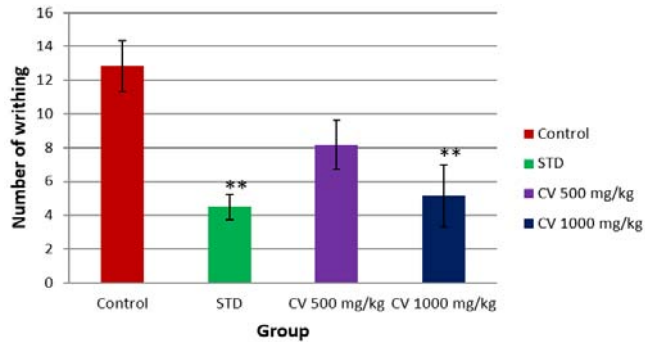

Figure 1: Effect of CV 500mg and CV 1000mg/kg in the acetic acid induced writhing test.

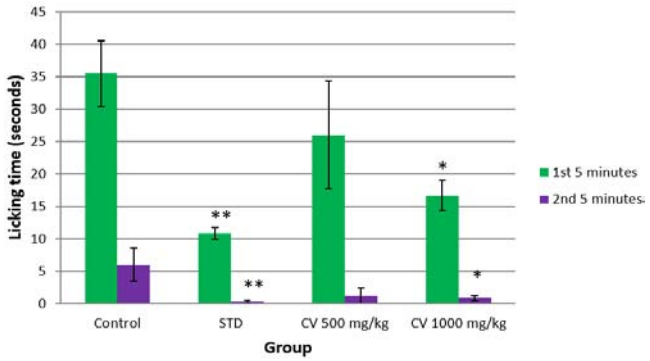

Figure 2: Effect of CV 500mg and CV 1000mg/kg in the formalin induced paw-licking test (1st 5 minutes and 2nd 5 minutes).

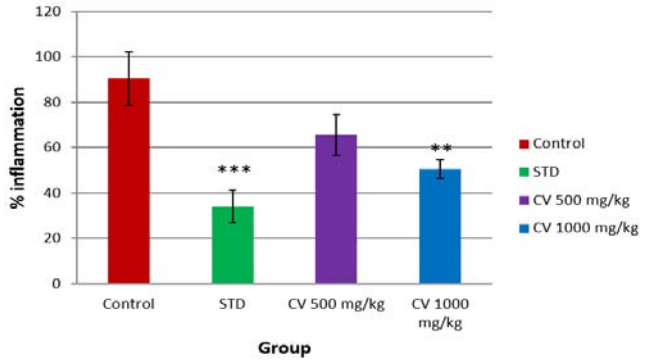

Figure 3: Effect of CV 500mg and CV 1000mg in xylene induced ear edema test.

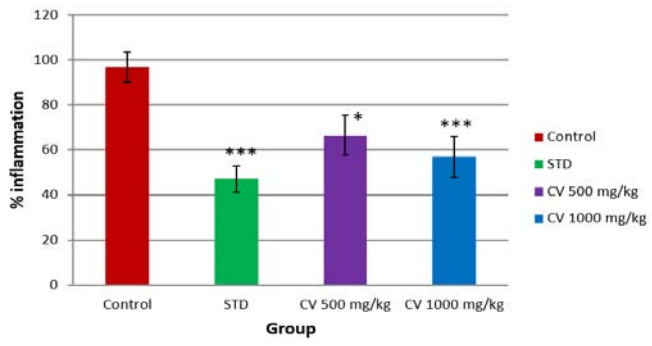

Figure 4: Effect of CV 500mg and CV 1000mg/kg in croton oil induced ear edema test.

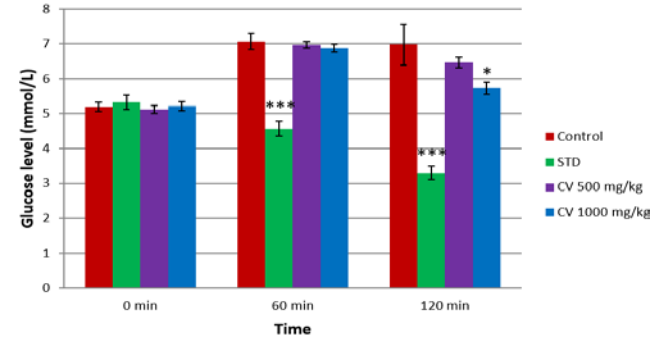

Figure 5: Effect of the ethanolic extract of CV 500mg and CV 1000mg/kg on the glucose loaded normal rats (OGTT).

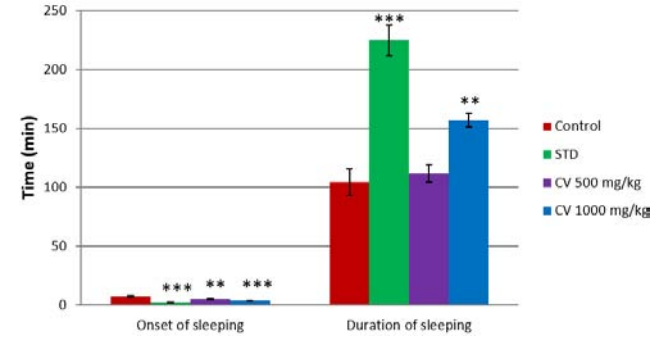

Figure 6: Effect of CV 500mg and CV 1000mg/kg on the onset of sleeping and duration of sleeping in pentobarbital induced sleeping time.

(Figure 2).

The result of xylene induced ear edema test showed that CV extract at the dose of 500mg/kg the effect was not significant but at 1000mg/kg, highly significantly ($p<0.01$), suppressed the ear swelling in mice. The rate of inhibition was 27.42% and 44.09% at 500mg and 1000mg/kg respectively. Diclofenac (100mg/kg) showed marked ($p<0.001$) anti-inflammatory activity with a 62.40% reduction of inflammation as compared to the control (Figure 3).

Antiinflammatory effect of CV in croton oil induced ear edema was shown in Figure 4. The CV extract reduced the inflammation at 500mg/kg significantly ($p<0.05$) and at 1000mg/kg dose effect was highly significant ($p<0.01$) as compared to the control group. The rate of inhibition of ear swelling at 500mg and 1000mg/kg was 31.25% and 41.11% respectively. The standard reference drug (diclofenac-Na, 100mg/kg) inhibited ear swelling highly significantly and it was 51.34%.

Ethanolic extract of *C. viscosum* exhibited hypoglycemic properties (Figure 5), lowering the blood glucose level insignificantly at 500mg/kg whereas at 1000mg/kg, reduced the acute hyperglycemic state significantly ($p<0.05$) by improving glucose tolerance in glucose loaded normal rats as compared to vehicle treated control group. In the same conditions, the hypoglycemic effect of glibenclamide was very highly significant ($p<0.001$).

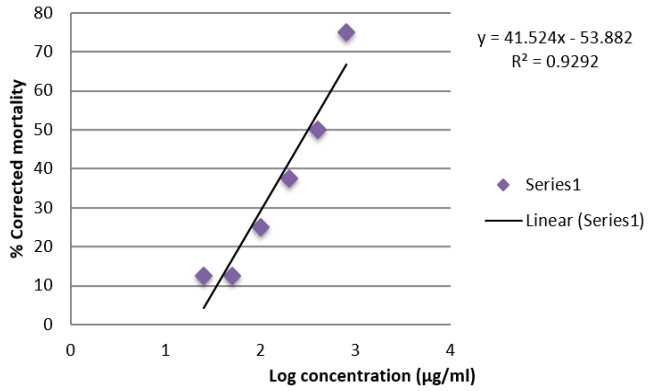

Figure 7: Graphical presentation of cytotoxic potential of ethanolic extract of *C. viscosum*.

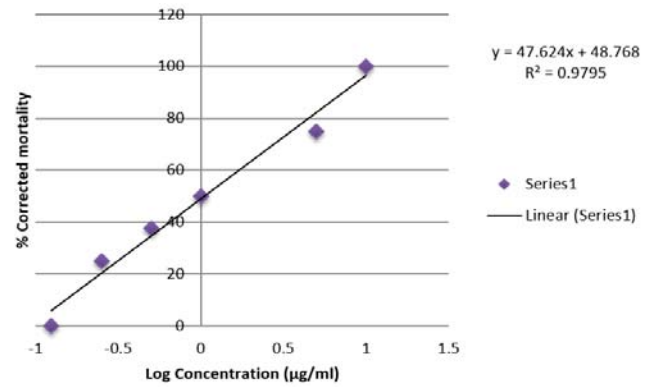

Figure 8: Graphical presentation of cytotoxicity of vincristine sulphate.

Ethanolic extract of CV decreased the onset of pentobarbital induced sleep and increased the total sleeping time highly significantly ($p<0.01$) in mice at 1000mg/kg dose (Figure 6).

The effect of *C. viscosum* leaves extract on the rate of mortality of brine shrimp larvae was presented in Figure 7 and 8. The CV extract displayed potent cytotoxic potential against *Artemia salina* (brine shrimp CV larvae) where the LC_{50} value was 316.23µg/ml and LC50 of standard drug vincristine sulphate was 1.07µg/ml.

Discussion

Plants are the essential foundation of medicine. Some important drugs that are still in use today are derived from traditional medicinal herbs. The hunt for new medicines has engaged ethnobotany and ethnopharmacology-a new route as an important source of knowledge, which led toward different sources and classes of compounds [21].

Many of the modern medicines are produced from plants. There is wide application of medicinal plants in the treatment and management of human diseases and ailments. But the claimed therapeutic reputations of such medicinal plants have to be verified in a scientific manner. In the present study one such plant *C. viscosum* leaves was taken for the study.

The analgesic activities were evaluated by two animal models. Acetic acid induced writhing model is commonly used for screening peripheral analgesics [22,23]. In general, acetic acid causes pain by liberating endogenous substances such as serotonin, histamine, prostaglandins (PGE2, PPGF2α), bradykinins and substance P, which stimulate nerve endings. Local peritoneal receptors are postulated to be involved in the abdominal constrictions response [24]. The reduction in acetic acid-induced writhes by *C. viscosum* suggested that the analgesic effect might be peripherally mediated *via* the inhibition of synthesis and release of PGs and other endogenous substances.

Formalin test is believed to be a more valid analgesic model which is biphasic and measures pain of both neurogenic (1st phase) and of inflammatory origin (2nd phase). The 1st phase (0-5min) measures centrally mediated effect and is insensitive to anti-inflammatory agents while the 2nd phase (15-30 min) which is qualitatively different from the first phase is dependent on peripheral inflammation and changes in central procession due to chemical mediators that stimulate nociception and thus induce pain [14]. This test measures the response to a long lasting nociceptive stimulus similar to clinical pain [25]. The ability of CV extract to inhibit first phase of the formalin test more prominently indicates its involvement in peripherally mediated neurogenic pain activity.

The application of mouse models of ear edema induced by different irritant agents (Croton oil, xylene, AA, phenol, histamine) have been widely used to identify the probable topical anti-inflammatory effect of the substance in study and to propose its possible mechanism of action [26]. Xylene causes instant irritation of the mouse ear, which leads to fluid accumulation and edema characteristic of the acute inflammatory response [27]. The results showed that CV extract at the dose of 1000mg/kg, significantly suppressed the ear swelling caused by xylene in mice.

Croton oil contains 12-o-tetracanoilphorbol-13-acetate (TPA) and other phorbol esters as main irritant agents. TPA is able to activate Protein Kinase C (PKC), which activates other enzymatic cascades in turn, such as Mitogen Activated Protein Kinases (MAPK), and Phospholipase A2 (PLA2), leading to release of Platelet Activation Factor (PAF) and AA. This cascade of events stimulates vascular permeability, vasodilation, polymorphonuclear leukocytes migration, release of histamine and serotonin and moderate synthesis of inflammatory eicosanoids by Cyclooxygenase (COX) and 5-Lipoxygenase (5-LOX) enzymes [28,29]. The crude extract of CV significantly reduced the inflammation, which may due to the inhibition of histamin release as well as eicosanoids synthesis.

Diabetes mellitus is a chronic metabolic disorder caused by partial or complete insulin deficiency, resulting in hyperglycemia leading to acute and chronic complications [30]. Postprandial glucose spike causes perturbation in endothelial cell function and increased blood coagulation [31]. Hyperglycemic states also increase products of glycosylation, which has a significant influence in development of diabetic induced vascular disease [32]. Therefore, management of hyperglycemic states is an important method of diabetes control.

The Oral Glucose Tolerance Test (OGTT) has long been used clinically for diabetes mellitus diagnosis and in research to evaluate the effectiveness of hypoglycaemic agents [33]. The action on glucose transporters is suggested here as a mechanism of action of plant extracts, considering that glucose is a rapid absorption sugar that

does not need the intervention of α-glucosidase for its absorption. A delay in glucose absorption indeed indicates another mechanism than the α-glucosidase inhibition previously reported for some herbs [34]. Since the extract has hypoglycemic effect in OGTT, the action may be mediated through the inhibition of glucose transporters or through the stimulation of pancreatic beta cell.

Pentobarbital-induced sleeping time test is the most widely used methods for central behavioral analysis and are sensitive ways to evaluate central stimulating activities of drugs and crude plant extracts [35,36]. Pentobarbital, a short-acting barbiturate, when given in appropriate dose, induces sedation in animals by stimulating the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) mediated postsynaptic inhibition through allosteric modification of $GABA_A$ receptors [37].

In the present study, CV decreased the time required for the onset of sleep and increased the duration of sleep as compared to control, which justifies that CV might have central depressant activity through the activation of GABA.

The brine shrimp assay is very useful tool for the isolation of bioactive compounds from plant extracts [38]. The brine shrimp lethality assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity, which in most cases correlates reasonably well with cytotoxic, anti-tumor properties and pesticidal activity [39]. The brine shrimp lethality assay has been successfully used as a simple biological test to guide the fractionation process of plant extracts in order to detect antitumour compounds [40]. In the present study, the assay revealed potent cytotoxic effect of *C. viscosum* ethanolic extract with LC_{50} of 316.23µg/ml.

Conclusion

The results obtained in this study concluded that *Clerodendrum viscosum* leaves extract possessed strong analgesic properties, showed good activity against acute inflammation. The results revealed that the plant extract has hypoglycaemic activity in glucose-loaded hyperglycemia in rats. *C. viscosum* demonstrated a little CNS depressant effect and exhibited potent cytotoxicity in brine shrimp lethality bioassay. Further studies are recommended to elucidate the precise mechanism of action and the potential of the plant extract.

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