Phyto-pharmacological Investigations of Leaves of *Cissus trifoliata* (L.)

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(Received: April 3, 2022; Accepted: June 10, 2022; Published (Web): June 22, 2022)

ABSTRACT: Repeated chromatographic separation and purification of dichloromethane soluble partitionate of methanol extract of leaves of *Cissus trifoliata* (L.) yielded a total of four compounds, namely β-sitosterol (**1**), β-sitostenone (**2**), epifriedelanol (**3**) and friedelin (**4**). The structures of the isolated chemical compounds were established by high field nuclear magnetic resonance (NMR) analyses, comparison with previously published data and co-TLC with authentic samples, whenever possible. Compounds **1**-**4** appeared to be the first time they were identified in this plant species. The methanol extract of *C. trifoliata* was assessed for central and peripheral analgesic activities by tail immersion and acetic acid-induced writhing reflex methods, respectively. The crude extract demonstrated significant central and peripheral analgesic efficacy at 200- and 400-mg/kg b.w. in mice model. The general toxicity of the extractives was assessed through a brine shrimp lethality bioassay, where the ethyl acetate (LC_{50}=0.88 μg ml^{-1}) and dichloromethane (LC_{50}=0.93 μg ml^{-1}) soluble fractions demonstrated considerable activity. The crude extract at 400 mg/kg (b.w.) also displayed significant antidiarrheal activity, whereas its dichloromethane soluble fractions exhibited moderate thrombolytic activity.

Key words: *Cissus trifoliata*, epifriedelanol, friedelin, analgesic, antidiarrheal, thrombolytic

INTRODUCTION

Plants are being explored as folk medicine around the world to heal a variety of ailments, notably obesity, arthritis, infections, cancer and diabetes. Plant extracts have medicinal potential due to their bioactive phytoconstituents that seem to have a distinct therapeutic window on the biological system.[1] Currently, the causes of an illness, along with specific plants that could be used to treat it, are gaining greater interest.[2] Bangladesh, like the rest of South Asia, has a long and fascinating history of natural medicine.[3] The bulk of these plants, nonetheless, haven't yet been chemically, pharmacologically or toxicologically assessed to discern their bioactive phytochemicals.[4]

Cissus trifoliata (L.) is a well-known medicinal plant in Mexico that has been used to alleviate infectious diseases and tumors. This can be found in the southern United States, Venezuela, Colombia, and possibly Ecuador. It can also be observed on a few Caribbean islands and southern part of Asia including Bangladesh. In Mexican folk remedies, a decoction of its stems is applied to the affected area or used as an infusion to treat gastrointestinal problems and tumors.[5,6]

A comprehensive study of the literature didn't disclose any previous scientific report on chemical and biological studies of plant leaf extracts to corroborate their meditative capabilities. As a result, the plant *C. trifoliata* was meticulously explored in this study for the isolation, purification and characterization of secondary metabolites as well as the assessment of pharmacological potentials by *in vivo* analgesic and antidiarrheal activities in mice model and *in vitro* lethality and thrombolytic activities.

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Dhaka Univ. J. Pharm. Sci. **21**(1): 69-75, 2022 (June)
DOI: https://doi.org/10.3329/dujps.v21i1.60398

MATERIALS AND METHODS

General experimental procedures. Gel permeation chromatography columns were loaded up with lipophilic Sephadex (LH-20), while analytical TLC (20×20 cm) and PTLC (20×20 cm) were performed on precoated (TLC Silica gel 60 F_{254}) plates with a thickness of 0.25 mm (Merck, Germany). UV light and/or Vanillin-H_2SO_4 spray reagent were used to visualize the spots. The ^{1}H NMR spectra were recorded in deuterated chloroform ($CDCl_3$) using a Bruker Advance 400 MHz NMR spectrometer.

Plant material collection and extraction. *C. trifoliata* leaves were collected from the hill tracts of Sylhet, Bangladesh in January, 2020. The plant was identified from the Bangladesh National Herbarium (DACB; Accession no- 59923). Sun-dried leaves were ground into a coarse powder in a high-capacity grinding machine. Methanol (2.5 L) was used to macerate the powdered component (400 gm) for 21 days. The crude extract was then retrieved by filtering the entire mixture via a fresh cotton plug and then a Whatman No.1 filter paper before being subjected to Heidolph Rotavapour at a low temperature (less than 40°C) and pressure to yield 16 gm of gummy mass. Concentrated crude extract of *C. trifoliata* (about 5 gm) was subjected to solvent-solvent partitioning using the modified Kupchan method[7] to yield *n*-hexane (HSF), dichloromethane (DMSF), ethyl acetate (EASF), and aqueous soluble partitionates.

Isolation and identification of compounds. Gel permeation chromatography over lipophilic Sephadex was used to fractionate the dichloromethane soluble fraction (LH-20). Table 1 shows the solvent systems used to separate the compounds in the column and a total of 60 test tubes were collected. TLC was used to screen the column fractions, and compound **1-3** was obtained by preparative TLC of fractions eluted with a combination of *n*-hexane, dichloromethane and methanol (2:5:1) using 5 percent ethyl acetate in toluene as the mobile phase, whereas compound **4** was purified by 25 percent ethyl acetate in toluene from the fractions eluted with the same mixture of solvents.

Table 1. Solvent systems used in Sephadex column for the analysis of dichloromethane soluble fraction of methanol extract of *C. trifoliata*.

Test tube No.	Solvent system	Proportion	Collected volume (ml)
1-40	*n*-Hexane: dichloromethane: methanol	2:5:1	80 ml
41-50	Dichloromethane: methanol	9:1	30 ml
51-55	Dichloromethane: methanol	1:1	20 ml
56-60	Methanol	100%	20 ml

Properties of the isolated compounds

β-sitosterol (1). White powder; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.35 (1H, d, *J*= 5.2 Hz, H-6), 3.54 (1H, m, H-3), 1.00 (3H, s, H-19), 0.91 (3H, d, *J*= 6.4 Hz, H-21), 0.84 (3H, t, *J*= 7.6 Hz, H-29), 0.82 (3H, d, *J*= 6.2 Hz, H-26), 0.80 (3H, d, *J*= 6.2 Hz, H-27), 0.67 (3H, s, H-18)

β-sitostenone (2). White powder; ^{1}H NMR (400 MHz, CDCl3): δ 5.72 (1H, s, H-4), 1.17 (3H, s, H-19), 0.90 (3H, d, *J*= 6.8 Hz, H-21), 0.84 (3H, d, *J*= 3.2 Hz, H-29), 0.82 (3H, d, *J*= 4 Hz, H-27), 0.81 (3H, d, *J*= 4 Hz, H-26), 0.70 (3H, s, H-18)

Epifriedelanol (3). White crystalline mass; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.73 (1H, d, H-3), 1.16 (3H, s, H-30), 1.00 (3H, s, H-26), 0.99 (3H, s, H-28), 0.98 (3H, s, H-27), 0.96 (3H, s, H-24). 0.94 (3H, s, H-29), 0.92 (3H, s, H-23), 0.85 (3H, s, H-25)

Friedelin (4). White powder; ^{1}H NMR (400 MHz, CDCl3): δ 2.34 (2H, m, H-2), 1.16 (3H, s, H-28), 1.05 (3H, s, H-27), 0.99 (3H, s, H-26), 0.95 (3H, s, H-30), 0.94 (3H, br. s, H-29), 0.87 (3H, s, H-23), 0.84 (3H, s, H-25), 0.77 (3H, s, H-24)

Biological assays

Drugs and reagents. Analytical grade reagents and chemicals were used in all the experiments. Beximco Pharmaceuticals Limited, Dhaka and Square Pharmaceuticals Limited, Dhaka kindly provided loperamide and diclofenac sodium, respectively as gift samples. Eptase (Streptokinase) (15,00,000 I.U. in each vial) was procured from Beacon Pharmaceuticals Ltd., Dhaka, Bangladesh.

Experimental animals. Breeding age Swiss albino mice (both male and female) were purchased from the International Center for Diarrheal Disease and Research in Bangladesh (icddr, b) for *in vivo* biological studies. They were housed in a laboratory controlled room with temperature ofn24 ± 2°C and relative humidity ofn60-70%. Mice were fed with rodent feed prepared and provided by icddr, b and water ad libitum. 12 Hours before and throughout the test period, food was withheld. These animals were kept in the same setting for at least 3-4 days to acclimatize to environmental changes before they were used in the experiments. The investigations were carried out in accordance to the guidelines of laboratory animal care.[8]

Study design for *in vivo* studies. For the various experimental models, mice were randomly assigned to four groups of four animals each. The mice in negative control group were given 1 percent Tween 80 in normal saline (10 ml/kg body weight). In contrast, the mice in positive control group for the experiment were provided standard medications. The experimental groups, assigned as third and fourth groups, received methanolic extract of *C. trifoliata* (MECT) 200- and 400-mg/kg b.w., respectively.

***In vivo* studies**

Central analgesic activity. The crude extract was assessed for its central analgesic potential using the tail immersion method[9], where diclofenac sodium served as the positive control. The standard was a 5 mg/kg diclofenac sodium solution made in normal saline and delivered orally.

Peripheral analgesic activity. The methanolic extract was also tested for its peripheral analgesic effectiveness using the acetic acid induced writhing method.[10] One percent acetic acid (0.1 ml/ 10g b. w.) was injected into the peritoneum of the experimental animals to trigger pain sensation. Here, diclofenac sodium (orally, 5 mg/kg of b. w.) was utilized as positive control, while 1 percent Tween 80 in normal saline was provided to the negative control group.

Antidiarrheal activity. The crude methanolic extract of *C. trifoliata* leaves was next tested for antidiarrheal activity in mouse model. Here, castor oil was used to induce diarrhea in mice.[11] The positive control group received 3 mg/kg of loperamide.[12]

***In vitro* studies**

Brine shrimp lethality bioassay. This technique was applied to evaluate the plant extracts for their cytotoxic potential using the method of Meyer *et al.*[13] Here, vincristine sulfate and DMSO were utilized as positive and negative controls, respectively.

Thrombolytic assay. The thrombolytic assay was performed utilizing approaches that had previously been used.[14] In this method, streptokinase (30 000 I.U.) and distilled water were used as positive and negative thrombolytic controls, respectively.

Statistical analysis. For statistical data analysis, triplicates of each sample were employed in all bioassays. Data were represented as mean ± SEM. The results were compared to those of the control group and were statistically evaluated (***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$). Microsoft Excel (version 2010) was used to conduct all statistical analyses.

RESULTS AND DISCUSSION

Characterization of compounds. Repeated chromatographic separation and purification of the dichloromethane soluble partitionate of crude methanolic extract of leaves of *C. trifoliata* yielded a total of four compounds (**1-4**). The chemical structures of the separated and

purified compounds were determined as two sterols namely β-sitosterol (**1**)[15] and β-sitostenone (**2**)[16] and two triterpenoids namely epifriedelanol (**3**)[17] and friedelin (**4**).[17] All the structures of the isolated compounds were confirmed by extensive NMR spectral analysis and comparing with previously published data and co-TLC with authentic samples, whenever possible (Figure 1).

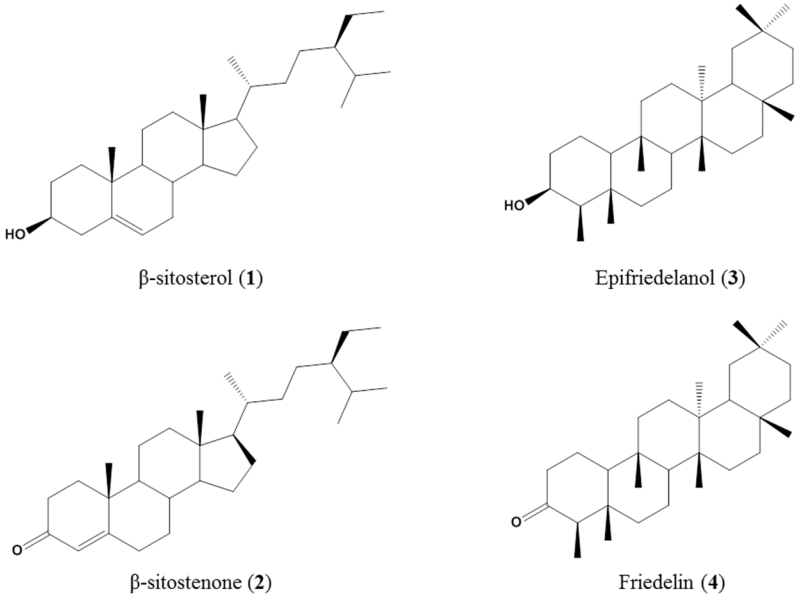

Figure 1. Chemical structures of compounds isolated from the leaves of *C. trifoliata*.

Central and peripheral analgesic activity. The methanol extract of *C. trifoliata* demonstrated significant central analgesic activity at both doses of 200- and 400- mg/kg b.w. Ninety minutes after the introduction of plant extracts, 400 mg/kg of MECT showed 196.55% elongation of response time whereas standard diclofenac sodium exhibited 445.17% elongation (Table 2).

Table 2. Central analgesic activity of MECT in mice.

Treatment	% time elongation (Mean ± SEM)		
	After 30 min	After 60 min	After 90 min
Diclofenac sodium (5 mg/kg)	139.55% ± 0.079***	197.86% ± 0.122***	445.17% ± 0.095***
MECT (200 mg/kg)	56.36% ± 0.103**	75.8% ± 0.053***	136.21% ± 0.224***
MECT (400 mg/kg)	75.91% ± 0.062***	109.25% ± 0.072***	196.55% ± 0.344***

Values are presented as Mean ± SEM (n = 4); *p < 0.05, **p < 0.01 and ***p < 0.001 were considered as significant; SEM = Standard error of mean

Evaluation of the statistical data verified that the crude MECT also exhibited significant peripheral analgesic activity for both 200- and 400- mg/kg doses. MECT at doses of 200- and 400- mg/kg exhibited an inhibition of abdominal writhing by 39.53% and 55.81%, respectively, whereas standard diclofenac sodium (5 mg/kg) displayed an inhibition of 77.91%. Abdominal writhing was diminished in a dose-dependent manner (Table 3).

Natural analgesics are being investigated as alternatives to manufactured medications since they have fewer adverse effects.[18] We used a tail immersion model and an acetic acid-induced writhing test to assess central and peripheral analgesic efficacy of *C. trifoliata*, respectively. Both of these methods are well established and

standard pharmacological practices for evaluating analgesia by natural compounds.[19] Analgesics with a central action work by raising the tolerance level and reconfiguring the physiological response to pain.[20] The inflammatory response is incited by intraperitoneal delivery of 1% acetic acid and thus, the synthesis of prostaglandins (particularly PGE2 and PGF2[21]) and histamine.[22] In our investigation, the studied fractions of *C. trifoliata* leaf extract at doses of 200- and 400-mg/kg b.w. reduced analgesia in Swiss albino mice significantly ($p< 0.05$). It's possible that they were operating via both central and peripheral pathways. To separate the relevant components, more phytochemical research is required.

Effect of MECT on castor oil induced diarrhea. MECT was also tested for antidiarrheal potential in a preclinical model. Although MECT at 200- and 400-mg/kg reduced the percentage of diarrheal feces by 19.15 and 27.66, respectively, the effect was not significant. In contrast, standard loperamide reduced diarrheal feces by 65.96% (Table 4).

Table 3. Peripheral analgesic activity of MECT in mice by acetic acid induced writhing test.

Test group	Mean writhing	% Inhibition of writhing (Mean ± SEM)
Control (1% Tween 80)	21.5	-
Diclofenac sodium (5 mg/kg)	4.75	77.91% ± 0.4146***
MECT (200 mg/kg)	13	39.53% ± 0.6124***
MECT (400 mg/kg)	9.5	55.81% ± 0.559***

Values are presented as Mean ± SEM (n = 4); *p < 0.05, **p < 0.01 and ***p < 0.001 were considered as significant; SEM = Standard error of mean

Table 4. Antidiarrheal effect of methanol extract of *C. trifoliata* leaves on castor oil induced diarrhea in mice.

Treatment	% Reduction in the number of diarrheal feces in mice			
	After 1 hour	After 2 hours	After 3 hours	After 4 hours
Loperamide (3 mg/kg)	50% ± 0.2887	21.3% ± 0.4787	64.45% ± 0.478**	65.96% ± 0.707***
MECT (200 mg/kg)	50% ± 0.2887	7.14% ± 0.4787	9.68% ± 0.2887	19.15% ± 0.645*
MECT (400 mg/kg)	75% ± 0.25	14.3% ± 1.224	25.81% ± 0.478	27.66% ± 0.645**

Values are presented as Mean ± SEM (n = 4); *p < 0.05, **p < 0.01 and ***p < 0.001 were considered as significant; SEM = Standard error of mean

Brine shrimp lethality bioassay. In the brine shrimp lethality bioassay, the lowest LC_{50} (0.88µg ml^{-1}) value was obtained with the ethyl acetate soluble fraction, whereas standard vincristine sulphate exhibited an LC_{50} value 0.56µg/ml (Table 5).

Table 5. Brine shrimp lethality bioassay and thrombolytic activity of different extractives of *C. trifoliata*.

Treatment	LC_{50} (µg/ml)	% Clot lysis
Vincristine sulfate	0.56	-
MECT	1.05	21.10%
HSF	34.36	15.32%
DMSF	0.93	32.29%
EA	0.88	17.23%
Blank	-	8.12%
SK	-	69.98%

Antitumorigenic drugs have been introduced from phytochemical sources that play a major role in the formation and progression of cancer at various stages. Induction of apoptosis in tumor cells is a major part of the mechanism.[23] The methanolic extract and its various partitionates of *C. trifoliata*, particularly dichloromethane and ethyl acetate soluble fraction (LC_{50} = 0.88 µg/ml, showed promising results in the current bioactivity studies when compared to vincristine sulfate, indicating that the test samples were biologically active.

Thrombolytic activity of *C. trifoliata*. In this study the MECT, HSF, DMSF and EASF exhibited 21.10%, 15.32%, 32.29% and 17.23% thrombolytic activity, respectively, while standard streptokinase demonstrated 69.98% thrombolytic activity.

Thrombolytic medications have been shown to be useful in both prevention and treatment of

various coronary heart diseases and stroke.[24] As they are natural, thrombolytic medicines derived from plants are frequently thought to be harmless. Thus, we evaluated the thrombolytic potential of methanolic extract and other partitionates of *C. trifoliata* leaf. The extractives exhibited considerably lower clot lysis activity compared to the standard streptokinase. However, further research is required to develop a cardioprotective drugs from this source.

CONCLUSION

β-sitosterol (**1**), β-sitostenone (**2**), epifriedelanol (**3**) and friedelin (**4**) were isolated after sequential separation and purification of dichloromethane soluble partitionate of methanolic extract *C. trifoliata* leaves. To the best of our knowledge, these chemicals are isolated for the first time from the examined plant. *In vivo* and *in vitro* biological results revealed significant central and peripheral analgesic activities as well as cytotoxic activity. Thus, further phytochemical study of this plant with greater emphasize may result in the identification of a new lead molecule(s).

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