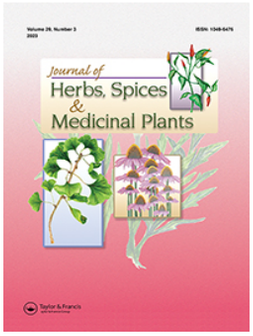

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Viscum monoicum: A New Source of Polyphenolic Compounds Responsible for Antioxidant and Anti-Inflammatory Activities

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ABSTRACT

Viscum monoicum has been used as a traditional medicinal plant for the treatment of many ailments that include pain, neuropharmacological disorders as well as various types of tumors. This study was designed to investigate the polyphenolic compound, antioxidant, and anti-inflammatory activities of the ethanolic extract of *Viscum monoicum* (EVM). The extract was subjected to HPLC-DAD analysis for the quantification of polyphenols. In addition, DPPH, ABTS free radical scavenging, reducing power, total antioxidant capacity, phenolic, and flavonoid content was measured for antioxidant activities of EVM. Besides, the anti-inflammatory activity of EVM was studied using carrageenan and histamine-induced rat paw edema at different doses. High levels of ellagic acid and (+)-catechin hydrate (410.15 and 340.88 mg/100 g extract, respectively) were determined for the first time through HPLC in EVM. IC_{50} of EVM was significant in DPPH and ABTS scavenging assay compared to that of ascorbic acid. The total antioxidant capacity, total phenolic, and total flavonoid contents were estimated and found significant amount equivalent to standard. At a dose of 400 mg kg^{-1}, a significant anti-inflammatory activity ($P<0.01$) was observed in rats at both carrageenan and histamine-induced edema test models with a reduction in the paw volume; in comparison to indomethacin. Therefore, the results suggest that polyphenolic compounds might be responsible for the antioxidant and anti-inflammatory activities of EVM.

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KEYWORDS
Viscum monoicum; HPLC; polyphenolic compounds; antioxidant; anti-inflammatory

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Introduction

In the majority of natural products, polyphenolic compounds are present and illustrate different types of biological potency such as antioxidant, anti-inflammatory activities, etc.[1] An excess of free radicals damages almost every biological molecule in live cells.[2] If not eliminated quickly, those free radicals can cause oxidative damage to functional macromolecules such as DNA, proteins, and lipids.[3] Overproduction of free radicals causes many illnesses, including cancer, atherosclerosis, inflammation, and neurological disorders. Free radical production can be reduced by antioxidants found in medicinal plants and diseases caused by oxidative stress.[4] Polyphenols found in medicinal plants help boost antioxidant activity[3] and act as anti-inflammatory agents.[5] *Viscum monoicum* Roxb. ex DC. (*V. monoicum*) belongs to the family of Santalaceae, which includes the three genera: *Viscum*, *Loranthus*, and *Dendrophthoe*.[6] *V. Monoicum* is found middle and upper zone of *Bruguiera sexangula* (L.) Poirwhich is the most preferred host species and upper zone of *Xylocarpus granatum* Koen which also the preferred host species.[7] A common name for *Viscum* is Shamulota (Bangla). It is a species of mangrove and a rare herbaceous, stem-parasite of trees, and shrubs. It is commonly found in different countries, like Bangladesh, Sri Lanka, Myanmar, Bhutan, Nepal, China, India, and Africa.[6] The plant can be used to treat hematemesis, neuritis, and rheumatism and also used to prevent fever, typhoid, jaundice, stomach disorders, hip inflammation, and fungal infection.[8] In the literature review, there is no sufficient information about the chemical composition of this plant but other species of *Viscum* such as *Viscum album* L. has been described in the literature as a sources of polyphenolic compounds such as, gallic acid, caffeic acid, protocatechuic acid, syringic acid, p-coumaric acid, ferulic acid, salicylic acid, vanilic acid, 4-hydroxy-benzoic acid, synapic acid, rosmarinic acid, naringenin, apiggenin, 3-O-Me-quercetin, myricetin, and kaempferol. On the other hand, *Viscum orientale* Willd also reported of polyphenolic compounds for example, gallic acid, vanillic acid, caffeic acid, ellagic acid, and quercetin.[9–11] *V. monoicum* has previously been reported as traditional remedies for antidiarrhoeal, analgesic, and antibacterial activities.[12] More research will be conducted on this plant. For these reasons, we attempted to investigate the major polyphenolic compounds, antioxidant, and anti-inflammatory activities of the ethanolic extract of *V. monoicum*.

Materials and Methods

Plant Material

V. monoicum is a perennial shrubby, stem-parasite of trees. Whole plants of *V. monoicum* were collected from the mangrove forest Sundarban, Bangladesh, in October 2017. The Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh, received a specimen of this plant (DACB 36543) for identification and future protection. The plants were cleaned and dried

carefully in a shed. After that, the dried sample was grinded by a mechanical grinder. The whole plant powder was reserved in an airtight bottle until the extraction procedure was performed.

Extraction

The powder of the whole plant sample was soaked in ethanol for two weeks and stirred occasionally. The extract was filtered through a clean cotton plug to remove plant debris and dried in a rotary vacuum evaporator (R-215, Buchi, Switzerland) at reduced pressure to get the crude extract. Finally, we have a 3.42% dried plant material extract.

The Test Animals

In most cases, male Wistar rats ranging from 179 to 205 g were utilized to test *in vivo* anti-inflammatory activity. The animals were kept in a standard laboratory setting (at the Pharmacology Laboratory of BCSIR, Chittagong). The temperature in the lab was kept at 25 ± 1°C with a 12/12 h light/dark cycle. The rats were taken fed a balanced diet and water *ad libitum*. Every experimental procedure adhered to the ethical committee of the Bangladesh Council of Scientific and Industrial Research (BCSIR) and globally established guidelines for the use and care of laboratory animals.

Chemicals and Drugs

Some chemicals and drugs were bought from Sigma-Aldrich (St. Louis, MO, USA) such as Gallic acid (GA), (+)-catechin hydrate (CH), vanillic acid (VA), caffeic acid (CA), (-)-epicatechin (EC), *p*-coumaric acid (PCA), rutin hydrate (RH), ellagic acid (EA), quercetin (QU), ascorbic acid, DPPH, ABTS, Folin-Ciocalteu reagent, indomethacin, and histamine phosphate. On the other hand, Acetonitrile (HPLC grade), methanol (HPLC grade), acetic acid (HPLC grade), ethanol, trichloroacetic acid (TCA), phosphate buffer (pH 6.6), potassium ferricyanide [$K_3Fe(CN)_6$], aluminum chloride ($AlCl_3$), sodium acetate, ferric chloride ($FeCl_3$), sodium phosphate, EDTA, tween 80, ammonium molybdate, and sodium carbonate were analytical grade and purchased from Merck (Darmstadt, Germany).

Quantification of Polyphenols of EVM by the HPLC

HPLC System

Polyphenols were quantified using Thermo Scientific DionexUltiMate 3000 Rapid Separation LC (RSLC) devices (Thermo Fisher Scientific Inc., MA, USA). Which combined with a quaternary rapid separation pump (LPG-

3400RS), Ultimate 3000RS autosampler (WPS-3000), a rapid separation diode array detector (RSDA), and a quaternary rapid separation pump (LPG (DAD-3000RS). Polyphenols were separated using a Dionex® Acclaim® C18 (4.6 × 250 mm; 5 m) column kept at 30°C using a temperature-controlled column chamber (TCC-3000). Finally, DionexChromeleon software used data collecting, peak integration, and calibrations (Version 6.80 RS 10).

Chromatographic Conditions

The phenolic component of the EVM was determined using HPLC-DAD.[13] The mobile phase consisted of the three solvents: solvent "A" (acetonitrile), solvent "B" (acetic acid solution, pH 3.0), and solvent "C" (methanol). The system was operated with the following gradient elution program: 0 min, 5% A/95%B; 10 min, 10%A/80%B/10%C; 20 min, 20%A/60%B/20%C, and 30 min, 100%A. There was a 5 min post-run at initial conditions for equilibration of the column. The flow rate and injection volume were fixed at 1 mL/min and 20 µL respectively. The wavelength was optimized for DAD detection to observe phenolic compounds at their respective maximum wavelengths as follows: λ 280 nm held for 18.0 min, changed to λ 320 nm and held for 6 min, and finally changed to λ 380 nm and held for the rest of analysis and the diode array detector was set at an acquisition range from 200 to 700 nm. GA, CH, VA, CA, and EC were detected and quantified at 280 nm, PCA, RH, and EA at 320 nm, and QU at 380 nm, respectively.

Preparation of Standards and Samples

Each polyphenolic compound stock standard was prepared in methanol by weighing 0.0050 g of the analyte into a 50 mL volumetric flask (100 gm L^{-1}). Except for caffeic acid (8 µg mL^{-1}) and quercetin (6 µg mL^{-1}), the mixed standard solution was made by diluting mixed stock standard solutions in methanol to a 20 µg mL^{-1} for each polyphenol. All standard solutions were maintained at 5°C in the refrigerator. The calibration curves of the standards were made by serial dilution of the stock standards (five sets of standard dilutions) with methanol yielded 1.25–20 µg mL^{-1} for GA, CH, VA, EC, PCA, RH, EA; 0.5–8.0 µg mL^{-1} for CA, and 0.375–6.0 µg mL^{-1} for QU. The calibration curves were built using chromatograms as peak area vs. standard concentration. A 5 mg mL^{-1} solution of EVM was produced in ethanol by vortex mixing (Branson, USA) for 30 min. The samples were kept at low temperatures (5°C) in the dark. Individual polyphenols were further identified by spiking the sample solution with phenolic standards. All solutions (mixed standards, sample, and spiked solutions) were filtered through a 0.20 µm nylon syringe filter (Sartorius, Germany) before being degassed in an ultrasonic bath (Hwashin, Korea) for 15 min before HPLC analysis.

Antioxidant Activities

DPPH Free Radical Scavenging Activity

Radical scavenging activity of extracts against the free radical DPPH was assessed.[14] A stock solution of the ethanolic extract of *V. monoicum* (500 μg mL^{-1}) was prepared in respective solvent systems. To obtain concentrations of 10, 20, 40, 60, 80, 100, and 250 μg mL^{-1}, seven-point dilutions were used. An equal amount of sample solution was mixed with an equal amount of methanolic DPPH solution (0.1 mM). The mixture was vortexed and kept at 25°C for 30 min in the dark. The absorbance of the combination was measured at 517 nm using a double beam UV/Visible spectrophotometer (Specord 205, Analytikjena, Germany) after 30 min of incubation. The radical scavenging activity was estimated using the following equation to get the inhibition percentage (I%):

$$I(\%) = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

Where A_{blank} is the absorbance of the control sample (which contains all reagents but not the test compound) and A_{sample} is the absorbance of the experimental sample (which contains the test compound and all reagents). The IC_{50} value (the sample concentration required to scavenge 50% of the DPPH free radical) was derived by plotting inhibition (%) versus extract concentration. All tests were done in triplicate, and the average of the findings was recorded. This research employed ascorbic acid as a standard.

ABTS radical scavenging activity test

The antioxidant activity was measured using the ABTS radical scavenging activity assay with certain adjustments.[15] The radical scavenging activity of ABTS was obtained by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate and allowing the mixture to remain at room temperature for 16 hours in the dark. The ABTS solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm at usage. 1 mL of each sample was mixed vigorously with 1 mL of ABTS solution at varied concentrations (10 to 250 μg mL^{-1}). After allowing the reaction mixture to cool to ambient temperature for 6 min, the absorbance at 734 nm was measured using the same spectrophotometer. The following formula was used to calculate the ABTS scavenging activity (I%):

$$I(\%) = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

Where A_{blank} is the absorbance of the control sample (which contains all reagents but not the test compound) and A_{sample} is the absorbance of the experimental sample (which contains the test compound and all reagents). The

IC_{50} value (the sample concentration required to scavenge 50% of the ABTS free radical) was derived by plotting inhibition (%) versus extract concentration. All tests were done in triplicate, and the average of the findings was recorded. This research employed ascorbic acid as a standard.

Reducing Power Assay

This assay was performed as described[16]: 1 mL of different strengths of the extract (10 to 250 µg mL^{-1}) were mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (1%). After 20 min of incubation at 50°C, a 2.5 mL solution of trichloroacetic acid (10%) was added to the mixture. It was then centrifuged for 10 min at 3000 rpm. After the centrifuged, 2.5 mL of the top layer of the mixture was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1%). Finally, the absorbance of the mixture was determined at 700 nm using the same spectrophotometer. We observed that increased absorbance of the reaction mixture indicated increased reducing power. All of the tests were repeated three times, with the results averaged. In this study, ascorbic acid was used as the standard reference chemical.

Total Antioxidant Capacity

The UV-Visible spectrophotometric technique was used to determine the total antioxidant capacity.[17] 0.1 mL of the extract (1 mg mL^{-1}) was mixed with the 1 mL of reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate, 4 mM ammonium molybdate mixture) in an Eppendorf tube. The tubes were incubated for 90 min at a constant temperature (95°C). After that, the solution was cooled to room temperature, and the absorbance was read at 695 nm against blank with the same spectrophotometer. A typical graph of ascorbic acid was used to compute ascorbic acid equivalents. The experiment was done in triplicates, and the results are given as ascorbic acid equivalents in mg per gm of extract.

Total Phenolic Content

Total phenolic content was determined using the Folin-Ciocalteu method.[18] 0.5 mL of extract (1 mg mL^{-1}) was combined with 5 mL Folin-Ciocalteu reagent (1:10 v/v distilled water) and 4 mL (75 g/l) Sodium carbonate in a vortex for 15 s. The mixture was left at 40°C for 30 min to allow for color development. At 765 nm, the absorbance was measured with the same spectrophotometer. Total phenolic content was calculated as mg of Gallic acid equivalent per gram using an equation derived from a standard Gallic acid calibration curve.

Total flavonoids content

The total flavonoids content was determined in the ethanol extract using the Aluminum chloride colorimetric method.[19] 5 mL ethanol extract (1

mg mL^{-1}) and 2.5 mL aluminum chloride reagent solution were combined. 133 mg aluminum chloride and 400 mg sodium acetate are combined in 100 mL DI water to make this reagent solution. After combining the extract and reagent solution, we waited 30 min at room temperature before measuring the absorbance of the reaction mixture using the same spectrophotometer at 430 nm. Using the equation obtained from the Quercetin calibration curve, total flavonoid content was calculated as mg of Quercetin equivalent per gram.

Anti-Inflammatory Activity

Carrageenan-Induced Oedema Test

Carrageenan-induced rat hind paw edema was used as the animal model of acute inflammation.[20] In this study, rats were placed into five groups, each with five individuals. 1% tween 80 in normal saline (10 mL kg^{-1}) was given to Group I (control). The 10 mg kg^{-1} body weight indomethacin was given orally to Group II (positive control). The EVM was given orally to Groups III, IV, and V at doses of 100, 200, and 400 mg kg^{-1} body weight, respectively. Acute inflammation was generated in all four groups by injecting 0.1 mL of carrageenan suspension in normal saline with 1% tween 80 in the right paw of the rats 1 h after oral administration of the tested materials. At 1, 2, 3, 4, and 5 h after the medication and extract were administered, the paw volume was measured with a micrometer screw gauge. The following expression was used to compute the percentage inhibition of the extract's inflammatory effect:

$$\%\text{inhibition of inflammation} = \frac{V_c - V_t}{V_c} \times 100$$

Where Vc is the average degree of inflammation by the control group and Vt is the average degree of inflammation of the test group.

Histamine-Induced Oedema Test

Sub-plantar application of 0.1% freshly produced histamine solution into the right hind paw of rats caused paw edema.[21] Twenty-five rats were separated into five groups of five for this experiment. 1% tween 80 in normal saline (10 mL kg^{-1}) was given to Group I (control). The 10 mg kg^{-1} body weight indomethacin was given orally to Group II (positive control). The EVM was given orally to Groups III, IV, and V at doses of 100, 200, and 400 mg kg^{-1} body weight, respectively. Acute inflammation was generated in all five groups by injecting 0.1 mL of histamine in normal saline with 1% tween 80 in the right hind paw of the rats 1 h after the oral administration of the investigated materials. At 1, 2, 3, 4, and 5 h after the standard and extract were administered, the paw volume was measured with a micrometer screw gauge. The

extract's percentage reduction of inflammatory impact was measured using the same technique to quantify carrageenan-induced paw edema.

Statistical Analysis

The information was reported as mean, standard deviation (S. D). The animal trials were statistically analyzed using one-way analysis of variance (ANOVA) and Dunnett's test using SPSS 11.5 software (Armonk, New York, USA). Differences between groups were considered significant at a level of $P < 0.05$.

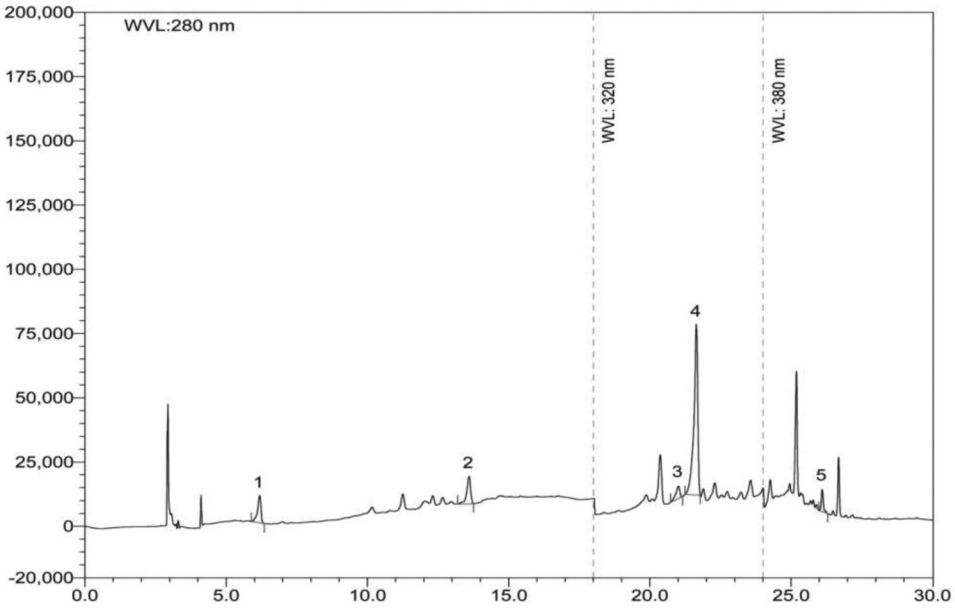

Figure 1. HPLC chromatogram of EVM. Peaks: 1, gallic acid; 2, (+)-catechin hydrate; 3, rutin hydrate; 4, ellagic acid; 5, quercetin.

Table 1. Contents of polyphenolic compounds in the EVM ($n = 5$).

	EVM	
Polyphenolic compound	Content (mg/100 g of dry extract)	% RSD
GA	72.93	1.61
CH	340.88	5.38
RH	69.77	1.49
EA	410.15	6.74
QU	19.06	0.68

EVM = Ethanol extract of whole plant of *Viscum monoicum*; RSD= Relative standard deviation; GA= Gallic acid, CH= (+)-Catechin hydrate, RH= Rutin hydrate, EA= Ellagic acid, QU= Quercetin.

Results

Identification and Quantification of Polyphenolic Compounds in EVM by HPLC

Using HPLC, we were identified and quantified individual polyphenolic compounds in the EVM. Figure 1 shows the chromatographic separations of phenolic compounds present in ethanol extract. As shown in Table 1, the content of each phenolic component was estimated using a calibration curve and reported as the mean of five determinations.

The experimental results indicated that EVM contained an especially high concentration of (+)-catechin hydrate and ellagic acid (340.88 and 410.15 mg/100 g of dry extract, respectively). It was also shown that gallic acid and rutin hydrate were detected at moderate concentration (72.93 and 69.77 mg/100 g of dry extract, respectively). Quercetin was also detected in low concentrations (19.06 mg/100 g of dry extract). The EVM did not detect the other polyphenolic compounds (vanillic acid; caffeic acid; (–)-epicatechin, and *p*-coumaric acid). It has been reported that *V. orientale* contain gallic acid (17.54), vanillic acid (8.99), caffeic acid (99.61), ellagic acid (4523.31), and quercetin (100.15) mg/100 g of dry extract. EVM contained higher amount of gallic acid but lower amount of elagic acid and quercetin compare to the *V. orientale*.[11]

Method Development

Previous research has found a direct correlation between antioxidant activity and total phenolic content in medicinal plants and phenolic compounds had a significant role in antioxidant activity.[22] The HPLC analysis, which uses a binary solvent system consisting of acidified water and a polar organic solvent to precisely assess polyphenolic levels, was developed with several separation systems.[23] We used the HPLC technique with modifications for method development.

Method Validation

The validation of this new HPLC technique was carried out by ICH guidelines.[24] Standard polyphenolics were used to determine linearity ranges, correlation coefficients, detection limits, quantitation limits, and recovery (Table 2). Standard polyphenolics exhibited good linearity over the evaluated range, with correlation coefficients (r^2) between 0.9956 and 0.9993. These compounds have detection and quantitation limits of 0.12–0.35 μg mL^{-1} and 0.37–1.24 μg mL^{-1}, respectively. In the recovery assay, samples were fortified with all 10 and 20 μg mL^{-1} standards. The recovery tests of all compounds range from 97.1–102.7%.

Table 2. Parameters of calibration graphs for the nine phenolic standards in this study.

Peak no.	Polyphenolic compound	Linearity range (μg mL^{-1})	Correlation coefficients (r^2)	Detection limit (μg mL^{-1})[a]	Quantitation limit (μg mL^{-1})[a]	Recovery (%)[b]
1	GA	1.25–20	0.9959	0.35	1.18	97.1 ± 2.01
2	CH	1.25–20	0.9965	0.33	1.17	96.6 ± 1.57
3	VA	1.25–20	0.9956	0.25	1.11	97.8 ± 2.05
4	CA	0.50–8.0	0.9969	0.17	0.48	100.5 ± 2.83
5	EC	1.25–20	0.9957	0.31	1.24	97.8 ± 2.32
6	PCA	1.25–20	0.9988	0.32	1.19	102.7 ± 2.61
7	RH	1.25–20	0.9979	0.26	1.12	101.3 ± 2.93
8	EA	1.25–20	0.9985	0.33	1.15	99.5 ± 2.17
9	QU	0.375–6.0	0.9993	0.12	0.37	101.5 ± 2.94

[a]Data were expressed as mean of triplicate measurements.
[b]Recovery are expressed as mean ± standard deviation carried out in *Viscum monoicum* ethanol extract.
GA= Gallic acid, CH= (+)-Catechin hydrate, VA= Vanillic acid, CA= Caffeic acid, EC= (-)-Epicatechin, PCA= *p*-Coumaric acid, RH= Rutin hydrate, EA= Ellagic acid, QU= Quercetin.

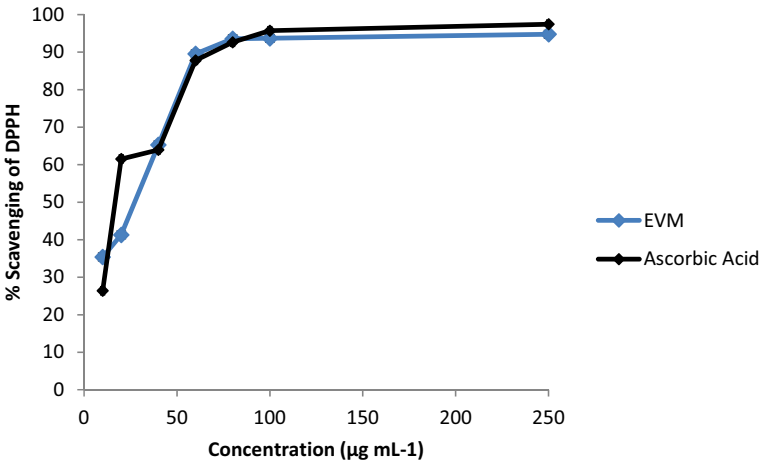

Figure 2. DPPH free radical scavenging activity of the EVM and standard.

Antioxidant Activities

The EVM was examined to see if it has any antioxidant properties. DPPH free radical scavenging, ABTS free radical scavenging activity, reducing power, total antioxidant capacity, total phenolic content and total flavonoid content were the six test techniques used in this investigation.

DPPH Free Radical Scavenging Activity

DPPH free radical scavenging activity of the EVM was found to be increased with the increase in concentration of the extract (Fig. 2). The extract exhibited 94.77% radical inhibitions at 250 μg mL^{-1}, whereas at the same concentration, the standard ascorbic acid exhibited 97.45% inhibitions. The IC_{50} value of the extract was significant (11.15 μg mL^{-1}) compared to the IC_{50} value of ascorbic

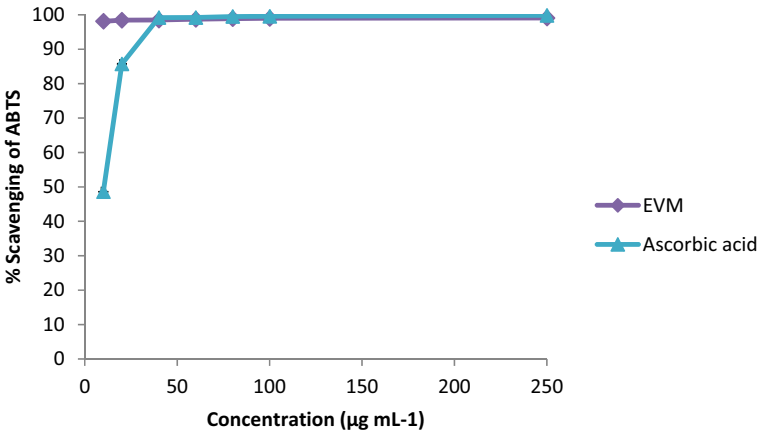

Figure 3. ABTS free radical scavenging activity of EVM and standard.

Table 3. Reducing power assay of the EVM and standards.

Concentration (µg mL^{-1})	Average absorbance at 700 nm of extract and standard	
	EVM	Ascorbic acid (standard)
10	0.047 ± 0.007	0.380 ± 0.012
20	0.070 ± 0.009	0.457 ± 0.017
40	0.151 ± 0.014	0.539 ± 0.023
60	0.184 ± 0.008	0.634 ± 0.037
80	0.288 ± 0.006	0.712 ± 0.013
100	0.370 ± 0.007	0.781 ± 0.029
250	0.750 ± 0.013	1.111 ± 0.019

The values are expressed as mean ± standard deviation ($n = 3$). EVM = Ethanol extract of whole plant of *Viscum monoicum*.

acid, 7.08 µg mL^{-1}. The IC_{50} value of EVM is higher than that of *V. orientale* (IC_{50} = 6.63 µg mL).[11]

ABTS Free Radicals Scavenging Activity

The scavenging powers of the EVM correlate with the increasing concentrations. At a minimum concentration (10 µg mL^{-1}), the extract showed greater inhibition (98.13%) than that of standard ascorbic acid, 48.60% (Fig. 3). The IC_{50} value of the extract was found significant (2.09 µg mL^{-1}) in comparison to ascorbic acid, 5.65 µg mL^{-1}. The IC_{50} value of EVM is higher than that of *Viscum album* (IC_{50} = 0.237 µg mL^{-1}).[25]

Reducing Power Assay

Ascorbic acid was employed as a positive control in the study of EVM's reducing power (Table 3). The maximum absorbance for EVM was found to be 0.750 at 250 µg mL^{-1} compared to standard ascorbic acid (1.111) at the

same concentration. The extract's absorbance increased when the concentration was increased.

Total Antioxidant Capacity

The total antioxidant capacity of the Phosphomolybdenum assay is based on the reduction of Mo (VI) to Mo (V) by the sample and the subsequent formation of green phosphate/Mo (V) complex at acidic pH. The overall antioxidant capacity of the EVM was found to be extremely high. The average absorbance was 0.3666 ± 0.01 at 695 nm and the total antioxidant capacity of the ethanolic extract was 703.4 ± 12.28 mg of ascorbic acid/g of extract.

Total phenolic content

In the EVM, the total phenolic content was 425.25 ± 10.08 mg g^{-1} gallic acid equivalent and it is fairly high. The total phenolic content is higher than that of *V. orientale* (73.4 mg g^{-1} gallic acid equivalent).[11]

Total flavonoid content

The total flavonoid content was also determined to be significant in EVM as the 4.84 ± 0.67 mg g^{-1} of quercetin equivalent per gm of dry extract. The total flavonoid content is lower than that of *V. orientale* (170.6 mg g^{-1} quercetin equivalent).[11]

Anti-Inflammatory Activity

Carrageenan-Induced Paw Edema

The anti-inflammatory activity of the EVM in carrageenan-induced edema tests is shown in Table 4. In this test, the positive control (Indomethacin) significantly ($p < 0.05$; $p < 0.01$) decreased the paw edema from 1 h to 5 h after carrageenan injection compared to saline with inhibition of 54.54% to 65.07%. A maximum edema paw volume of 1.46 ± 0.25 mm was observed in control rats 5 h after the carrageenan injection. Rats with the extract at 400 mg kg^{-1} body weight significantly decreased ($p < 0.05$; $p < 0.01$) the carrageenan-induced edema paw volume from 1 h to 5 h compared to the standard drug indomethacin at a dose of 10 mg kg^{-1} body weight. The inhibition percentage of the edema paw volume by the 400 mg kg^{-1} body weight of the extract was also found to be statistically significant compared with the indomethacin treated animals at 1, 2, 3, 4, and 5 h. The highest reduction in the paw volume by the 400 mg kg^{-1} body weight was 61.64%, comparable to that of the indomethacin (65.07%) at 5 h.

Table 4. Effects of EVM and indomethacin on carrageenan-induced edema paw volume in Wistar rats.

Treatment	Dose (mg kg^{-1})	Right hind paw volume (% Inhibition)				
		1 h	2 h	3 h	4 h	5 h
Vehicle	10 (mL kg^{-1})	0.99 ± 0.12	1.09 ± 0.18	1.29 ± 0.16	1.39 ± 0.24	1.46 ± 0.25
Indomethacin	10	0.45 ± 0.15 (54.54)*	0.49 ± 0.16 (55.05)*	0.53 ± 0.14 (58.91)**	0.52 ± 0.19 (62.59)**	0.51 ± 0.14 (65.07)**
EVM	100	0.76 ± 0.04 (23.23)*	0.79 ± 0.12 (27.52)*	0.88 ± 0.13 (31.78)*	0.93 ± 0.14 (33.09)**	0.89 ± 0.15 (39.04)*
EVM	200	0.73 ± 0.14 (27.17)*	0.68 ± 0.13 (37.61)*	0.73 ± 0.17 (43.41)*	0.76 ± 0.15 (45.32)*	0.72 ± 0.17 (50.68)**
EVM	400	0.63 ± 0.16 (36.36)*	0.59 ± 0.15 (45.87)*	0.57 ± 0.12 (55.81)**	0.55 ± 0.19 (60.43)**	0.56 ± 0.13 (61.64)**

Each value is presented as the mean ± SEM ($n = 5$). EVM = Ethanol extract of whole plant of *Viscum monoicum*.
* indicate$p < 0.05$ compared with control group (Dunnett's test).
** indicate $p < 0.01$ compared with control group (Dunnett's test).

Table 5. Effect of EVM and indomethacin on histamine-induced edema paw volume in Wistar rats.

		Right hind paw volume (% Inhibition)				
Treatment	Dose (mg kg^{-1})	1 h	2 h	3 h	4 h	5 h
Vehicle	10 (mL kg^{-1})	1.12 ± 0.18	1.29 ± 0.16	1.38 ± 0.15	1.42 ± 0.21	1.56 ± 0.25
Indomethacin	10	0.55 ± 0.13 (50.89)*	0.58 ± 0.12 (55.04)*	0.59 ± 0.13 (57.25)**	0.52 ± 0.14 (63.38)**	0.50 ± 0.17 (67.95)**
EVM	100	0.89 ± 0.12 (20.54)*	0.99 ± 0.13 (23.26)*	0.97 ± 0.17 (29.71)*	0.95 ± 0.18 (33.09)*	0.96 ± 0.10 (38.46)*
EVM	200	0.74 ± 0.10 (33.93)*	0.79 ± 0.18 (38.76)*	0.84 ± 0.20 (39.13)*	0.82 ± 0.12 (42.25)**	0.81 ± 0.15 (48.08)**
EVM	400	0.62 ± 0.15 (44.64)*	0.67 ± 0.17 (48.06)**	0.68 ± 0.13 (50.72)*	0.66 ± 0.15 (53.52)**	0.64 ± 0.13 (58.97)**

Each value is presented as the mean ± SEM ($n = 5$). EVM = Ethanol extract of whole plant of *Viscum monoicum*.
* indicate$p < 0.05$ compared with control group (Dunnett's test).
** indicate $p < 0.01$ compared with control group (Dunnett's test).

Histamine-Induced Paw Edema

Table 5 shows the anti-inflammation effect of the EVM using histamine-induced paw edema tests. In the histamine-induced edema test, a maximum edema paw volume of 1.56 ± 0.25 mm was observed in control rats 5 h after the histamine injection. Rats pre-treated with the extract at 400 mg kg^{-1} body weight significantly decreased ($p < 0.05$; $p < 0.01$) the histamine-induced edema paw volume from 1 h to 5 h compared to the standard drug indomethacin at a dose of 10 mg kg^{-1} body weight. The percentage inhibition of the edema paw volume by the extracts 400 mg kg^{-1} body weight was also statistically significant ($p < 0.05$; $p < 0.01$) compared favorably with the indomethacin treated animals at 1, 2, 3, 4, and 5 h. The maximum reduction in the paw volume by the 400 mg kg^{-1} body weight was 58.97% compared to the indomethacin (67.95%) at 5 h.

Discussion

HPLC analysis was done for the correlation between pharmacological activity and the responsible compound of this plant. Based on HPLC studies, (+)-catechin hydrate and ellagic acid were found in EVM for the first time in significant amounts, indicating the anti-oxidant and anti-inflammatory activity. Previous studies illustrated that catechin hydrate and ellagic acid pertain high antioxidant and anti-inflammatory activities.[26,27] In addition, gallic acid, rutin hydrate, and quercetin were also present in the plant extracts, and these three compounds had formerly shown good anti-inflammatory activity.[28,29,30]

DPPH is a relatively stable free radical scavenger that uses hydrogen proton donation to transform unpaired electrons into paired ones. The ability of plant extracts to donate hydrogen protons to the lone pair electron in radicals was determined in this work using DPPH radical scavenging. In all solvent extracts, the inhibition was higher at increasing concentrations. The approaches have demonstrated the extracts' efficacy in a concentration-dependent way.[31] The presence of phytoconstituents in the plant extracts may explain the antioxidant activity shown in DPPH radical scavenging experiment.

The ABTS test is commonly used to determine the antioxidant capacity of single compounds and complex plant combinations.[32]

The transformation of Fe^{3+} into Fe^{2+} was also observed to determine reducing power ability. The reducing ability of a compound generally depends on the presence of reductones (antioxidants), which exert the antioxidant response by donating hydrogen atom and breaking the free radical chain.[33] The antioxidant principles present in EAV cause the reduction of Fe^{3+}/ferricyanide complex to its ferrous form, and thus shows reducing power ability.

In the ethanolic extract, total antioxidant capacity was found significantly compared to ascorbic acid per gm of extract.

According to total phenolic content, the high value in the ethanol extract might be attributable to the high quantity of phenolic compounds present in the extract. Because of their hydroxyl groups, phenols are significant phytochemical components because of their scavenging abilities.[34] Flavonoids are compounds found in nature that have anti-inflammatory activities *in vitro* and *in vivo*.[35] Flavonoids also have anti-inflammatory qualities because they inhibit enzymes involved in generating inflammatory chemical mediators.[36] Phytochemical constituents, predominantly polyphenols, such as phenolic acids, flavonoids, phenylpropanoids, etc., contribute the most to the free radical scavenging and antioxidant activities of plants. Saponins and certain flavonoids have been identified as possible free-radical scavengers, and their ability to neutralize the DPPH radical is linked to their chemical structure.[37] As a result, the existence of these phenolics, flavonoids, and other antioxidants in the EVM might explain its antioxidant activity.

A biphasic response is observed in the inflammatory response caused by carrageenan in rats. Edema is formed due to the fast synthesis of inflammatory mediators such as histamine, serotonin, and bradykinins in these biphasic reactions. This is known to be the first phase. Consequently, the second phase releases prostaglandins and nitric oxide with a peak at 3 h, which is produced by an inducible isoform of cyclooxygenase (COX-2) and nitric oxide synthase (iNOS).[38] This study aimed to diminish the oedomatogenic reaction in rats elicited by carrageenan, and it was discovered that pre-treating the extract and administering it orally reduced the response. As a result, we can draw a link between the extract's anti-inflammatory activities and the regulation of inflammatory mediator intracellular signaling pathways.

Histamine is a key inflammatory mediator, a powerful vasodilator, and a booster of vascular permeability.[39] Histamine is subcutaneously injected into a rat, which spreads out in the body like a wheel. This increases the vascular permeability of the host capillary venules in the skin. Substances that oppose the activity of histamine receptors cause the area of the wheel formed to reduce. This could be because the anti-inflammatory activity of the extract is confirmed by its antihistamine activity. The antihistaminic effect of the extract enhances the concentration of the extract. The extract inhibits the formation, release, or action of the inflammatory mediators and thus, effectively suppresses the production of edema by histamine. This study shows that the EAV possesses a significant antioedematogenic effect ($P < 0.01$) on paw edema induced by carrageenan and histamine when compared with the standard drug (indomethacin) in treated rats. This extract's anti-inflammatory activity may be similar to that of indomethacin.

Conclusion

The EVM revealed strong acute anti-inflammatory activity since it considerably decreased the production of edema caused by carrageenan and histamine. The

presence of phytoconstituents such as polyphenolic constituents (ellagic acid, catechin hydrate, gallic acid, rutin hydrate, and quercetin) may be responsible for the EVM's effectiveness as acute anti-inflammatory and antioxidant agents. The polyphenolic compounds described above have been identified and reported for the first time. As a result, the EVM has primary antioxidant and anti-inflammatory properties, which support its usage as a traditional folk treatment for inflammation, pain, boils, and other ailments. However, a more thorough investigation is required to discover the extract's actual mechanism(s) of action.

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Author Contributions

Khondoker Shahin Ahmed performed experiments and wrote the manuscript; Maisha Farzana, Shaikh Emdadur Rahman, Ismet Ara Jahan, Tanzir Ahmed Khan, Muhammad Abdullah Al-Mansur and Nadia Sultana helped with the interpretation of data; Hemayet Hossain conceptualized the project and revised the manuscript.

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