Phytochemical and Biological Studies of Bark Extract of *Miliusa velutina* (Dunal) Hook. f. & Thomson

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ABSTRACT: The main objective of the current research was phytochemical and biological studies of the stem bark of *Miliusa velutina* (Dunal) Hook. f. & Thomson (Annonaceae). Four purified compounds *i.e*., friedelin, lupeol, β-sitosterone and caffeic acid were isolated by repeated chromatographic separation and purification of *M. velutina*. The compounds were identified by analysis of NMR spectral data. The crude dichloromethane extract of stem bark of *M. velutina* (DEMV) along with its Kupchan partitionates *i.e.*, petroleum ether (PESF), ethyl acetate (EASF), chloroform (CSF) and aqueous (AQSF) soluble fraction were screened for antioxidant, cytotoxic, thrombolytic and antibacterial activities. During the antioxidant activity assay, the AQSF revealed maximum activity with IC_{50} value of 71.67 µg/ml. The cytotoxicity of plant samples was determined by brine shrimp lethality bioassay, where the maximum cytotoxic activity has been observed for EASF (LC_{90} = 9.01 µg/ml). In the thrombolytic activity test, the crude dichloromethane extract demonstrated significant efficacy with 46.27% inhibition of clot lysis. In antibacterial screening, the CSF exhibited noticeable inhibitory activity against *Shigella boydii* with the zone of inhibition 15 mm compared to the standard ciprofloxacin (zone of inhibition = 47 mm).

Key words: *Miliusa velutina*, Friedelin, Lupeol, β-sitosterone, Caffeic acid, Antioxidant, Cytotoxic, Thrombolytic, Antimicrobial.

INTRODUCTION

Medicinal plants are a valuable source of bioactive molecules with therapeutic potential, and plant-based systems continue to play a pivotal role in maintaining human health.[1] According to the World Health Organization (WHO) data, amongst the total population, 65% of people predominately depend upon traditional and plant-oriented medicines in their basic treatment.[1]Although the global demand of plant-based medicine is rising day by day, the literature survey reveals that only 1% of them has been identified through scientific investigations and accepted for commercial uses.[2] Therefore, the present studies were designed to characterize the secondary metabolites isolated from the stem bark of *M. velutina*.[3] Besides, the biological investigations were also performed to assess the rationale of its use in traditional medicines.

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Miliusa velutina (Dunal) Hook. f. & Thomson (Family: Annonaceae; Bengali name: Gandhagajari) is a deciduous tree (8-11 m in height), which is distributed throughout the regions of Bangladesh, India, China, Malaysia.[3] The genus *Miliusa* consists of approximately 40 species. But very few species have been studied to a significant extent. The essential oil of *M. tomentosa* exhibited pain-relieving property.[4] Bioactive alkaloids such as 10-methoxyliriodenine and 10-hydroxyliriodenine isolated from *M. cf. banacea* showed toxicity against DNA repair.[5] The plant also contains cytotoxic acetogenin[6], an aporphine alkaloid, (+)-isocorydine α-*N*-oxide[7] and four other alkaloids, reticuline, liriodenine and norcorydine.[3,7] Recently, homogentisic acid derivatives, miliusanal, miliusanones[8], and bicyclic lactones[9] named velutinones A-H isolated from this plant have been shown to display antimalarial activity and toxicity against the cancer cell lines.[8,9]

Although several researchers have worked on the isolation of secondary metabolites from *M. velutina*, a few articles are published based on its pharmacological properties.[6-9] Therefore, in continuation of our previous work,[10-12] we investigated the stem bark extract of *M. velutina* to isolate and characterize secondary metabolites and subsequent evaluation of antioxidant, cytotoxic, thrombolytic and antimicrobial properties of its extractives.

MATERIALS AND METHODS

General experimental procedures. ^{1}H NMR spectra were recorded in $CDCl_3$ on Bruker 400 MHz NMR. The chemical shifts are reported in ppm with respect to TMS. Preparative TLCs were carried out over silica gel $60F_{254}$ coated glass plates, and 1% vanillin-sulfuric acid reagent was used to detect the compounds.

Collection and extraction of plant sample. Stem bark of *M. velutina* was collected from the Bhawal National Park, Gazipur, in January 2017. The plant was taxonomically identified in Bangladesh National Herbarium, Dhaka, Bangladesh where a voucher specimen has been deposited with accession No. DACB-28061. The dust-free, sun-dried plant samples were pulverized and macerated in 3.0 L dichloromethane at room temperature for 15 days. Whatman No. 1 filter paper was used to filter the mixture. The filtrate was concentrated to dryness with a rotary evaporator. The concentrated crude extract was subjected for modified Kupchan partitioning[13] protocol into petroleum ether, ethyl acetate, chloroform and aqueous fractions.

Isolation of chemical constituents. For rapid fractionation, the crude dichloromethane extract of stem bark of *M. velutina* was fractionated by vacuum liquid chromatographic (VLC) tehnique[14] utilizing silica gel 60H and petroleum ether, petroleum ether-ethyl acetate, ethyl acetate-methanol in order of increasing polarities. This provided thirty two VLC fractions. Following TLC screening of VLC fractions, two compounds, *i.e*., compound MV-1 and MV-2 were isolated from the different VLC fractions 9 and 14. On the other hand, VLC fraction 16 was subjected for gel permeation chromatography over lipophilic Sephadex (LH-20). Preparative TLC of column fractions 4-7 gave compound MV-3, which is a mixture of two compounds (MV-3a and MV-3b**)**.

Antioxidant activity. The antioxidant potential of the plant samples in methanol solution (2 ml) was screened by a commonly used DPPH free radical scavenging assay method.[15]

Cytotoxic activity. Brine shrimp lethality bioassay[16,17] was carried out to evaluate the cytotoxic activity of the test samples. In this rapid bioassay, vincristine sulfate (VS) and dimethylsulfoxide (DMSO) were used as a positive and negative control, respectively.

***In vitro* thrombolytic activity.** The *in vitro* assay method described by Prasad *et al.*[18] was used to evaluate the efficacy of the plant samples as the thrombolytic agents. In this experiment, streptokinase (SK) was used as the reference standard and distilled water as a negative control.

Antimicrobial activity. The most common disc diffusion method[19,20] was adopted to evaluate the antimicrobial activity of the plant samples. Here, antibacterial drug ciprofloxacin (30 μg) and the antifungal agent fluconazole (30 μg) were used as the controls agents.

Properties of isolated compounds. Friedelin (MV-1): White solid crystals; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.43 (1H, m, H_b-2), 2.29 (1H, m, H_a-2), 1.70 (1H, m, H_a-1), 1.20 (3H, s, H-28), 1.07 (3H, s, H-27), 1.03 (3H, s, H-26), 1.02 (3H, s, H-29), 0.97 (3H, s, H-30), 0.90 (3H, d, *J* = 6.4 Hz, H-23), 0.89 (3H, s, H-25), 0.75 (3H, s, H-24).

Lupeol (MV-2): White amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.71 and 4.59 (1H, br. s each, H-29), 3.21 (1H, m, H-3), 2.40 (1H, m, H-19), 1.70 (3H, s, H-30), 1.05 (3H, s, H-26), 0.99 (3H, s, H-23), 0.97 (3H, s, H-27), 0.85 (3H, s, H-25), 0.81 (3H, s, H-28), 0.78 (3H, s, H-24).

β-sitosterone (MV-3a): Yellowish solid; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.75 (1H, br. s, H-6), 1.20 (3H, s, H-19), 0.93 (3H, d, *J* = 6.8 Hz, H-21), 0.87 (3H, t, *J* = 7.6 Hz, H-29), 0.86 (3H, d, *J* = 6.8

Hz, H-26), 0.84 (3H, d, *J* = 6.0 Hz, H-27), 0.73 (3H, s, H-18).

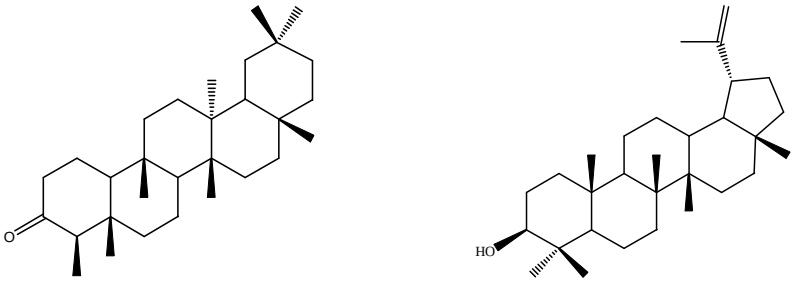
Caffeic acid (MV-3b): Yellowish amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.61 (1H, d, *J* =16.0 Hz, H-7), 7.09 (1H, dd, *J* = 8.0, 1.6 Hz, H-6), 7.06 (1H, d, *J* = 1.6 Hz, H-2), 6.93 (1H, d, *J* = 8.0 Hz, H-5), 6.32 (1H, d, *J* = 16.0 Hz, H-8).

RESULTS AND DISCUSSION

After repeated chromatographic separation and purification of dichloromethane extract of the stem bark of *M. velutina*, we obtained four compounds (Figure 1). The isolated compounds were characterized by analysis of their high-resolution ^{1}H NMR spectroscopic data and comparison with published values.

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound MV-1 showed eight methyl group signals at δ 1.20, 1.07, 1.03, 1.02, 0.97, 0.90, 0.89 and 0.75. These were attributed to Me-17, Me-13, Me-14, Me-20α, Me-20β, Me-4, Me-9 and Me-5, respectively. A ketonic moiety rather alcoholic functional group was at C-3 position was affirmed by the absence of peak of oxymethine proton resonance around δ 3.0-3.5. These and other spectral characteristics shown in the material and method section are consistent with that reported for fridelin.[21] Thus, MV-1 was identified as fridelin, which was substantiated by co-TLC with the authentic sample.

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound MV-2 displayed a double doublet (J=11.2, 7.2 Hz) at δ 3.21, which can be assigned to the oxymethine proton, H-3 in the triterpene nucleus. The spectrum also demonstrated the presence of six tertiary methyl groups at δ 1.05 (H-26), 0.99 (H-23), 0.97 (H-27), 0.85 (H-25), 0.81 (H-28) and 0.78 (H-24). The multiplet of one proton intensity at δ 2.40 was assigned to H-19. An isopropenyl side chain with signals at δ 1.70 (3H, s, H-30) and two olefinic protons at δ 4.59 and 4.71 (1H, br. s each) demonstrates its relationship with lupane-type triterpenoids. The above spectral data are comparable to those published for lupeol.[22,23] Based on these data, the identity of MV-2 was ascertained as lupeol.



MV-1 (Friedelin) **MV-2 (Lupeol)**

MV-3a (β-sitosterone) **MV-3b (Caffeic acid)**

Figure 1. Compounds MV-1, MV-2, MV-3a and MV-3b isolated from *M. velutina*.

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound MV-3 revealed a mixture of two compounds, *i.e.* β-sitosterone or sitost-6-en-3-one (MV-3a) and caffeic acid or 3,4-dihydroxy-*trans*-cinnamic acid (MV-3b). The signals attributable to **MV-3a** revealed a broad singlet at δ 5.75 for the

olefinic proton, H-6 of the steroidal nucleus[21] and six methyl signals at 1.20 (3H, s, H-19), 0.93 (3H, d, *J* = 6.8 Hz, H-21), 0.87 (3H, t, *J* = 7.6 Hz, H-29), 0.86 (3H, d, *J* = 6.8 Hz, H-26), 0.84 (3H, d, *J* = 6.0 Hz, H-27), 0.73 (3H, s, H-18). The spectral characteristics of MV-3a are in close agreement to those observed for β-sitosterone or sitost-6-en-3-one.[21]

The ^{1}H NMR spectral signals assignable to the second compound MV-3b displayed a broad singlet at δ 7.06 (H-2) and two doublets (*J* = 8.0 Hz) centered at δ 6.93 (H-5) and 7.09 (H-6), each integrating for one proton indicated a 1,3,4-trisubstituted aromatic moiety in compound MV-3b. Two downfield doublets at δ 7.61 (1H, *J* = 16.0 Hz) and 6.32 (1H, *J* =16.0 Hz) revealed the presence of a pair of *trans*-coupled olefinic protons at H-7 and H-8, respectively. The above spectral features are in close agreement to those observed for caffeic acid[21,24] and thus MV-3b was characterized as caffeic acid**.** Its identity was further confirmed by co-TLC with previously isolated sample.

The DPPH radical scavenging assay is one of the most widely used method to evaluate the antioxidant potentials of plant extracts. Therefore, this method was employed to assess the antioxidant potential of *M. velutina* stem bark extractives via measuring the DPPH free radical scavenging ability. The results of antioxidant activity summarized in Figure 2 showed the concentration-dependent percentage inhibition of DPPH radical (Figure 2a) and IC_{50} values of DEMV, PESF, CSF, EASF, AQSF and standard at different concentrations (Figure 2b). *M. velutina* showed a significant antioxidant property that can be compared with the standard ASA at different concentrations tested. The percentage of DPPH scavenging activity of the tested plant samples increased with concentrations. As shown in Figure 2b, among all the extracts tested, AQSF of *M. velutina* showed the highest DPPH scavenging activity with IC_{50} values of 71.67 µg/ml followed by PESF. The DPPH scavenging activity of stem bark extract of *M. velutina* indicates that the stem bark may contain various active substances especially polyphenol with antioxidant activity.[25]

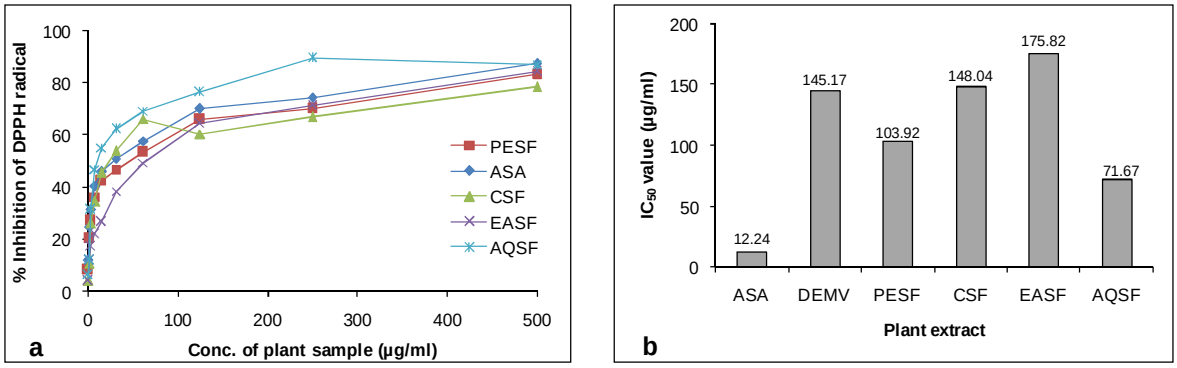


Figure 2. Concentration-dependent % inhibitions of DPPH radical scavenging activity (a) and IC_{50} of DEMV, PESF, CSF, EASF, AQSF and standard ASA (b).

Brine shrimp (*Artemia salina*) lethality bioassay is a common technique employed for preliminary toxicity screening of plant extracts.[17] We used percentage of shrimps died of the toxic effect of the samples in the Y axis, and the logarithmic values of concentrations in the X axis to plot the desired curve. We sketched the best-fit line to calculate the lethal concentration (LC_{90}) values of the test materials by doing regression analysis. Compared to standard vincristine sulfate, all the test samples showed the significant brine shrimp larvicidal activity. The EASF exhibited the maximum LC_{90} values of 9.01 µg/ml which was more potent cytotoxic than vincristine sulfate (LC_{90} of 10.48 µg/ml). The second highest cytotoxic extract was the DEMV (LC_{90} = 13.18 µg/ml), that was also significant compared to

reference drug. Moreover, the other three plant samples CSF, AQSF and PESF showed noticeable LC_{90} values as 22.32, 27.00 and 36.45 µg/ml, respectively (Table 1). The cytotoxic activity exhibited by the plant samples was promising and this clearly indicated the presence of potent bioactive compounds in the extract which requires further investigation.[26]

Table 1. Cytotoxic and thrombolytic activities of crude extract and different partitionates of *M. velutina.*

Sample code	LC_{90} (µg/ml)	% Lysis of clot
DEMV	13.18	46.27
PESF	36.45	39.42
CSF	22.32	36.81
EASF	9.01	38.13
AQSF	27.00	36.68
VS	10.48	--
SK	--	68.68

Here, DEMV = Dichloromethane crude extract of stem bark of *M. velutina*, PESF = Petroleum ether soluble fraction, CSF = Chloroform soluble fraction, EASF = Ethyl acetate soluble fraction and AQSF = Aqueous soluble fractions, VS= Vincristine sulfate, SK = Streptokinase.

The thrombolytic activity of the dichloromethane extract of *M. velutina* and its different Kupchan fractions (PESF, CSF, EASF and AQSF) was assessed to screen its cardio protective potentiality. The anti-thrombus property of the plant samples has been expressed as % of clot lysis that is represented in Table 1. In this experiment, the addition of 100 µl of 3000 I.U. streptokinase exhibited 68.68% lysis of blood clot after 90 min incubation at 37°C. The negative control (distilled water) showed very negligible % clot lysis (0.12%). In this study, the DEMV exhibited highest thrombolytic activity (46.27%) followed by PESF (39.42%) and EASF (38.13%). This might be an important finding which might have important implications in cardiovascular health.

With fewer adverse effects, plant-based extracted samples may have promising antimicrobial properties. Therefore, the development of antimicrobial agents from plant sources is an ongoing research interest globally.[27] In this study, the crude dichloromethane extract of stem bark of *M. velutina* and its organic fractions, at 400 µg/disc, were tested for antimicrobial screening using the disc diffusion method.[19,20] The observed zone of inhibition of the tested samples ranged from 9 to 15 mm and 9 to 13 mm in antibacterial and antifungal assay, respectively (Table 2).

Table 2. Antimicrobial activities of different partitionates of *M. velutina.*

Test microorganisms	Diameter of zone of inhibition (mm)			
	PESF	CSF	AQSF	Standard
Bacteria				Ciprofloxacin
Eschericha coli	9	--	9	48
Pseudomonas aeruginosa	10	12	10	48
Sarcina lutea	10	10	10	49
Shigella boydii	11	15	11	47
Sh. dysenteriae	--	10	--	50
Staphylococcus aureus	9	--	9	50
Fungus				Fluconazole
Aspergillus niger	9	9	--	46
Candida albicans	10	10	10	46
Saccharomyces cerevisiae	13	10	10	46

Among the extractives, the PESF, CSF, and AQSF exhibited mild to moderate antimicrobial activity (Table 2). The CSF revealed the highest inhibition against the growth of tested microbes having a zone of inhibition ranged from 9 mm to 15 mm with maximum activity against *Shigella boydii* (zone of

inhibition = 15 mm). The findings of this study indicate that different samples of *M. velutina* have mild to moderate antimicrobial potential to inhibit the growth of different tested microbial strains.

CONCLUSION

The phytochemical analysis of the dichloromethane extract of the stem barks of *M. velutina* afforded four compounds, which were identified as friedelin, lupeol, β-sitosterone and caffeic acid. The results of *in vitro* pharmacological studies indicated that the tested samples of *M. velutina* have notable antioxidant, cytotoxic, thrombolytic, and moderate antimicrobial activities. In any case, it is vital to investigate the bioactive metabolites responsible for these pharmacological activities. The bioactivities of the extractives of *M. velutina*, as shown in our study, endorse the utilizations of this plant in the traditional system of medicine.

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