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Chemical Constitutions and Hemolytic Activity of *Macrotyloma uniflorum* Linn

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Abstract

In the present investigation, attempt was taken to chromatographic separation and isolation of dichloromethane extract of aerial parts of *Macrotyloma uniflorum* Linn afforded a total of two compounds, methyl ester of hexadecanoic- and ethyl ester of hexadecanoic acid mixture (**I**), and n-hexadecanoic acid (**II**). The structures of these compounds were elucidated by spectroscopic analyses, including UV, IR, ^{1}H-NMR, ^{13}C-NMR and mass spectroscopy. In addition, the fractionated crude extracts dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), 1-butanol (1-BuOH) and aqueous (H_2O) from aerial parts of *M. uniflorum* were tested for hemolytic assay. Among all tested extracts, only the extract of 1-butanol exhibited the significant hemolytic activity.

Key words: *Macrotyloma uniflorum*, extract, spectroscopy, isolation, dichloromethane, hemolytic

INTRODUCTION

Prior to World war second, a series of natural products isolated from higher plants became clinical agents and a number are still in use today. The use of plants as medicines goes back to early man. Certainly the great civilizations of the ancient Chinese, Indians, and North Africans provided written evidence of man ingenuity in utilizing plants for the treatment of a wide variety of diseases [1]. The importance of medicinal plants and traditional health systems in solving the health care problems is gaining increasing attention and because of this resurgence of interest, the research on plants of medicinal importance is rapidly increasing at the international level. However, this is occurring while natural habitats in countries of origin are being lost. Medicinal plants have long been the subjects of human curiosity and need. It is estimated that there are about 2,500,000 species of higher plants and the majority of these have not been examined in detail for their pharmacological activities [2]. Plants are the natural reservoir of many antimicrobial [3] antimalarial [4] anticancer [5] and drug [6] agents.

Macrotyloma uniflorum Linn (Bengali name- Kurti kalai; English name- horse gram; Family- Fabaceae) is a herbaceous plant with annual branches, suberect or twining, leaflets 2.5-5 cm and widely distributed throughout Bangladesh but abundant in Rajshahi and Dinajpur districts [7]. It is famous for its medicinal uses because different parts of the plants are used for the treatment of heart conditions, asthma, bronchitis, leucoderma, urinary discharges and for treatment of kidney stones [8]. Literature survey showed that Dolichin A & B, pyroglutaminylglutamine along with some flavonoids were isolated from this plant [9-10]. Indeed, *M. uniflorum* could play a role in antioxidation [11] as when this plants were exposed to toxic levels of lead, several enzymes showed a pivotal role against oxidative injury. *M. uniflorum* has the greatest potential for further utilization as nutraceuticals, forage, and food for malnourished and drought-prone areas of the world [12]. Herbal medicine is part and parcel of the much needed health care in most of the developing countries including Bangladesh. As a part of our investigations on the medicinal plants of Bangladesh, we investigated *M. uniflorum* and isolated Kaempferol-3-*O*-β-D-glucoside, β-sitosterol and stigmasterol [13], phenolic compounds [14], cytotoxicity assessment [15] and antimicrobial activities [16] of this plant. We herein, report the isolation of methyl ester of hexadecanoic- and ethyl ester of hexadecanoic acid (**I**), and n-hexadecanoic acid (**II**) and hemolytic activity for the first time from the aerial parts of *Macrotyloma uniflorum* Linn growing in Bangladesh.

MATERIALS AND METHODS

Plant Material

Macrotyloma uniflorum (Fabaceae) was collected from the village, Susunda of Muradnagar, Comilla, Bangladesh in March 2002. The botanical identification was made by Prof. Salar Khan (University of Dhaka) and voucher specimen was

deposited at the Bangladesh National Herbarium (BNH) (DACB accession No. 28264).

General

UV spectra were recorded on a Shimdzu UV-160 A spectrophotometer whereas IR spectra were taken on a Shimadzu IR-470 spectrophotometer. ^{1}H-NMR, ^{13}C-NMR spectra were obtained from Bangladesh council of scientific and industrial research (BCSIR) (400 MHz Bruker NMR spectrophotometer with TMS as the internal reference). Mass spectra were also obtained from BCSIR, Dhaka, Bangladesh. Silica gel (G-60, 70-230 mesh, particle size 0.043-0.063 mm) was used for column chromatography. TLC was done on precoated aluminum sheets (Silica gel 60 F_{254}, Merck) using solvent systems S_1: CH_2Cl_2-MeOH (4:20) and S_2: CH_2Cl_2-EtOAc (4:0.8)..

Extraction of Plant Materials

The harvested plant samples (aerial parts) were cleaned, chopped into small pieces and air dried followed by drying at 40^{0}C in an oven and were ground into powder. The powdered plant was (3.5 Kg) was successively extracted with aqueous 80% ethanol (18 L×3 times, 24 h) at room temperature. The extract was filtered and the filtrate was evaporated to dryness at 40^{0}C under vacuum and finally freeze-dried to obtain crude ethanolic extract of 484 g (13.82%) as solid material.

The ethanol extract (480 g) was suspended in water (~2000 mL) and the suspension was transferred into a separating funnel. The aqueous suspension was successively partitioned with dichloromethane (CH_2Cl_2, *ca*. 2000 mL×3), ethyl acetate (EtOAc, *ca*.1500 mL×3) and 1-butanol (1-BuOH, *ca*.1500 mL×3). The CH_2Cl_2, EtOAc, 1-BuOH and aqueous extracts were evaporated separately and lastly freeze-dried. The extractive yield (%) of all extracts is shown in Table 1. The extracts were tested for their hemolytic assay.

Isolation of Compounds

The CH_2Cl_2 soluble extract (40 g) was chromatographed over silica gel column and eluted with hexane followed by CH_2Cl_2, EtOAc and MeOH to afford seven fractions (D_1F_1-D_1F_7). The fraction D_1F_1 (7.5 g) was refractionated on a silica gel column and eluted with mixture of solvent increasing polarity (dichloromethane, ethyl acetate and methanol) and five fractions (D_1F_1'-D_1F_5') were obtained. Compound **I** was obtained from the fraction D_1F_2' after purification by treating with n-hexane. The fraction D_1F_3 (10.0 g) was further fractionated on a silica gel column chromatography using CH_2Cl_2, EtOAc and MeOH as eluants and six fractions (D_2F_1-D_2F_6) were obtained. Fractions (D_2F_3-D_2F_6) gave single spot with tailing. These were yellowish green colored due to associated chlorophyll. The chlorophyll was removed by charcoal treatment. The decolorized fraction (D_2F_3-D_2F_6) were fractionated on a silica gel column and eluted with a mixture of CH_2Cl_2-EtOAc (4:0.8) and four fractions (D_3F_1-D_3F_4) were obtained. Among them the compound **II** (0.15 g) was obtained from the fraction D_3F_3.

Compound I

Yellow semi solid, R_f 0.59 (TLC, S_1); UV ($CHCl_3$) λ_{max} : 236 nm; IR (KBr, cm^{-1}) ν_{max} : 3430 (-OH), 2920 (-CH), 1734 and 1660 (C=O), 1457 (=CH_2); ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 5.28 (3H, broad *s*), 4.52 (1H, *d*, *J* = 6.83 Hz), 4.05 (1H, *d*, *J* = 7.08), 3.58 (3H, *s*), 2.74 (1H, *m*), 2.21 (3H, *d*, *J* = 6.68 Hz), 1.99 (2H, *m*), 1.53 (3H, *m*), 1.22 (48H, broad *s*), 1.02 (2H, broad *s*), 0.80 (9H, *d*, *J* = 6.84 Hz); ^{13}C-NMR (400 MHz, $CDCl_3$): δ_C 174.36, 173.94, 142.21, 134.85, 134.66, 131.76, 130.01, 130.08, 129.86, 129.63, 128.16, 127.99, 127.87, 127.69, 127.08, 124.49, 124.35, 118.30, 61.25, 60.19, 59.92, 51.45, 51.17, 39.68, 37.41, 37.38, 37.20, 36.70, 34.45, 34.17, 33.02, 33.01, 32.87, 32.74, 32.60, 32.01, 29.78, 29.68, 29.54, 29.45, 29.19, 28.34, 25.69, 25.20, 22.77, 22.69, 21.0, 19.64, 19.60, 15.90, 14.14, 14.01 and 13.96; MS m/z (rel. int.): 270 $(M)^+$ ($C_{17}H_{34}O_2$), 239, 227, 199, 185, 171, 143, 87, 74, 55, 43 and 29. The another spectrum MS m/z (rel. int.): 284 $(M)^+$ ($C_{18}H_{36}O_2$), 239, 157, 101, 84, 73, 55, 43 and 29.

Compound II

Light yellow semi solid, R_f 0.56 (TLC, S_2); UV ($CHCl_3$) λ_{max} : 235 nm; IR (KBr, cm^{-1}) ν_{max} : 3330 (-OH), 2856 (-CH), 1704 (C=O), 1487 (=CH_2); ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 5.34 (3H, *s*), 2.79 (2H, *m*), 2.32 (2H, *t, J* = 6.92 and 13.96 Hz), 2.05 (2H, *m*), 1.61 (2H, *m*), 1.27 (14H, broad *s*); ^{13}C-NMR (400 MHz, $CDCl_3$): δ_C 180.33, 131.98, 130.27, 130.05, 128.33, 128.30, 128.14, 127.98, 127.83, 127.19, 34.18, 32.09, 31.61, 29.77, 29.65, 29.53, 29.45, 29.34, 29.23, 29.15, 29.12, 27.27, 25.69, 25.61, 24.74, 22.78, 22.66, 20.63, 14.25, 14.10, 14.05 and 13.92; MS m/z (rel. int.): 256 $(M)^+$ ($C_{16}H_{32}O_2$), 213, 157, 129, 115, 97, 83, 73, 60, 43 and 29.

Hemolytic assay

The test was performed in96-well plates following the method described by [17]. Each well received 100µl of 0.85 NaCl solution containing 10mM $CaCl_2$. The first well was the negative control that contained only the vehicle (distilled water or DMSO 10%), and in the second well, 100µl of test substance that was diluted in half was added. The extracts were tested at concentration ranging from 10 to 2500µg/ml. The serial dilution continued until the 11th well. The last well received 20µl of 0.1% Triton X-100% (in 0.85% saline) to obtain 100% hemolysis (positive control). Then each well received 100µl of a 2% suspension of mouse erythrocytes in 0.85% saline containing 10mM $CaCl_2$. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed and the liberated hemoglobin was measured spectroscopically as absorbance at 540 nm.

The EC_{50} values and their 95% confidence intervals

(CI 95%) were obtained by nonlinear regression using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA).

RESULTS AND DISCUSSION

The aerial parts of *M. uniflorum* were extracted with ethanol at room temperature followed by solvent-solvent partitioning with CH_2Cl_2 and water. Column chromatography fraction of CH_2Cl_2 soluble fractions followed by TLC over silica gel yielded compound **I** and **II**. Compounds were identified by spectral analyses including UV, IR, ^{1}H-NMR, ^{13}C-NMR and MS.

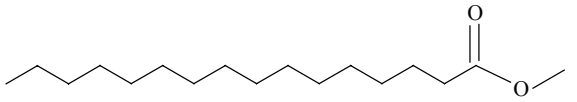
Table 1. Percentage of different extracts from *M. uniflorum*

Extracts	Amounts (g)	Yield[a] (%)
Dichloromethane (CH_2Cl_2)	40.0	1.14
Ethyl acetate (EtOAc)	48.0	1.37
1-Butanol (1-BuOH)	110.0	3.14
Aqueous (H_2O)	58.5	1.67

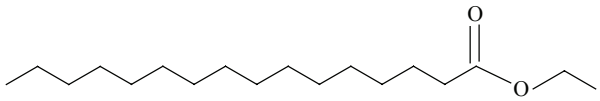
[a]Percentage extract yield (w/w) was estimated as dry extract/dry material weight×100

The Compound **I** was obtained as a yellow semi solid, gave a single black spot with vanillin-H_2SO_4 acid spray reagent on TLC plates. The UV spectrum showed a λ_{max} 236 nm which indicated that the compound **I** does not contain any conjugation. IR spectrum at 3430 cm^{-1} for –OH group, 2920 cm^{-1} due to aliphatic –CH stretching and the absorption bands at 1734 and 1660 cm^{-1} were indicated the presence of >C=O group and ester carbonyl group respectively. In the ^{1}H-NMR spectrum of the compound **I** showed signals at δ 5.28 (3H, broad singlet) was assigned to –CH linkage and signals at δ 4.52 (1H, *d, J* = 6.83 Hz), 4.05 (1H, *d, J* = 7.08 Hz) and 2.74 (1H, m) were due to the presence of –CH group. The signal δ 3.58 (3H, *s*) for –OCH_3 group, δ 1.22 (14 H, broad singlet) & 1.02 (2H, broad singlet) indicated the presence of methylene group in fatty acid ester. A doublet at δ 0.80 (9H, *d, J* = 6.84 Hz) assigned the methyl group had adjacent >CH group in the compound.

The ^{13}C-NMR showed the presence of 53 carbons of the compound. 174.36 and 173.36 ppm disappeared in DEPT spectrum supported the presence of two ester carbonyl carbon in the compound **I**. DEPT spectrum for six methyl carbon which signals at 19.60, 19.64, 15.90, 14.14, 14.01 and 13.96 ppm. Fifteen methylene carbon signals at 61.25, 60.19, 51.45, 51.17, 39.68, 37.38, 37.20, 33.02, 32.74, 32.01, 29.78, 29.68, 29.54, 25.20, 21.0 ppm and eighteen methine carbon signals at 131.76, 130.08, 130.01, 128.16, 127.99, 127.87, 127.69, 127.08, 124.35, 59.92, 37.41, 33.01, 32.60, 29.45, 29.19, 25.69, 22.77 and 22.69 ppm. By subtracting these carbon signals from the total ^{13}C-NMR spectrum, the remaining eleven signals were assigned to eleven quaternary carbons. The mass spectrum of the compound **I** gave molecular ion peak at m/z 270 and 284 corresponding to the molecular formula $C_{17}H_{34}O_2$, and $C_{18}H_{36}O_2$, respectively. So, above spectral data of the compound **I** it would be a mixture of two compounds methyl ester of hexadecanoic acid ($C_{17}H_{34}O_2$) (Figure 1a) and ethyl ester of hexadecanoic acid ($C_{18}H_{36}O_2$) (Figure 1b). This mixture of compound **I** which was not possible to separate by our conventional chromatography, it may require more spectral studies.



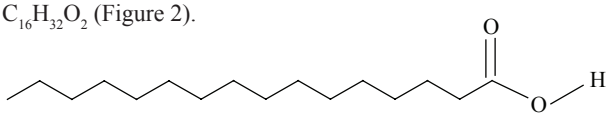
Methyl ester of hexadecanoic acid



Ethyl ester of hexadecanoic acid

Figure-1

The compound **II** was obtained as semi solid and UV spectrum displayed absorption maximum at 235 nm, indicated that it does not contain any conjugation. The IR spectrum at 3330 cm^{-1} for –OH, and 1704 cm^{-1} indicated the presence of >C=O group. The ^{1}H-NMR data showed a signal at δ 5.34 (3H, *s*) was assigned to olefinic proton and signals at δ 2.79 (2H, *m*) and 2.32 (2H, *t, J* = 6.92 and 13.96 Hz) were due to oxymethine proton. The signals at δ 2.05 (2H, *m*) and 1.61 (2H, *m*) were methine proton and the chemical shift at 1.27 (14H, broad *s*) indicated that this compound contain 14 methelene proton. The ^{13}C-NMR indicated the presence of 30 carbons of the compound. The signals at 180.33 ppm for the presence of >C=O group of –COOH. DEPT spectrum showed that two methyl carbon which signals at 14.33 and 14.18. Eighteen methylene carbon signals at 34.18, 32.09, 31.61, 29.77, 29.65, 29.53, 29.45, 29.34, 29.23, 29.15, 29.12, 27.27, 25.69, 25.61, 24.74, 22.78, 22.66 and 20.63 ppm and nine methine carbons signals at 131.98, 130.27, 130.05, 128.33, 128.30, 128.14, 127.98, 127.83 and 127.19 ppm. On the basis of UV, IR, ^{1}H-NMR, ^{13}C-NMR and mass spectral data it was concluded that the compound **II** was a n-hexadecanoic acid as formula $C_{16}H_{32}O_2$ (Figure 2).



n-Hexadecanoic acid

Figure-2

Table 2. Hemolytic activity of *M. uniflorum* extracts on mouse erythrocyte (2%)

Extracts	EC_{50} (μg/ml) CI 95%
Dichloromethane (CH_2Cl_2)	>2400
Ethyl acetate (EtOAc)	846
1-Butanol (1-BuOH)	200
Aqueous (H_2O)	>2500

The results obtained from the hemolytic assay are presented in Table 2. The extract obtained from 1-butanol was the most active in this assay (EC_{50} = 200 μg/ml), followed by the extract from ethyl acetate, which presented EC_{50} values of 846 μg/ml. The other tested extracts were inactive in this assay. In conclusion, this study shows that the *M. uniflorum* plant could be considered as potential sources of therapeutic agents. Further studies are necessary for chemical characterization of the active principle and more extensive biological evaluations.

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