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Chemical Constituents and Hemolytic Activity of *Macrotyloma uniflorum* L.

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Abstract: The bioactivity guided separation of the dichloromethane extract of the aerial parts of *Macrotyloma uniflorum* Linn. resulted in the isolation of methyl ester of hexadecanoic and ethyl ester of hexadecanoic acid mixture (I) and n-hexadecanoic acid (II). The structures of the isolated compounds were elucidated by spectroscopic analysis, including UV, IR, ^{1}H-NMR, ^{13}C-NMR and mass spectroscopy. In addition, the fractionated crude extract of 1-butanol exhibited the significant hemolytic activity by using mouse erythrocytes.

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 (Phillipson, 2001). 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 2500000 species of higher plants and the majority of these have not been examined in detail for their pharmacological activities (Ram *et al*., 2004). Plants are the natural reservoir of many antimicrobial (Cowan, 1999) antimalarial (Schwikkard and van Heerden, 2002) anticancer (Kintzios, 2006) and drug (Rates, 2001) 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 (Kirtikar and Basu, 1998). 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

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treatment of kidney stones (Ghani, 2003). Literature survey showed that Dolichin A and B, pyroglutaminylglutamine along with some flavonoids were isolated from this plant (Incham *et al*., 1981; Handa *et al*., 1990). Indeed, *M. uniflorum* could play a role in antioxidation (Reddy *et al*., 2005) as when this plants were exposed to toxic levels of lead, several enzymes showed a pivotal role against oxidative injury. *Macrotyloma uniflorum* has the greatest potential for further utilization as nutraceuticals, forage and food for malnourished and drought-prone areas of the world (Morris, 2008). Herbal medicine is part and parcel of the much needed health care in most of the developing countries including Bangladesh. However, in previous phytochemical studies, Kaempferol-3-O-β-D-glucoside, β-sitosterol and stigmasterol (Kawsar *et al*., 2003) and phenolic compounds (Kawsar *et al*., 2008a) were isolated from *M. uniflorum*. Recently, the cytotoxicity (Kawsar *et al*., 2008b) and antimicrobial activities (Kawsar *et al*., 2008c) of this plant has been reported. Therefore, in the present study was conducted to isolation of compounds and hemolytic activity by using mouse erythrocytes for the first time from the aerial parts of *M. 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°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°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

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

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 in 96 well plates following the method described by (Costa-Lotufo *et al*., 2002). Each well received 100 µL of 0.85 NaCl solution containing 10 mM $CaCl_2$. The first well was the negative control that contained only the vehicle (distilled water or DMSO 10%) and in the second well, 100 µL of test substance that was diluted in half was added. The extracts were tested at concentration ranging from 10 to 2500 µg mL^{-1}. The serial dilution continued until the 11th well. The last well received 20 µL of 0.1% Triton X-100% (in 0.85% saline) to obtain 100% hemolysis (positive control). Then each well received 100 µL of a 2% suspension of mouse erythrocytes in 0.85% saline containing 10 mM $CaCl_2$. 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

Repetitive chromatography of the dichloromethane soluble extract of *M. uniflorum* afforded two compounds I and II. Compound I was obtained as a yellow semi solid and UV spectrum showed a λ_{max} 236 nm which indicated that it does not contain any conjugation. The IR spectrum suggested hydroxyl (3430 cm^{-1}), aliphatic –CH stretching (2920 cm^{-1}), carbonyl (1734 cm^{-1}) and ester carbonyl (1660 cm^{-1}). Its ^{1}H-NMR spectra revealed that signals at δ 5.28 (3H, broad singlet) for -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) and 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. ^{13}C-NMR data of 2 showed the presence of 53 carbons and DEPT spectrum confirmed that the peaks at δ 174.36 and 173.36 were due to two ester carbonyl carbons. Six methyl carbon peaks at δ 19.60, 19.64, 15.90, 14.14, 14.01 and 13.96; fifteen methylene peaks at δ 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 and eighteen methine peaks at δ 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. By subtracting these carbon signals from the total ^{13}C-NMR spectrum, the remaining eleven signals were assigned to eleven quaternary carbons. The 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. On the basis of these spectral data, compound I was identified as a mixture of two compounds methyl ester of hexadecanoic acid ($C_{17}H_{34}O_2$) (Fig. 1a) and ethyl ester of hexadecanoic acid ($C_{18}H_{36}O_2$) (Fig. 1b). This mixture of compound I which was not possible to separate by our conventional chromatography, it may require more spectral studies.

Compound II was isolated as a semi solid and UV spectrum (235 nm) indicated that it does not contain any conjugation. IR spectrum for -OH (3330 cm^{-1}) and for >C = O (1704 cm^{-1}) groups. ^{1}H-NMR data revealed a signal at δ 5.34 (3H, s) for olefinic proton and two signals at δ 2.79 (2H, m) and 2.32 (2H, t, J = 6.92 and 13.96 Hz) for oxymethine protons. 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 the compound contained 14 methylene protons. ^{13}C-NMR spectrum of the compound had 30 carbon signals indicating that the compound contained 30 carbons. The peak at 180.33 for the presence of >C = O group of -COOH. DEPT experiment showed that two methyl carbons peak at 14.33 and 14.18. Eighteen methylene carbons peak at 34.18, 32.09, 31.61, 29.77, 29.65, 29.53, 29.45, 29.34,

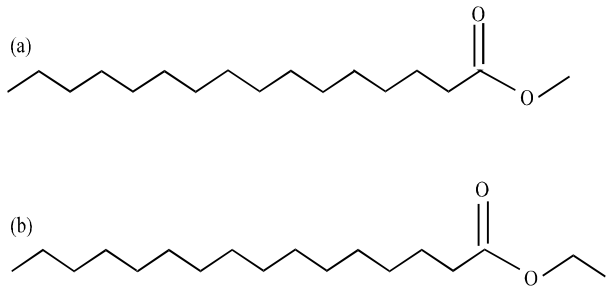

Fig. 1: Structure of the compound I (a: methyl ester of hexadecanoic acid and b: ethyl ester of hexadecanoic acid)

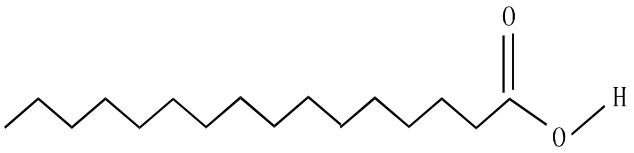

Fig. 2: Structure of the compound II (n-hexadecanoic acid)

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

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

The total hemolysis was obtained with 50 μL of Triton X-100 1% and 1 h incubation. The EC_{50} and 95% confidence interval (CI 95%) were obtained by non-linear regression. Extracts with an EC_{50} value lower than 250 μg mL^{-1} were considered active

29.23, 29.15, 29.12, 27.27, 25.69, 25.61, 24.74, 22.78, 22.66 and 20.63; nine methine carbons peak at 131.98, 130.27, 130.05, 128.33, 128.30, 128.14, 127.98, 127.83 and 127.19. The molecular ion peak at m/z 256. The molecular formula of the compound was ascertained as $C_{16}H_{32}O_2$ (Fig. 2). The above fact indicated that the compound might be an n-hexadecanoic acid.

The n-hexadecanoic-, methyl/ethyl ester of hexadecanoic acids are considered as fatty acids and these play important role in biological process (Aleryani *et al*., 2005; Bao *et al*., 2002). Like other plants, *Litsea glutinosa* (Chowdhury *et al*., 2008), *Suaeda maritime* (Leach *et al*., 1990), *Alpinia hainanesis* and *Alpinia katsumadai* (Nan *et al*., 2004), *Macrotyloma uniflorum* was also found to contain n-hexadecanoic acid. *Macrotyloma uniflorum* L. plant is relatively high in iron, but the availability of the iron is reduced by the phylates, tannins and oxalic acid it contains. *M. uniflorum* is also a good source of protein (Borhade *et al*., 1984) and appears to be a fairly good source of calcium (Sudha *et al*., 1995). The carbohydrate content of the whole *M. uniflorum* is 56.3% and dehulled ash content 2.92% and this plant is also good sources of antioxidant (Siddhuraju and Manian, 2007). Recently, *M. uniflorum* extracts were found to be effective in the inhibition of calcium oxalate crystallization (Das *et al*., 2005). Proteinase inhibitor was also isolated from this plant inhibited specifically the enzymes trypsin and chymotrypsin (Ramasarma *et al*., 1995). The plant proteinase inhibitors of serine proteinases play a dominant role in natural plant defense and infection processes.

The extract obtained from 1-butanol was the most active in this assay (EC_{50} = 200 μg mL^{-1}), followed by the extract from ethyl acetate, which presented EC_{50} values of 846 μg mL^{-1} (Table 2). 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.

CONCLUSION

The specific aims were to isolation of compounds from *M. uniflorum*, methyl ester of hexadecanoic- and ethyl ester of hexadecanoic acid mixture and n-hexadecanoic acid were identified by using spectroscopic methods. The 1-butanol extract showed the significant hemolytic activity by mouse erythrocytes. The presence of compounds in our results is of great interest for the investigation of members of this genus where it constitutes a possible chemotaxonomic marker.

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