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, 1H-NMR, 13C-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 (Schwilkard 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 (Kirkor 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 healthcare 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 Munshidagram, 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 Shimadzu UV-160 A spectrophotometer whereas IR spectra were taken on a Shimadzu IR-470 spectrophotometer. 1H-NMR, 13C-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 0.043-0.63 mm) was used for column chromatography. TLC was done on precoated aluminum sheets (Silica gel 60 F254, Merek) using solvent systems S1: CH2Cl2-MeOH (4:20) and S2: CH2Cl2-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 (CH2Cl2, ca. 2000 mL×3), ethyl acetate (EtOAc, ca. 1500 mL×3) and 1-butanol (1-BuOH, ca. 1500 mL×3). The CH2Cl2, 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 CH2Cl2 soluble extract (40 g) was chromatographed over silica gel column and eluted with hexane followed by CH2Cl2, EtOAc and MeOH to afford seven fractions (D1,F1-D1,F7). The fraction D1,F1 (7.5 g) was reinfractionated on a silica gel column and eluted with mixture of solvent increasing...
Table 1: Percentage of different extracts from M. uniflorum

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Amounts (g)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichloromethane (CHCl₃)</td>
<td>40.0</td>
<td>1.14</td>
</tr>
<tr>
<td>Ethyl acetate (EtOAc)</td>
<td>48.0</td>
<td>1.37</td>
</tr>
<tr>
<td>1-butanol (1-ButOH)</td>
<td>110.0</td>
<td>3.14</td>
</tr>
<tr>
<td>Aqueous (H₂O)</td>
<td>58.5</td>
<td>1.67</td>
</tr>
</tbody>
</table>

*Percentage extract yield (w/w) was estimated as dry extract/dry material weight

polarity (dichloromethane, ethyl acetate and methanol) and five fractions (D₃F₇-D₃F₁) were obtained. Compound I was obtained from the fraction D₃F₇ after purification by flash chromatography with n-hexane. The fraction D₃F₇ (10.0 g) was further fractionated on a silica gel column chromatography using CHCl₃-CH₂OAc and MeOH as eluants and six fractions (D₃F₁-D₃F₄) were obtained. Fractions (D₃F₁-D₃F₆) gave a single spot with tailing. These were yellowish green colored due to associated chlorophyll. The chlorophyll was removed by charcoal treatment. The dechlorinated fraction (D₃F₇) were fractionated on a silica gel column and eluted with a mixture of CH₂Cl₂-CH₂OAc (4:0.8) and four fractions (D₃F₁-D₃F₄) were obtained. Among them the compound II (0.15 g) was obtained from the fraction D₃F₇.

**Compound I**

Yellow semi solid, Rₛ 0.59 (TLC, S₅); UV (CHCl₃) λₑƛₑƛₑ : 236 nm; IR (KBr, cm⁻¹) v max : 3450 (–OH), 2920 (–CH), 1734 and 1660 (C = O, 1457 (–CH₂); ¹H-NMR (400 MHz, CDCl₃): δₗ 5.28 (3H, s, broad δ), 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); ¹³C-NMR (400 MHz, CDCl₃): δₗ 174.36, 173.94, 142.21, 134.85, 134.66, 131.76, 131.01, 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, 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⁺) (C₁₅H₂₃O₂), 239, 227, 199, 185, 171, 143, 87, 74, 55, 43 and 29. The another spectrum MS m/z (rel. int.): 284 (M⁺) (C₁₆H₂₃O₂), 239, 157, 101, 84, 73, 55, 43 and 29.

**Compound II**

Light yellow semi solid, Rₛ 0.56 (TLC, S₅); UV (CHCl₃) λₑƛₑƛₑ : 235 nm; IR (KBr, cm⁻¹) v max : 3330 (–OH), 2856 (–CH), 1704 (C = O), 1487 (–CH₂); ¹H-NMR (400 MHz, CDCl₃): δₗ 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); ¹³C-NMR (400 MHz, CDCl₃): δₗ 180.33, 131.98, 130.27, 130.05, 128.33, 128.30, 128.14, 127.98, 127.83, 127.19, 34.18, 32.09, 31.99, 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⁺) (C₁₀H₁₂O₂), 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 CuCl₂. 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 CuCl₂. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed and the liberated hemoglobin was measured spectrophotometrically as absorbance at 540 nm.
The EC50 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 λmax 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 ¹H-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 –OCH₃ 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. ¹³C-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 methane 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 ¹³C-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 C₁₉H₃₀O₂ and C₁₉H₂₈O₂, respectively. On the basis of these spectral data, compound I was identified as a mixture of two compounds methyl ester of hexadecanoic acid (C₁₉H₃₆O₂) (Fig. 1a) and ethyl ester of hexadecanoic acid (C₁₉H₃₈O₂) (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 yellow 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. ¹H-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. ¹³C-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,

![Structure of the compound I](image)

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%)

<table>
<thead>
<tr>
<th>Extracts</th>
<th>EC₅₀ (μg mL⁻¹)</th>
<th>CI 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichloromethane (CH₂Cl₂)</td>
<td>&gt;2400</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate (EtOAc)</td>
<td>840</td>
<td></td>
</tr>
<tr>
<td>1-butanol (1-ButOH)</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>Aqueous (H₂O)</td>
<td>2800</td>
<td></td>
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</tbody>
</table>

The total hemolysis was obtained with 50 μL of Triton X-100 1% and 1 hr incubation. The EC₅₀ and 95% confidence interval (CI 95%) were obtained by non-linear regression. Extracts with an EC₅₀ 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 methane 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 C₁₆H₃₁O₂ (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 (Aleyami et al., 2005; Bao et al., 2002). Like other plants, Litsea glutinosa (Chowdhury et al., 2008), Suaeda maritima (Lech et al., 1990), Alpinia hainanensis and Alpinia katsumadai (Nan et al., 2004), Macrotlyloma uniflorum was also found to contain n-hexadecanoic acid. Macrotlyloma 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). Protease 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 (EC₅₀ = 200 μg mL⁻¹), followed by the extract from ethyl acetate, which presented EC₅₀ 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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REFERENCES


