Research Article

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Isolation and identification of flavonoid glycosides and natural phthalate derivative from the leaves extract of *Melothria heterophylla* (Lour.) Cogn.

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ABSTRACT

Two known flavonoid glycosides (luteolin-7-O-glucoside and apigenin-7-O-glucoside) and phthalate derivative (dioctyl phthalate) were isolated and characterized for the first time from the methanolic leaves extract of *Melothria heterophylla* (Lour.) Cogn. a well known traditional plant in Mizoram, India for the treatment and management of inflammation, fever, malaria, sprain and pain. The structure of these compounds were established with the aid of spectroscopic methods (UV, Mass, IR, ^{13}C NMR and ^{1}H NMR spectroscopy).

KEYWORD: *Melothria heterophylla* (Lour.) Cogn.; Cucurbitaceae; Flavonoid glycoside; Luteolin 7-O-glucoside; Apigenin 7-O-glucoside; Dioctyl phthalate

1. INTRODUCTION

Melothria heterophylla (Lour.) Cogn. (Cucurbitaceae) commonly known as *nauawimu* is a perennial herb, wild glabrous climber with slender, angular branches with yellow white flower which has been used in traditional folklore medicine for their anti-inflammatory, analgesic and antipyretic activities, purgative, sedative, anti-diabetic and anti-malarial effects and also is claimed to be useful in treating rheumatism, toothache and respiratory disorders by the tribal people of Mizoram, India[1]. Plant derived natural products such as flavonoids, terpenes and alkaloids have received considerable attention in recent years due to their diverse pharmacological properties including anti-inflammatory, antipyretic and analgesic activities[2]. Literature survey revealed that steroids and a mixture of lignoceric, tricosanoic and behenic acid was isolated from this plant and the anthelmintic activity of the aerial parts of the plant and the inhibitory effects of an antioxidant constituents from the plant on matrix metalloproteinase-1 expression in UV-radiated human dermal fibroblasts were also reported[3, 4, 5]. In the present study, we report the isolation and characterization of two known flavonoid glycosides (luteolin- 7-O-glucoside and apigenin 7-O-glucoside) and phthalate derivative (dioctyl phthalate) from the methanolic leaves extract of *Melothria heterophylla* (Lour.) Cogn.

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2. MATERIALS AND METHODS

2.1. Instrument:

Melting points were determined on a melting point apparatus (JSGW, Model-3046). The UV spectra were recorded by using Jasco-Spectrophotometer Model V-530 in MeOH solution. The IR Spectra of the isolated compounds were determined in KBr discs and film using IR Prestige-21, FTIR Shimadzu Spectrophotometer. ^{1}H NMR and ^{13}C NMR Spectra of the isolated compounds were recorded in Bruker-DPX-300 (Switzerland; 300 mHz) Spectrophotometer at DMSO-d6 using TMS as internal standard. Mass spectra of the isolated compounds were recorded by using a Time of Flight-Mass Spectrophotometer: Qtof Micro Y A263 (Switzerland). For cc, Silica gel silica gel G (E. Merk & Co Ltd) was used. Silica Gel 60-120 mesh size (E. Merk & Co Ltd) was used for analytical TLC

2.2. Plant material

Leaves of *Melothria heterophylla* (Lour.) Cogn. were collected from Mualpui area, Aizawl, Mizoram during July 2008. The plant material was identified and authenticated by Dr P.Venu (Scientist'F'), Botanical Survey of India, Kolkata (Reference No: CHN/I-I/60/2010/Tech.II/277 Dated: 30-07-2010). A specimen having voucher number MH-018 (2009) was deposited in the herbarium of the Department of Pharmacy, Regional Institute of Paramedical and Nursing Sciences (RIPANS), Aizawl, Mizoram for future references. The collected fresh leaves of the plant were cleaned, washed with distilled water, dried in ventilated room under shade for three weeks, pulverized using grinder and passed through 40-mesh sieve to get fine powder.

2.3. Extraction and isolation

The air- dried and powdered leaves (2.0 kg) were extracted at room temperature successively by soxhlet apparatus using petroleum ether (60-80^{0}C b.p) followed by methanol (MeOH). The extraction was carried out exhaustively and the solvents were recovered by distillation under reduced pressure using rotary vacuum evaporator to obtain crude petroleum ether extract (113 g, 4.5 %; w/w) and MeOH extract (300 g, 12 %; w/w). In the present study, the crude methanol extract of *Melothria heterophylla* was suspended in water and partitioned with n-hexane (5:5). The residual aqueous layer was again fractionated with ethyl acetate (EtOAc) in order to remove chlorophyll, neutral substances and other impurities. The EtOAc soluble fraction was then further spotted on the TLC plates and chromatogram was developed in chromatographic chambers using selected solvent systems at room temperature. The visualization of spots on TLC plates was carried out either under UV chamber at 254nm (short wavelength) or at 365nm (long wavelength) using antimony trichloride in chloroform (20%) as spraying reagent. Several solvent systems were studied for effective separation of the components, but EtOAc: MeOH (7:3) showed the best separation. Five distinct spots as well as fluorescences (light blue, dark yellow, bright yellow, pale yellow and light pink) with different Rf values (0.81, 0.73, 0.58, 0.43 and 0.38) were visualized which shows the presence of flavonoids in the crude EtOAc soluble fraction of the MeOH extract of the plant. Then further isolation of the active constituents was done with EtOAc soluble fraction of the MeOH extract of the plant. EtOAc soluble fraction was dissolved in a minimum amount of EtOAc, adsorbed on silica gel to form a slurry, air dried till free flowing and chromatographed over silica column (using silica gel, 60-120 mesh size; 10-20 times the weight of extract). The column was eluted successively with EtOAc and MeOH of increasing order of polarity (10:0; 9:1; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 2:8; 1:9 and 0:10). The solvent elution rate was adjusted at 40 drops per minute. Fractions of approximately 50 ml each were collected and monitored by TLC. Similar fractions were pooled together and were named Fraction A, B, C and D. Fraction C showing three distinct spots (R_f values 0.73, 0.58 and 0.43) on the TLC plate was selected for re-column. Then fraction C was concentrated and re-chromatographed on silica gel column using a gradient of $CHCl_3$: MeOH (10:0; 9:1; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 2:8; 1:9 and 0:10). Sub fractions number 15-25 eluted with $CHCl_3$ and MeOH (9:1 ratio) having same R_f values were combined, concentrated and again re-chromatographed on silica gel column for further purification to get compound C (yellow amorphous powder, R_f =0.73). Sub fractions number 40-55 eluted with $CHCl_3$: MeOH (8:2 ratio) having same R_f values were combined, concentrated and again re-chromatographed on silica gel column for further purification to get compound A (yellowish thick oil, R_f =0.58). Sub fractions number 70-78 eluted with chloroform and methanol (7:3 ratio) having same R_f values were combined, concentrated and again re-chromatographed on silica gel column for further purification to get compound B (pale yellow needle shape crystal, R_f =0.43).

3. RESULTS

3.1. Compound A .

A yellowish thick oil; UV (MeOH) nm λ_{max}(log ε) : 220 (4.16), 270 (3.80); IR υ^{KBr}_{Max}cm^{-1}: 2940, 2840, 2780, 1750, 1450, 1270, 1120, 1050, 950; ESI-MS: positive-ion m/z 429.10 [M+K]; ^{1}H NMR (300 MHz, DMSO-d_6): *d* 7.69 (2H, *dd*, J=7.5,1.6 Hz, H-2, H-5), 7.52 (2H, *dd*, *J* = 7.5, 1.6 Hz, H-3, H-4), 4.15 (2×2H, *m*, H-1, H-11"), 1.67 (2H, *m*, H-2', H- 2"), 1.27-1.47 (8×2H, *m*, H-3', H-4', H-5', H-7' and H-3", H-4", H-5", H- 7"), 0.92 (2×3H, *t*, *J* = 7.3 Hz, H-8', H-8") and 0.89 (2×3H, *t*, *J* = 7.2 Hz, H-6', H- 6"); ^{13}C NMR (300 MHz, DMSO-d_6): *d* 166.97 (2×C=O), 131.69 (C-1, 6), 131.58 (C-3, 4), 128.64 (C-2, 5), 67.73 (C-1', 1"), 38.94 (C-2', 2"), 29.78 (C-3', 3"), 28.34 (-C 4', 4"), 23.23 (C-7', 7"), 22.37 (C-5', 5"), 13.87 (C-6', 6"), 10.78 (C-8', 8").

3.2. Compound B

A pale yellow needle shape crystal (MeOH); m.p. 230-237° C; UV (MeOH) nm λ_{max}(log e): 270 (3.99), 333 (4.06); IR υ^{KBr}_{Max}cm^{-1}: 3450, 3445 (-OH), 1645, 1640 (C=O) and 1600, 1495 cm-^{1}(C=C); ESIMS: positive-ion m/z 432.12 [M+H]; ^{1}H NMR (300 MHz, DMSO-d_6): *d* 7.94 (2H, *d*, J = 8.4 Hz, H-2',6'), 6.94 (2H, *d*, *J* = 8.4,Hz, H-3',5'), 6.87(1H, *s*, H-3), 6.83 (1H, *bs*, H-8), 6.43 (1H, *bs*, H-6), 5.08 (1H, *d*, *J* = 6.8 Hz, Glu H-1), 3.25-5.08 (6H, *m*, sugar protons).^{13}C NMR (300 MHz, DMSO-d_6): *d* 181.7 (C-4), 164.0 (C-2), 162.3 (C-7), 161.3 (C-4'), 160.9 (C-5), 156.8 (C-9), 128.4 (C-2', 6'), 120.7 (C-1), 115.9 (C-3', 5'(, 105.3 (C-10), 103.0 (C-3), 99.2 (C-6), 94.4 (C-8) and sugar: 97.7 (C-1"), 77.1 (C-5"), 76.9 (C-3") 73.0 (C-2"), 69.4 (C-4"), 60.4 (C-6").

3.3. Compound C

A yellow amorphous powder (MeOH); m.p. 266-268 ^{0}C; UV (MeOH) nm λ_{max}(log e): 255 (3.51), 349 (4.34); IRυ^{KBr}_{Max}cm-^{1}: 3365 (-OH), 1652 (C=O), 1591 (C=C), 1565, 1455 cm-^{1}; ESIMS: positive-ion m/z 449.20 [M+H]; ^{1}H NMR (300 MHz, DMSO-d_6): *d* 12.98 (1H, *s*, OH-5), 7.46 (1H, *dd*, *J* = 8.3, 2.0 Hz, H-6'), 7.43 (1H,*d*, J = 2.0 Hz, H-2'), 6.92 (1H, *d*, *J* = 8.3 Hz, H-5×), 6.79 (1H, *d*, *J* = 1.9 Hz, H-8), 6.75 (1H, *s*, H-3), 6.45 (1H, *d*, *J* = 1.9 Hz, H-6), 5.09 (1H, *d*, *J* = 7.3 Hz, H-1"), 3.73-3.17 (5 H, *m*, H-2", 3", 4", 5"and 6"); ^{13}C NMR (300 MHz, DMSO-d_6): *d* 181.8 (C-4), 164.3 (C-2), 162.8 (C-7), 161.0 (C-5), 156.8 (C-9), 149.8 (C-4'), 145.7 (C-3'), 121.2 (C-1'), 119.0 (C-6'), 115.8 (C-5') 113.4 (C-2'), 105.2 (C-10), 103.0 (C-3), 99.7 (C-1"), 99.4 (C-6), 94.6 (C-8), 77.0 (C-5"), 76.3 (C-3"), 73.0 (C-2"), 69.4 (C-4"), 60.5 (C-6").

4. DISCUSSION:

4.1. Compound A:

Compound A obtained as yellowish thick oil showed molecular ion peak at m/z 429.10 [M+K] ESIMS: positive-ion mode corresponding to the molecular formula $C_{24}H_{38}O_4$. This was further supported by ^{13}C NMR Spectral analysis which displayed 12 signals for all carbon atoms in the molecule including two carbonyl groups at d 166.97 (2×C=O) and the quaternary carbon at d *C* 131.69 showed phthalate moiety. The ^{1}H NMR spectra showed the signals for phthalate moiety: δ *H* 7.69 (dd, *J* = 7.5, 1.6 Hz), δ *H* 7.52 (dd, *J* = 7.5, 1.6 Hz) and δ *C* 128.64; and δ *C* 131.58 showed the phthalate moiety as evidenced from the correlations between the carbonyl groups and the hydroxylated methine protons at d 4.13 and 4.15.

On comparison with the literature, it was revealed that the physical and spectral data (UV, IR, Mass and NMR spectrum) of dioctyl phthalate isolated earlier from *Ferula rutabensis*[6], *Mentha longifolia* [7] and

Alchornea cordifolia (Schumach. & Thornn.) Mull. Arg.[8] were in good agreement with those recorded for dioctyl phthalate in the present study. Therefore, it was concluded that the isolated compound A is ***Dioctyl phthalate.*** The ^{1}H NMR spectrum of yellowish thick oil The ^{1}H NMR spectra showed the signals for phthalate moiety: δ *H* 7.69 (dd, *J* = 7.5, 1.6 Hz),δ *H* 7.52 (dd, *J* = 7.5, 1.6 Hz) and δ *C* 128.64; and δ *C* 131.58; and the quaternary carbon at δ *C* 131.69 and two carbonyl groups at δ 166.97 (2×C=O), showed the phthalate moiety as evidenced from the correlations between the carbonyl groups and the hydroxylated methine protons at δ 4.13 and 4.15. It can also be noted that this compound is more effective than ß-sitosterol, whose anti-inflammatory properties are widely reported in literature[8]. Dioctyl phthalate also showed antimicrobial activity[9]**.**

4.2. Compound B

Compound B obtained as pale yellow needle shape showed molecular ion peak at m/z 429.10 [M+K] ESIMS: positive-ion mode corresponding to the molecular formula $C_{24}H_{38}O_4$. The ^{1}H NMR spectrum of yellow amorphous powder showed The ^{1}H-NMR spectrum of isolated compound B (pale yellow needle shape crystal) in DMSO-d6 contained two distinctive groups of resonances. Those at δ 7.96 (2H) and 6.95 (2H) (both as d, J= 9 Hz), 6.87 (1H, s), 6.84 (1H) and 6.45 (1H) (both as d, J = 2.2 Hz), represented the flavone nucleus protons ; The ^{13}C NMR data showed the presence of a ketone carbonyl (δ 181.8), two olefinic carbons (δ 164.3 and 103.0), and four hydroxyl carbons (δ 162.8, 161.0, 149.8 and 145.7)[10]**.** The ^{13}C NMR chemical shifts of this moiety were consistent with the identification of compound B as a derivative of apigenin[11].The second group, appearing between δ 3.1and 5.06, comprised the resonances of a glucosidic moiety. The anomeric proton resonance observed at δ 5.06 (1H, d, J = 7 Hz) indicated the ß-glucose. The proton signals at δ 12.96 (1H, s) (downfield-shifted due to the hydrogen bonding with 4-oxo group) and 10.41(1H, s) were indicative for the hydroxyl groups located at C-5 and C-4'. The molecular formula $C_{21}H_{20}O_{10}$ calculated from ESIMS quasimolecular ion peaks at m/z 433.12 ([M+H] provided the identity of compound B as apigenin -7-O-glucoside, the ^{1}H and ^{13}CNMR resonance signals of which are in good accordance with those reported for the apigenin-7-O-diglycosides[12]**.** On comparison with the literature on flavonoids of apigenin, it was revealed that the physical and spectral data(UV, IR, Mass and NMR spectrum) of apigenin-7-O-glucoside isolated earlier from *Vitex agnus castus*[13]**,** *Ammoides atlantica*[14]**,** *Erica cinerea*[15]**,** *Mentha longifolia*[7]**,** *Isatis microcarpa*[16]**,** *Sonchus erzincanicus* Matthews[17] and *Acanthus integrifolius*[18] are in good agreement with those recorded for apigenin-7-O-glucoside in the present study. Therefore, it was concluded that the isolated compound B is ***apigenin-7-O-glucoside***. Antibacterial, anti-inflammatory, antihistaminic, antispasmodic and anti-aggregant activity of apigenin was also reported[19].

4.3. Compound C

The ^{1}H NMR spectrum of yellow amorphous powder showed a down field sign at d 12.98 (1H, s) indicating the presence of a chelated hydroxyl at C-5 position. It also showed two meta-coupled doublets (J=1.9 Hz) at d 6.79 and 6.45 each integrating for one proton, and were assigned to H-8 and H-6, respectively of ring A of 5, 7-dihydroxyflavonoids[20]. The presence of ABX system at 7.46 (add, J=8.3, 2.0 Hz), 7.43 (d, J=2.0 Hz) and 6.92 (d, J=8.3 Hz), characteristic of 1, 2, 4-trisubsituted phenyl unit[21]. The only singlet at d 6.75, integrating for one proton, was attributed C-3 to proton of flavonoids[22]. These spectral data revealed the presence of luteolin skeleton[11, 23]. In addition, the ^{1}H NMR spectrum showed a series of signals between d 3.73-3.15, attributable to a sugar moiety. The coupling constant (J=7.3 Hz of the anomeric proton located at δ 5.09 and the ^{13}C NMR chemical shifts of the sugar carbons (δ 99.7, 77.0, 76.3, 73.0, 69.4 and 60.5) revealed the presence of ß-O-glucoside unit in luteolin-7-O-glucoside. The ^{13}C NMR data showed the presence of a ketone carbonyl (δ 181.8), two olefinic carbons (δ 164.3 and 103.0), and four hydroxyl carbons (δ 162.8, 161.0, 149.8 and 145.7)[11]**.** On comparison with the literature on flavonoids of luteolin, it was revealed that the physical and spectral data (UV, IR, Mass and NMR spectrum) of luteolin-7-O-glucoside isolated earlier from callus cultures of *Soymida febrifuga*[24]**,** *Ammoides atlantica*[14]**,** *Isatis microcarpa*[16]**,** *Cakile maritime*[25]**,** *Sonchus erzincanicus* Matthews[17] and *Ballota andreuzziana*[26] were in good agreement with those recorded for luteolin-7-O-glucoside in the present study. Therefore, it was concluded that the isolated compound C is ***luteolin-7-O-glucoside.*** Luteolin also possesses antioxidant effects and pro-oxidant effects[27]. The anti-inflammatory activity of luteolin in acute and chronic models in mice has been reported[28]. The antispasmodic and mild sedative property of luteolin-7-*O*-glucoside was reported[29]. Luteolin-7-*O*-glucoside showed potent xanthinoxidase inhibitory activity *in vitro*[30]**.** Luteolin-7-O-glucoside also showed potent antimicrobial and antioxidant activity[25, 26].

5. CONCLUSION

According to our investigation, it can be concluded that the leaves of *M. heterophylla* are rich in flavonoids as two known flavonoid glycosides (luteolin-7-O-glucoside and apigenin-7-O-glucoside) were isolated and characterized from the methanolic leaves extract of *Melothria heterophylla* (Lour.) Cogn. The present findings proved and validated the traditional use of this plant for the management and treatment of pain, inflammation and fever and for its sedative activity. Even though luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside and dioctyl phthalate were already reported from the other sources, this is the first report for the isolation and structural elucidation of bioactive compounds from the methanolic leaves extract of *Melothria heterophylla* (Lour.) Cogn.

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