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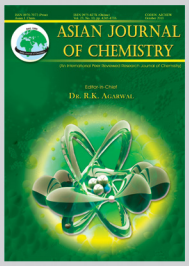

Phytochemical Investigation of the Stem Bark of *Phlogacanthus thyrsiflorus* (Roxb.) Nees.

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The stem bark of *Phlogacanthus thyrsiflorus* (Acanthaceae) was subjected to phytochemical investigation. The powdered stem bark was extracted with methanol and then partitioned between chloroform and petroleum ether. Two labdane diterpenes namely 19-hydroxy-labda 8(17), 13-diene-15, 16-olide and ent-labd-8(17), 13-dien-15,16-olide-19-oic acid (pinusolidic acid) and one triterpene betulin were isolated from the chloroform and petroleum ether fraction of the methanolic extract by column chromatography followed by preparative thin layer chromatography. The structure of the compounds were established by high field NMR methods (^{1}H NMR, ^{13}C NMR) as well as by comparison with reported compounds.

Key Words: Labdane diterpene, 19-Hydroxy-labda 8(17), 13-Diene-15, 16-Olide, Pinusolidic acid, Triterpene, Betulin.

INTRODUCTION

Phlogacanthus thyrsiflorus (Acanthaceae) locally called ram basak is a large shrub found usually in the sub tropical Himalayas, Bihar, North Bengal and Assam[1]. The leaves are 15-20 cm long. They are unequal and sword shaped. The flowers are orange or red. They occur in compact, compound flower arrangements at the end of branches[2].

The whole plant is used in wooping cough and menorrhagia. A decoction of leaves is given for diseases of spleen and liver and for fevers[1]. Flowers are used as antidote to pox, prevents skin diseases like sores, scabies[3]. The ethanolic extracts of leaves of this plant have been reported to have significant central and peripheral analgesic activity[4]. The methanolic extract of leaves of this plant showed antimicrobial activity[5].

The leaf part upon phytochemical investigation revealed two new compounds namely phlogantholide-A-19-O-β-D-glucopyranoside (a diterpene lactone glucoside)[6] and phlogantholide-A (a diterpene lactone)[7] along with some known compounds β-sitosterol, lupeol and betulin[8].

The present communication reports the isolation of two labdane diterpenes namely19-hydroxy-labda 8(17), 13-diene-15,16-olide and ent-labd-8(17), 13-dien-15,16-olide-19-oic acid (pinusolidic acid) and one triterpene, betulin from the methanolic extract of the stem bark of *Phlogacanthus thyrsiflorus.*

EXPERIMENTAL

NMR spectra were obtained on a Bruker Avance (400 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer using the residual solvent peaks as internal standard. Column chromatography was conducted on silica gel (Merck, mesh 70-230) and sephadex LH-20. TLC and preparative thin layer chromatography were carried out using Merck silica gel 60 PF_{254} on glass plates at a thickness of 0.5 mm and spots were visualized under UV light (254 nm) and spraying with 1 % vanillin-H_2SO_4 followed by heating at 110 ºC for 5-10 min.

General procedures: The stem bark of *Phlogacanthus thyrsiflorus* was collected from Sylhet, Bangladesh, in September 2009 and authentification of the plant sample was confirmed by the taxonomist of the National Herbarium of Bangladesh, Mirpur, Dhaka. A voucher specimen was deposited (accession number is 35388) in the herbarium for further reference.

The stem bark after cutting into small pieces were dried under sun for several days. The plant materials were then oven dried for 24 h at considerably low temperature (40 ºC) for better grinding. The dried samples were then ground in coarse powder using high capacity grinding machine. The coarse powder was then stored in air-tight container with marking for identification and kept in cool, dark and dry place for future use.

Extraction and isolation: About 500 g of stem bark powder was soaked in 3 L of methanol in a large conical flask for 7 days with occasional shaking and stirring. The whole mixture was then filtered off through a cotton plug followed by Whatman filter paper no.1 and the filtrate thus obtained was concentrated at 40 ºC with a rotary evaporator. The weight of the dried residue was 8 g, 7.5 g of the extract was then fractionated with petroleum ether and chloroform which afforded petroleum ether (2 g) and chloroform (3 g) soluble fraction.

An aliquot of the chloroform soluble partitionate (2.5 g) was fractionated by column chromatography over silica gel (Kieselgel 60, mesh 70-230) using ethyl acetate and toluene mixture in order of increasing polarities. A total of 106 fractions were collected, each 20 mL. Preparative thin layer chromatography of column fractions eluted with 25 % ethyl acetate in toluene over silica gel was performed using toluene: ethyl acetate (75:25) and this afforded compound **1** (6 mg) and compound **2** (5 mg).

Few portions (500 mg) of the petroleum ether fraction were subjected to column chromatography using sephadex LH-20 as stationary phase. A mixture of *n*-hexane, dichloromethane and methanol was used as mobile phase in an order of increasing polarity. A total of 50 fractions were collected, each 3 mL. Preparative thin layer chromatography of column fractions eluted with (*n*-hexane:dichloromethane:methanol = 2:5:1) was performed using toluene : ethyl acetate (95:5) and this afforded compound **3** (8 mg).

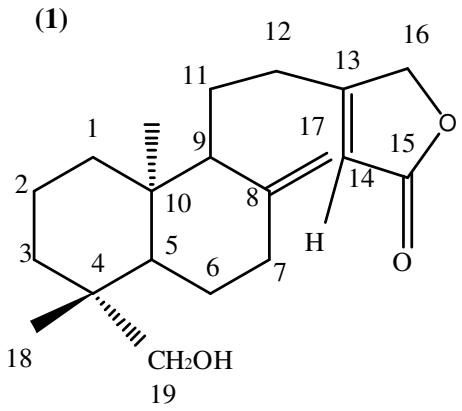

Fig. 1. 19-hydroxy-labda 8(17), 13-diene-15,16-olide (Ref.9)

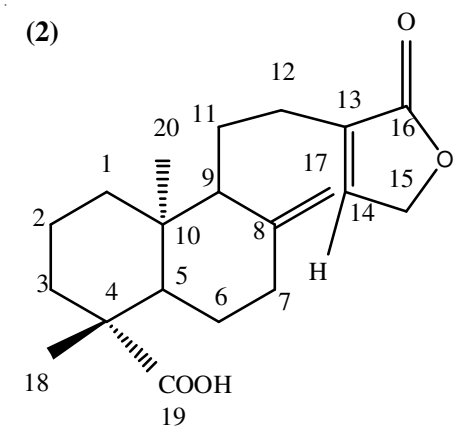

Fig. 2. Ent-labd-8(17), 13-dien-15,16-olide-19 oic acid (pinusolidic acid) (Ref. 9)

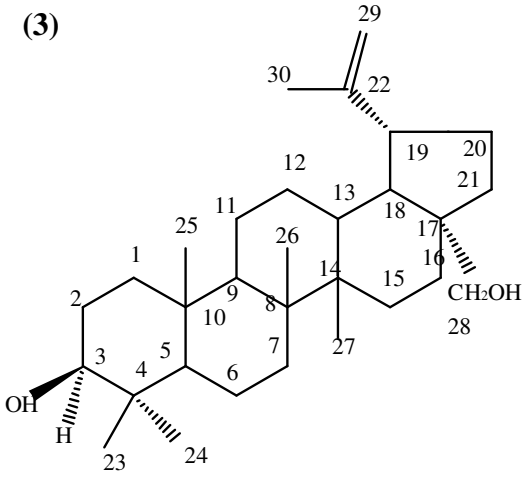

Fig. 3. Betulin (Ref. 10)

Detection method

19-hydroxy-labda 8(17), 13-diene-15,16-olide(1): (6 mg) (Fig. 1); Transparent needles; brick red colour spot was visualized under UV light (254 nm) on TLC plate upon spraying with 1 % vanillin-H_2SO_4 followed by heating at 110 ºC for 5-10 min. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.84 (1H, s) (H-14), 4.68 (1H, d, *J* = 17.6 Hz), 4.73 (1H, d, *J* = 17.6 Hz) (H-16), 4.52 (1H, s), 4.87 (1H, s) (H-17), 0.98 (1H, s) (H-18), 3.72 (1H, d, *J* = 10.8 Hz), 3.39 (1H, d, *J* = 10.8 Hz) (H-19), 0.67 (1H, s) (H-20). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 39.15 (t) (C-1), 18.96 (t) (C-2), 38.54 (t) (C-3), 39.71 (s) (C-4), 56.25 (d) (C-5), 24.48 (t) (C-6), 35.39 (t) (C-7), 147.42 (s) (C-8), 56.30 (d) (C-9), 38.90 (s) (C-10), 21.43 (t) (C-11), 27.48 (t) (C-12), 167.5 (s) (C-13), 115.28 (d) (C-14), 170.88 (s) (C-15), 65.09 (t) (C-16), 106.78 (t) (C-17), 27.09 (q) (C-18), 73.12 (t) (C-19), 15.25 (q) (C-20).

Ent-labd-8(17), 13-dien-15,16-olide-19 oic acid (Pinusolidic acid) (2) (Fig. 2): (5 mg); white amorphous powder; pink colour spot was visualized under UV light (254 nm) on TLC plate upon spraying with 1 % vanillin-H_2SO_4 followed by heating at 110 ºC for 5-10 min. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.08 (1H, s) (H-14), 4.75 (2H, s) (H-15), 4.58 (1H, s), 4.89 (1H, s) (H-17), 1.24 (3H, s) (H-18), 0.61 (3H, s) (H-20). ^{13}C NMR (125 MHz,$CDCl_3$): δ_C 39.2 (d) (C-1), 19.89 (d) (C-2), 38.64 (d) (C-3), 40.5 (s) (C-4), 55.74 (d) (C-5), 24.68 (t) (C-6), 38.05 (d) (C-7), 147.4 (s) (C-8), 56.3 (d) (C-9), 44.1 (s) (C-10), 21.9(t)(C-11),26.06(d)(C-12), 134.9 (s)(C-13), 143.9 (d) (C-14), 70.1 (t) (C-15), 174 (s) (C-16), 106.8 (t) (C-17).

Betulin (3) (Fig. 3): (8 mg); white amorphous powder; purple colour spot was visualized under UV light (254 nm) on TLC plate upon spraying with 1 % vanillin-H_2SO_4 followed by heating at 110 ºC for 5-10 min. ^{1}H NMR(400 MHz, $CDCl_3$): δ 3.18 (1H, dd, *J* = 10.2, 5.2 Hz) (H-3), 0.96 (3H, s) (H-23), 0.75 (3H, s) (H-24), 0.82 (3H, s) (H-25), 0.97 (3H, s) (H-26), 1.02 (3H, s) (H-27), 3.32 (1H, d, *J* = 10.8 Hz), 3.79 (1H, d, *J* = 10.8 Hz) (H-28), 4.57 (1H, s), 4.67 (1H, s)(H-29), 1.67 (3H, s) (H-30).

RESULTS AND DISCUSSION

A total of three compounds were isolated from chloroform and petroleum ether fractions of the methanolic extract

of the stem bark of *Phlogacanthus thyrsiflorus* by chromatographic separation and purification over silica gel. The structures of the isolated compounds were elucidated by high field NMR (^{1}H NMR, ^{13}C NMR) as well as by comparison with the reported compounds.

From the ^{1}H NMR spectrum (400 MHz, $CDCl_3$), compound 1 was elucidated as 19-hydroxy-ent-labd- 8(17), 13-dien-15,16-olide. The spectrum showed one doublet of one proton intensity at δ 3.72 (J = 10.8 Hz) and another one proton doublet at δ 3.39 (J = 10.8 Hz). The signal confirmed the position of a hydroxymethylene group at position 19. The spectrum displayed two singlet at δ 4.52 and δ 4.87 (1H each) assignable to protons at C-17. Two doublet of one proton at δ 4.68 and δ 4.73 (J = 17.6) assignable to proton at C-16. The spectrum displayed two singlets at δ 0.67 and δ 0.98 (3H each) assignable to protons of methyl groups at C-20 and C-18 respectively. By comparing the ^{1}H NMR spectral data with those reported[9] for related compound, it was established as a labdane diterpene namely 19-hydroxy-ent-labd- 8(17), 13-dien-15,16-olide.

The ^{13}C NMR data of compound **1** displayed signals for five non-protonated carbon at δ_C 39.71 (C-4), δ_C 38.90 (C-10), δ_C 167.50 (C-13), δ_C 147.42 (C-8), δ_C 170.88 (C-15), three methines at δ_C 56.3 (C-9), δ_C 56.25 (C-5), δ_C 115.28 (C-14), nine methylenes at δ_C 21.43 (C-11), δ_C 24.48 (C-6), δ_C 27.48 (C-12), δ_C 35.39 (C-7), δ_C 38.54 (C-3), δ_C 39.15 (C-1), δ_C 65.09 (C-16), δ_C 106.78 (C-17), δ_C 73.12 (C-19), two methyls at δ_C 27.09 (C-18) and δ_C 15.25 (C-20). By comparing the ^{13}C NMR data with reported data[6], the structure was established as 19-hydroxy-ent-labd- 8(17), 13-dien-15,16-olide. This is the first report of isolation of this compound from this plant.

From the ^{1}H NMR spectrum (400 MHz, $CDCl_3$), compound **2** was elucidated as ent-labd- 8(17), 13-dien-15,16-olide-19-oic acid (pinusolidic acid). The spectrum showed three singlet of one proton intensity at δ 7.08, δ 4.89 and δ 4.58 confirming the position of one proton at C-14, two protons at C-17 for the exomethylene group respectively. The signal for two protons at C-15 appeared as a singlet at δ 4.75. The spectrum displayed two singlets at δ 0.61 and δ 1.24 (3H each) assignable to protons of tertiary methyl groups at C-20 and C-18 respectively. By comparing the ^{1}H NMR spectral data with those reported[7] for related compound, it was established as a labdane diterpene namely ent-labd- 8(17), 13-dien-15,16-olide-19-oic acid (pinusolidic acid).

The ^{13}C NMR data of compound **2** displayed signals for five non-protonated carbon at δ_C 174 (C-16), δ_C 134.9 (C-13), δ_C 147.4 (C-8), δ_C 44.1 (C-10), δ_C 40.5 (C-4), three methines at δ_C 56.3 (C-9), δ_C 55.74 (C-5), δ_C 143.9 (C-14), nine methylenes at δ_C 106.8 (C-17), δ_C 21.9 (C-11), δ_C 39.2 (C-1), δ_C 19.89 (C-2), δ_C 38.64 (C-3), δ_C 24.68 (C-6), δ_C 38.05 (C-7), δ_C 26.06 (C-12), δ_C 70.09 (C-15). By comparing the ^{13}C NMR data with reported data[7], the structure was established as ent-labd- 8(17), 13-dien-15,16-olide-19-oic acid (pinusolidic acid). This is the first report of isolation of this compound from this plant.

From the ^{1}H NMR spectrum (400 MHZ, $CDCl_3$), compound **3** was elucidated as betulin. The spectrum showed one isopropenyl moiety at δ 1.67 (C-30) indicating a lupine skeleton, one proton singlet at δ 4.67 and one proton singlet at δ 4.57 indicating the presence of two exocyclic methylene protons at (C-29). Two doublets of one proton centered at δ 3.32 (J = 10.8 Hz) and δ 3.74 (10.8 Hz) is evident for the presence of methylene proton attached to hydroxyl group at (C-28). The ^{1}H NMR spectrum revealed a double doublet at δ 3.18 (J = 10.2 Hz and 5.2 Hz) assigning for the proton at C-3, the presence of a number of methyl protons appeared as five singlets at δ 0.75 (C-24), δ 0.82 (C-25), δ 0.96 (C-26), δ 0.97 (C-23), δ 1.02 (C-27). The above spectral features are in close agreement to those reported for betulin[10] and hence the structure was confirmed as betulin.

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