- Short communication
Bangladesh J. Bot. **51**(1): 185-189, 2022 (March) DOI: https://doi.org/10.3329/bjb.v51i1.58836

LUPANE TRITERPENOIDS, PHYTOSTEROL, PHENOLIC ACID AND FATTY ACID FROM LEAVES OF *BOUEA OPPOSITIFOLIA* (ROXB.) MEISN.

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Keywords: *Bouea oppositifolia*, Betulin, Lupenone, Lupeol, Stigmasterol, *p*-Coumaric acid, Oleic acid

Abstract

Phytochemical investigation of petroleum ether (PESF) and dichloromethane soluble (DCMSF) fractions designated as PESF and DCMSF, respectively of methanol extract of leaves of *Bouea oppositifolia* (Roxb. Meisn. (an endangered species of Bangladesh) yielded six compounds, which were characterized as betulin (**1**), lupeol (**2**), lupenone (**3**), stigmasterol (**4**), *p*-coumaric acid (**5**), and oleic acid (**6**) by high field NMR studies and comparison with published values. The identity of the isolated compounds were further substantiated by co-TLC.

Since antiquity, plants and plant-derived herbal products have been used as drugs to alleviate different ailments (Balunas and Kinghorn 2005). Besides, plant-derived molecules also serve as templates for the designing, synthesis or semi-synthesis of other therapeutic agents.

Bouea oppositifolia (Roxb.) Meisn., locally known as Mali aam belonging to Anacardiaceae, is a tree that grows up to 33 meters in height in the hot, tropical monsoon climate such as in Bangladesh, Myanmar, Thailand, Laos, Cambodia, Vietnam, Malaysia, and Indonesia (Martin *et al*. 1987). The plant is mostly found in Khagrachari and Chittagong districts of Bangladesh which is considered as a critically endangered species (Rahman 2017). In Thailand, the fruits of this plant are used to treat phlegm and constipation, the root is used in fever, common cold and applied as a detoxifier in folkloric medicines (Neamsuvan *et al.* 2014). Ripe fruits are eaten and green fruits are used in cooking by the local people of Bangladesh. Recently, the antidiarrheal and analgesic activities of *B. oppositifolia* (Roxb.) have been reported in mice model (Islam *et al.* 2020). Neranon *et al.* (1998) investigated the methanolic extract of the twigs of *B. oppositifolia* and isolated phenolic compounds identified as astilbin, gallic acid methyl ester, epicatechin, and fustin. Both the unripe and ripe fruits of *B. macrophylla* (another species of *Bouea* genus) revealed the presence of 82 and 121 volatile compounds, respectively by gas chromatography-mass spectrometry (Rajan and Bhat, 2017).

As part of the continuing research on medicinal plants of Bangladesh (Shajib *et al.* 2017, Ibrahim *et al.* 2018, Chowdhury *et al.* 2019, Soma *et al.* 2019), in the present study the leaf extract of *B. oppositifolia* was evaluated, and isolation and structure elucidation of six secondary metabolites are reported for the first time.

The leaves of *B. oppositifolia* were collected from Remakri in Thanchi Upazila of Bandarban District in South Eastern Bangladesh in November 2018. The plant was taxonomically identified

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in Bangladesh National Herbarium, Mirpur, Dhaka, where a voucher specimen (Accession no. DACB-56286) has been deposited for future reference. The leaves were cleaned with water and sun-dried for several days. Then the dried leaves were ground to a coarse powder by using a high capacity grinding machine at the Department of Pharmaceutical Chemistry, University of Dhaka. The powdered leaf (650 g) was taken in a brown reagent bottle (4 L) and soaked in 2.5 L of distilled methanol. The container with its content was sealed with its cap and kept for 15 days to facilitate complete mixing, accompanying occasional shaking and stirring. The whole mixture was then filtered through a fresh cotton plug followed by second filtration with Whatman No. 1 filter paper. The filtrate was evaporated with a vacuum rotary evaporator below 40° C until to give a gummy mass (73.5 g). A portion (5 g) of the crude extract was partitioned using the modified Kupchan partitioning protocol (Van Wagenen *et al.* 1993) into petroleum ether (1.8 g), dichloromethane (1.3 g), chloroform (0.98 g), and aqueous (0.22 g) soluble materials.

The dichloromethane and petroleum ether soluble fractions (DCMSF and PESF, respectively) were subjected to size exclusion chromatography (SEC) over lipophilic Sephadex (LH-20) using *n*-hexane-dichloromethane-methanol (2:5:1) as the mobile phase. A total of 50 fractions (each around 5 ml) were collected from dichloromethane soluble partitionate. Based on their TLC behavior, fractions 21-24, 4, 9, 11, 14 and 18 were subjected to preparative thin-layer chromatography (PTLC-20×20 cm) over silica gel (Kiselgel F_{254}) using appropriate solvent systems comprising of 20, 5, 10, 10, 15, 15% ethyl acetate in toluene to yield six purified phytoconstituents designated by **1**-**6**. Compounds **1**-**5** were detected on PTLC plates by spraying Vanillin-H_2SO_4 at both sides of the plates after covering remaining portion with clean glass plates. On the other hand, compound **6** was detected by UV light (254 nm).

Characterization of secondary metabolites:

^{1}H NMR spectra were recorded for all the purified samples in $CDCl_3$ with the help of Ultra Shield Bruker (400 MHz) instrument from Jahangirnagar University. The chemical shift values are reported in ppm with reference to the TMS signal. All solvents and reagents used in the experiment were of analytical grade and procured from Merck, Germany.

Betulin (**1**): colorless mass (Three times run, 10.1 mg); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.78 (3H, s, H3-24), 0.84 (3H, s, H3-25), 0.95 (3H, s, H3-23), 0.96 (3H, s, H3-27), 1.00 (3H, s, H3-26), 1.66 (3H, s, H3-30), 3.18 (1H, dd, *J* = 11.2 Hz, 4.8 Hz, H-3), 3.34 (1H, d, *J* = 10.8 Hz, Hb-28), 3.80 (1H, d, *J* = 10.4 Hz, Ha-28), 4.60 (1H, s, Ha-29) and 4.70 (1H, s, Hb-29).

Lupeol (**2**): colorless gum (Two times run, 8.2 mg); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.78 (3H, s, H3-24), 0.82 (3H, s, H3-28), 0.93 (3H, s, H3-26), 0.95 (3H, s, H3-23), 1.02 (3H, s, H3-25), 1.70 (3H, s, H3-30), 2.38 (1H, m, H-19), 3.20 (1H, dd, *J* = 11.2 Hz, 6.4 Hz, H-3), 4.59 (1H, br. s, Ha-29) and 4.70 (1H, br. s, Hb-29).

Lupenone (**3**): colorless mass (Two times run, 8.4 mg); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.77 (3H, s, H3-27), 0.82 (3H, s, H3-26), 0.85 (3H, s, H3-24), 0.93 (3H, s, H3-23), 0.95 (3H, s, H3-28), 1.04 (3H, s, H3-25), 1.67 (3H, s, H3-30), 2.38 (1H, m, H-19), 4.59 (1H, br. s, Ha-29), and 4.71 (1H, br. s, Hb-29).

Stigmasterol (**4**): colorless mass (Three times run, 6.4 mg); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.70 (3H, s, H3-19), 0.83 (3H, m, H3-26), 0.85 (3H, m, H3-27), 0.87 (3H, t, H3-29), 0.94 (3H, d, *J* = 7.0 Hz, H3-21), 1.03 (3H, s, H3-18), 3.52 (1H, m, H-3), 5.00 (1H, dd, *J* = 12 and 8 Hz, H-23), 5.14 (1H, dd, *J* = 12 and 8 Hz, H-22), 5.37 (1H, d, *J* = .8 Hz, H-6).

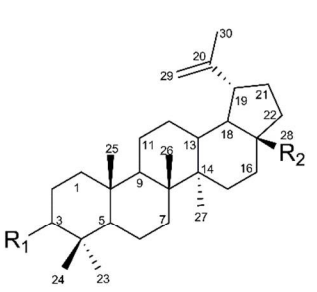
p-Coumaric acid (**5**): colorless gum (Two times run, 5.1 mg); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.28 (1H, d, *J* = 16 Hz, H-2), 6.84 (2H, d, *J* = 8.4 Hz, H-5, H-9), 6.86 (2H, d, *J* = 8.4 Hz, H-6, H-9) and 7.61 (1H, d, *J* = 16 Hz, H-3).

Oleic acid (**6**): colorless gum (Four times run, 5.6 mg); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.95 (3H, t, *J* = 8.4 Hz, H3-18), 1.33 (1H, m, H-16), 1.57 (2H, m, H-(4-7), H-(12-15)), 1.60 (1H, m, H-3), 2.10 (2H, m, H-8 or H-11), 2.48 (1H, t, *J* = 8.4 Hz, H-2) and 5.42 (1H, q, H-9 or H-10).

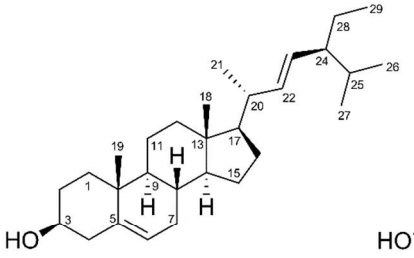
Repeated chromatographic separation and purification of the petroleum ether and dichloromethane soluble fractions of *B. oppositifolia* leaf extract afforded six secondary metabolites (**1-6**). Preparative thin-layer chromatography (PTLC) of the 21st to 24th dichloromethane soluble fractions yielded compound **1** using 20% ethyl acetate in toluene as solvent. Similarly, PTLC screening of fractions 4, 9, 11, 14 and 18 from the petroleum ether soluble partitionate yielded compounds **2** to **6** using the solvent systems comprising of 5, 10, 10, 15 and 15% ethyl acetate in toluene, respectively. The structures of the purified compounds were solved by careful interpretation of their ^{1}H-NMR spectroscopic data and comparison with existing literature values. The identities of the compounds were further confirmed by co-TLC with previously isolated authentic samples.

The ^{1}H NMR spectral data of compound **1**, **2** and **3** were indicative of lupene-type pentacyclic triterpenoid skeleton. Characteristic double doublets near δ 3.18 (*J* = 11.2 Hz and 4.8 Hz) revealed the presence of oxymethine proton at C-3 for both compound **1** and **2**, whereas the absence of this signal in case of compound **3** indicated a carbonyl carbon at C-3. Furthermore, all three ^{1}H NMR spectra of these compounds demonstrated proton signals indicative of one exomethylene group (δ 4.60 and 4.70, each 1H, br s), one vinylic methyl group (δ 1.66, s) and five tertiary methyl groups (δ 0.78, 0.84, 0.95, 0.96 and 1.00). Subsequently, comparative study with published values allowed us to identify the compounds as betulin (**1**), lupeol (**2**) and lupeone (**3**), respectively (Aktar *et al.* 2009, Noor *et al.* 2014, Parvin *et al.* 2009). The identity of triterpenes **1**-**3** was further substantiated by co-TLC with authentic compounds.

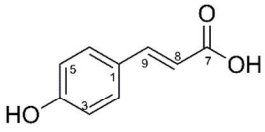
The ^{1}H NMR spectrum of compound **4** was consistent with that of a steroidal nucleus. A multiplet at δ 3.52 revealed the oxymethine group at C-3. Moreover, three olefinic protons were represented by a doublet at δ 5.37 (1H, d, *J* = 8 Hz) and two double doublets at δ 5.00 (dd, *J* = 12 and 8 Hz) and 5.14 (dd, *J* = 12 and 8 Hz) could be assigned to the typical H-6, H-23 and H-22, respectively. Subsequently, comparative studies with the published data led us to characterize this compound as stigmasterol (**4**) (Parvin *et al.* 2009). Co-TLC of compound **4** with previously isolated stigmasterol further supported its identity as stigmasterol.



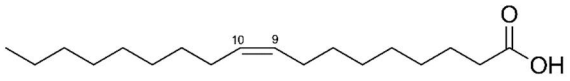
R_1 = OH, R_2 = CH_2OH Betulin (**1**)
R_1 = OH, R_2 = CH_3 Lupeol (**2**)
R_1 = O, R_2 = CH_3 Lupenone (**3**)



Stigmasterol (**4**)



p-Coumaric acid (**5**)



Oleic acid (**6**)

A pair of *trans*-coupled protons represented by doublets at δ 6.28 (d, *J* = 16.0 Hz) and 7.61 (d, *J* = 16.0 Hz) were revealed from the ^{1}H NMR spectral data of compound **5**. Furthermore, two sets of *ortho*-coupled signals at δ 6.84 (d, *J* = 8.4 Hz) and 6.86 (d, *J* = 8.4 Hz) indicated *para*-substitution on the aromatic ring. Thus, the compound was identified as *p*-coumaric acid (**5**). This was further supported by comparison with published values (Hussain *et al.* 2008) as well as by co-TLC with the authentic sample.

The ^{1}H NMR spectral data of compound **6** represented a long chain fatty acid compound with a methyl triplet at one end, a carboxylic acid moiety at the other end and a double bond in the middle of the structure. This was subsequently characterized as oleic acid (**6**) (Barison *et al*., 2010).

In conclusion, the present phytochemical study with the leaves of of *B. oppositifolia* afforded three triterpenoids (betulin, lupeol and lupenone), one sterol (stigmasterol), a phenolic compound (*p*-coumaric acid) and a fatty acid (oleic acid). This is the first report of isolation of these constituents from this endangered plant species growing in Bangladesh.

Acknowledgements

Last author (MAR) is grateful to the Ministry of Education, Government of the People's Republic of Bangladesh for a research grant (LS 2017326) to him for the period of July 2017-June 2020 to accomplish the work.

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(Manuscript received on 07 july 2021; revised on 13 December 2021)