Short Communication

Dhaka Univ. J. Sci. 61(2): 211-212, 2013 (July)

Isolation of Betulinic Acid and 2,3-Dihydroxyolean-12-en-28-oic Acid from the Leaves of *Callistemon linearis*

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(Received : 12 October 2011; Accepted : 2 March 2013)

I. Introduction

Callistemon linearis (Bengali name- Brushful; Family- *Myrtaceae*) is a beautiful evergreen shrubs and small trees with 34 species. They are commonly known as bottle brushes because of their cylindrical brush like flowers resembling a traditional bottle brush. They are found in the more temperate regions of Australia and seven species of *callistemon* have been introduced in India as an ornamental tree.[1] Previous phytochemical studies of different *callistemon* species revealed the presence of different monoterpenes, sesquiterpenes and flavonoids.[2] Antimicrobial and antioxidant activities of methanolic extract obtained from *Callistemon linearis DC* Leaf have been studied by an Indian researcher.[3] The leaf extracts of plant contain carbohydrate, glycoside, flavonoids, saponin, phytosterol, phenolic compounds and volatile oil containing four components namely *n*-dec-3-ene; 3-carene; 1,8-cineol and gamaterpinine.[4] The paper deals with the isolation and characterization of compounds from the methanol extract of dried powdered leaves and antioxidant screening of different extracts of *Callistemon linearis.*

II. Materials and Methods

General experimental procedure

The ^{1}H NMR spectra were recorded in $CDCl_3$ on a Bruker AMX-400 (400 MHz) spectrometer with TMS as an internal standard. Silica gel PF_{254} (70-230 mesh) was used for VLC, TLC and column chromatography. All solvents and reagents except petroleum ether were purchased from E. Merck. Petroleum ether was collected through distillation of petrol.

Plant Materials

Fresh leaves of *Callistemon linearis* were collected from Chittagong BCSIR. A voucher specimen has been deposited in the Bangladesh National Herbarium, Dhaka (DACB-35514) for identification. The leaves of the plant were cut into small pieces and air dried for several days. The pieces were then dried in oven for 24 hours at 40 °C for better grinding. The oven dried leaves were then ground into a coarse powder.

Extraction and Isolation

About 600 g of the coarse powder was extracted with methanol at room temperature with occasional shaking and stirring, which was then filtered through Whatman No.1 filter paper. The filtrate was then concentrated at 50 °C in a rotary evaporator and a dry mass of methanol extract (40.6 g) was obtained. A portion of the methanol extract (23 g) was subjected to Vacuum Liquid Chromatography (VLC) using petroleum ether, ethyl acetate and methanol mixtures of increasing polarities to give 32 fractions.

TLC screening of the fractions 19-23 revealed the presence of identical spots on TLC plate and thus, these five fractions were combined together and fractionated again by paring through sephadex column. The fractions 15-17 obtained from this sephadex column were found to give identical spots on TLC plate. These fractions were combined together and subjected to preparative thin layer chromatography (PTLC) using toluene: ethyl acetate (85:15) as eluent. From the developed PTLC plates, six bands (A, B, C, D, E and F) were found under UV lamp at 254 nm and showed purple color after spraying at one side of the plate with vanillin-sulfuric acid followed by heating in an oven. Each band was then scrapped and filtered off, using ethyl acetate. Among the six bands, band B and C were further subjected to PTLC and each of them showed three bands under UV lamp (B1, B2, B3 from band B and C1, C2, C3 from band C) when eluted with toluene: ethyl acetate (97:3) and (95:5), respectively. All these bands were then scrapped and filtered using ethyl acetate and the dried mass were subjected to ^{1}H NMR spectroscopy to check their purity. The ^{1}H NMR spectra reveled that only B1 and C2 were in the pure form, whereas, others were found to have impurities. Thus, the bands B1 and C2 were recognized as compound-1 and compound-2, respectively. Both the compounds were about 4 mg in weight.

Antioxidant activity test

The antioxidant activity (free radical scavenging activity) of the crude ethyl acetate, carbon tetrachloride, petroleum ether and methanol extracts on the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was estimated.[5] Two mL of ethanol solution of each crude extract (2 mg) at different concentrations such as 500, 250, 125, 62.5, 31.25,

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15.625, 7,831, 3.906, 1.953 and 0.977 *M*g-mL^{-1} were mixed with 3 mL ethanolic solution of DPPH (40µg-mL^{-1}). After 30 min reaction period at room temperature in dark place, absorbance was measured at 517 nm against ethanol as blank by UV/Visible spectrophotometer. The antioxidant potential was assayed from the bleaching of purple colored ethanol solution of DPPH radical by extracts as compared to that produced by the standard antioxidant agents of *tert*-butyl-1-hydroxytoluene (TBHT) and ascorbic acid (ASA). The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH Scavenged (\%)} = A_{control} - A_{sample} / A_{control} \times 100$$

Where, $A_{control}$= Absorbance of DPPH alone

A_{sample} = Absorbance of DPPH along with different concentrations of extracts

IC_{50} was calculated from equation of line obtained by plotting a graph of concentration versus % inhibition and the best-fit line was obtained from the curve data by means of regression analysis.

III. Results and Discussion

The structures of the isolated compounds (Compound-1 and Compound-2) were determined by ^{1}H NMR analyses as well as by comparing with the previously reported values.[6,7] The ^{1}H NMR spectrum of compound-1 revealed the presence of an allylic methyl group at δ 1.68 (3H, s) and terminal methylene protons at δ 4.60 (1H, br.s) and δ 4.73 (1H, br.s). It also showed five methyl singlets at δ 0.75, 0.82, 0.93, 0.96, 0.97 and one singlet of secondary hydroxyl group at δ 1.68. Two multiplates were observed at δ 3.00 and δ 3.18, which are assigned to H-19 and H-3, respectively. On the other hand, the ^{1}H NMR spectrum of compound-2 showed olefinic resonances at δ 5.27 and δ 3.89 and two oxygenated methanes at δ 3.20 and δ 2.80. The rest of the protons were overlapping aliphatic CH, CH_2 and CH_3 groups. Seven methyl singlets were observed at δ 0.75, 0.77, 0.89, 0.90, 0.92, 0.98 and 1.13. By comparing the ^{1}H NMR spectra with that of published one, compound-1 was identified as betulinic acid[6] and compound-2 was identified as 2,3-dihydroxy olean-12-en-28-oic acid[7] (Fig. 1.).

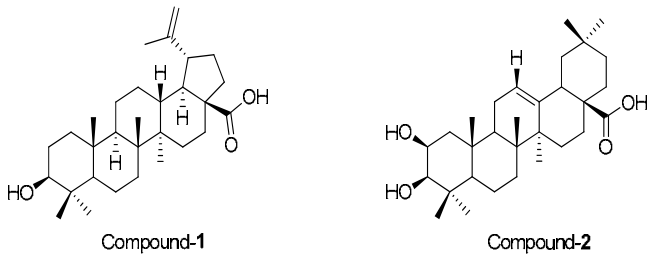

Fig. 1. Structure of Compound-1 and Compound-2.

Betulinic Acid(1): needle shaped crystals; ^{1}H-NMR (400 MHz, $CDCl_3$)**:** δ 3.18 (dd, 1H, H-3, 4.8Hz), 3.00 (m, 1H, H-19), 1.68 (s, 3H, H-30), 0.75 (s, 3H, H-24), 0.97 (s, 3H, H-26), 0.93 (s, 3H, H-23), 0.96 (s, 3H, H-27), 0.82 (s, 3H, H-25).

2,3-Dihydroxy olean-12-en-28-oic acid (2): needle shaped crystals; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.27 (m, 1H, H-12), 3.20 (m, 1H, H-2), 2.80 (m, 1H, H-3), 0.75 (s, 3H, H-25), 0.77 (s, 3H, H-24) , 0.89 (s, 3H, H-29), 0.90 (s, 3H, H-23), 0.92 (s, 3H, H-26), 0.98 (s, 3H, H-30), 1.13 (s, 3H, H-27s).

Four crude extracts of *Callistemon linearis* in ethyl acetate, carbon tetrachloride, petroleum ether and methanol extracts were subjected to free radical scavenging activity to evaluate the antioxidant potential of *Callistemon linearis*, where *tert*-butyl-1-hydroxytoluene (TBHT) and ascorbic acid (ASA) were used as reference standard. It has been observed that ethyl acetate extract showed the highest antioxidant activity with IC_{50} value of 0.45µg-mL^{-1}, which has stronger antioxidant activity than that of the reference standard TBHT (1.65µg-mL^{-1}) and might be consider as an important source of natural antioxidant. On the other hand, carbon tetrachloride and methanol extracts showed moderate antioxidant activities, whose IC_{50} values were 84.41µg-mL^{-1} and 35.00µg-mL^{-1}, respectively. IC_{50} value of petroleum ether extract could not be calculated because of lower values of inhibition than 50%.

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