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Secondary metabolites (Triterpenes) from *Couroupita guianensis*

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SUMMARY

The *n*-hexane and carbon tetrachloride soluble fractions of a methanolic extract of the stem bark of the *Couroupita guianensis* furnished three compounds, identified as β-amyrin (**1**), betulin-3β-caffeate (**2**) and lupeol-3β-caffeate (**3**). The structures of the isolated compounds were deduced by extensive spectroscopic analysis as well as by comparison with published values. Compounds **1-3** were subjected to antioxidant screening through free radical scavenging activity by DPPH (1,1-diphenyl-2-picrylhydrazyl), where compound 2 showed moderate antioxidant activity with IC_{50} value 108.0 μg/ml.

Key words: *Couroupita guianensis*; Lecythidaceae; β-amyrin; Betulin-3β-caffeate; Lupeol-3β-caffeate

INTRODUCTION

The plant *Couroupita guianensis* (English name- Cannon ball tree, local names-Nagalingam, Shibalingam) belonging to the family of Lecythidaceae is an evergreen tree and it is planted in road sides of different districts of Bangladesh (Haque, 1986). The plant of this genus is used for treating skin diseases of livestock by the local Indian (Usher, 1984). Various parts of *C. guianensis* are known to exhibit antifungal activity (Khan *et al*., 2003). Previous chemical investigations of *C. guianensis* revealed the occurrences of 6,12-dihydro-6,12-dioxoindolo-[2,1-*b*]quinazoline (tryptanthrin), indigo, indirubin, isatin (Bergman *et al*., 1985), couropitone, β-amyrin, β-amyrone, β-amyrin acetate, stigmasterol, ergosta-4,6,8(14),22-tetraen-3-one, β-sitosterol and its glycoside (Anjaneyulu, 1998; Dictionary of natural products, 2001). This paper details the isolation and structure elucidation of three triterpenoids β-amyrin (**1**), betulin-3β-caffeate (**2**) and lupeol-3β-caffeate (**3**). Although β-amyrin has been previously documented from *C. guianensis*, this is the first report of occurrence of betulin-3β-caffeate (**2**) and lupeol-3β-caffeate (**3**) from this plant.

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MATERIALS AND METHODS

General experimental procedure

NMR spectra were acquired by using Ultra Shield Bruker DPX 400 NMR instruments. The ^{1}H and ^{13}C

spectra were recorded in $CDCl_3$ and chemical shifts are reported in ppm with respect to residual non deuterated solvent signals. Mass spectra were recorded on a LCT premier KD146 mass spectrometer using positive mode ESI technique.

Plant material

The plant *C. guianensis* was collected from Sher-e-Bangla Agricultural University campus, Sher-e-Bangla Nagar, Dhaka-1207 in May 2006 and was taxonomically identified by Dr. Mahbuba Khanam, Director, Bangladesh National Herbarium, Ministry of Environment and Forest, Dhaka, Bangladesh where a voucher specimen has been maintained for this collection (accession number is DACB-32,062). The stem barks were cut into small pieces, cleaned, dried and pulverized.

Extraction and isolation

The powdered stem bark (410 g) of *C. guianensis* was soaked in 1.5 l methanol for 5 days with occasional shaking and stirring. Then the extract was filtered and concentrated using a rotary evaporator at low temperature and reduced pressure. A portion (5 g) of the concentrated methanol extract was fractionated by the modified kupchan (Vanwagenen *et al*., 1993) procedure into *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble fractions. Evaporation of solvents afforded *n*-hexane (1.1 g), carbon tetrachloride (1.6 g), chloroform (1.5 g) and aqueous soluble (0.6 mg) materials. The *n*-hexane and carbon tetrachloride soluble materials were separately subjected to vacuum liquid chromatography (VLC) over silica gel 60H and the columns were eluted with *n*-hexane-ethyl acetate mixtures of increasing polarities with 20 fractions collected for 100 ml each. Evaporation of solvents from the VLC fraction eluted with 40% ethyl acetate in *n*-hexane of the *n*-hexane soluble materials provided crystalline mass of compound 1 (20 mg). VLC fraction eluted with 35% ethyl acetate in *n*-hexane obtained from the carbon tetrachloride soluble materials were further fractionated by gel permeation chromatography over lipophilic Sephadex LH-20 using *n*-hexane-dichloromethane-methanol (2:5:1) mixture as the mobile phase. TLC of these fractions demonstrated the presence of two prominent bands in sub-fractions f-26 and f-34. Sub-fractions f-26 and f-34 were again subjected to preparative TLC over silica gel PF_{254} using *n*-hexane ethyl-acetate acetic acid (85:15:few drops) and (75:25:few drops) as the developing solvents to yield compound **2** (17.7 mg) and compound **3** (7.6 mg), respectively.

Compounds isolated

β-amyrin (**1**): White amorphous; $C_{30}H_{50}O$ (ESIMS: *m/z* $[M+H]^+$ 427.4282); ^1H NMR (400 MHz, $CDCl_3$) δ 5.12 (1H, t, *J* = 3.5, H-12), 3.23 (1H, dd, *J* = 11.0, 5.0, H-3â), 1.12 (3H, s, H_3-27), 0.98 (3H, s, H_3-23), 0.96 (3H, s, H_3-26), 0.92 (3H, s, H_3-24), 0.86 (3H, s, H_3-30), 0.86 (3H, s, H_3-29), 0.82 (3H, s, H_3-28), 0.78 (3H, s, H_3-25).

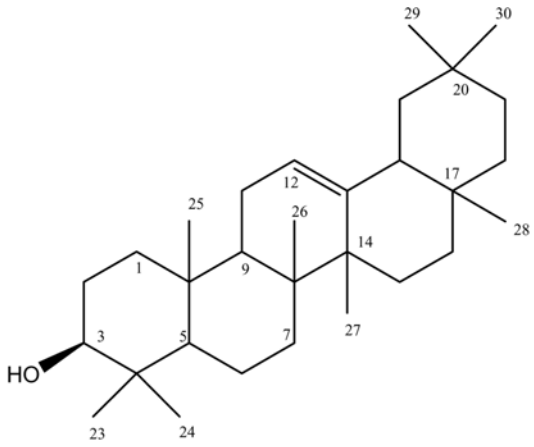
Betulin-3β-caffeate (**2**): Brown solid mass; $C_{39}H_{56}O_5$ (ESIMS: *m/z* $[M+H]^+$ 605.3871); ^1H NMR (400 MHz, $CDCl_3$): δ 7.54 (1H, d, *J* = 15.0 Hz, H-3'), 7.09 (1H, br s, H-6''), 6.98 (1H, d, *J* = 8.0 Hz, H-2''), 6.85 (1H, d, *J* = 8.0 Hz, H-5''), 6.24 (1H, d, *J* = 15.0 Hz, H-2'), 4.67 (1H, br. s, H_a-29), 4.60 (1H, m, H_α-3), 4.58 (1H, br s, H_b-29), 3.81 (1H, d, *J* = 10.8 Hz, H_a-28), 3.35 (1H, d, *J* = 10.8 Hz, H_b-28), 2.37 (1H, m, H-19), 1.68 (3H s, H_3-30), 1.01 (3H, s, H_3-26), 0.98 (3H, s, H_3-27), 0.90 (3H, s, H_3-23), 0.87 (3H, s, H_3-25), 0.86 (3H, s, H_3-24). ^{13}C NMR: δ 38.4 (C-1), 23.8 (C-2), 81.3 (C-3), 38.1 (C-4), 55.4, (C-5), 18.2 (C-6), 34.2 (C-7), 40.9 (C-8), 50.3 (C-9), 37.1 (C-10), 27.0 (C-11), 25.2 (C-12), 37.3 (C-13), 42.7 (C-14), 20.8 (C-15), 29.2 (C-16), 47.8 (C-17), 48.8 (C-18), 47.8 (C-19), 150.3 (C-20), 29.7 (C-21), 34.1 (C-22), 28.0 (C-23), 16.7 (C-24), 16.2 (C-25), 16.0 (C-26), 14.8 (C-27), 60.7 (C-28), 109.8 (C-29), 19.1 (C-30), 167.8 (C-1'), 114.4 (C-2'), 146.6 (C-3'), 127.4 (C-1''), 122.3 (C-2''), 144.0, (C-3''), 146.6 (C-4''), 116.0 (C-5''), 122.3 (C-6'').

Lupeol-3β-caffeate (**3**): Brown solid mass; $C_{39}H_{56}O_4$ (ESIMS: *m/z* $[M+H]^+$ 589.4429); ^1H NMR (400MHz, $CDCl_3$): δ 7.54 (1H, d, *J* = 15.0 Hz, H-3'), 7.10 (1H, br

s, H-6''), 6.99 (1H, d, *J* = 8.0 Hz, H-2''), 6.86 (1H, d, *J* = 8.0 Hz, H-5''), 6.25 (1H, d, *J* = 15.0 Hz, H-2'), 4.68 (1H, br. s, H_a-29), 4.59 (1H, m, H_α-3), 4.57 (1H, br s, H_b-29), 2.36 (1H, m, H-19), 1.68 (3H s, H_3-30), 1.03 (3H, s, H_3-26), 0.98 (3H, s, H_3-27), 0.90 (3H, s, H_3-23), 0.87 (3H, s, H_3-25), 0.87 (3H, s, H_3-24), 0.78 (3H, s, H_3-28). ^{13}C NMR: δ 38.4 (C-1), 23.8 (C-2), 81.3 (C-3), 38.1 (C-4), 55.4 (C-5), 18.2 (C-6), 34.2 (C-7), 40.9 (C-8), 50.4 (C-9), 37.1 (C-10), 21.0 (C-11), 25.1 (C-12), 38.1 (C-13), 42.8 (C-14), 27.4 (C-15), 35.6 (C-16), 43.0 (C-17), 48.3 (C-18), 48.0 (C-19), 150.9 (C-20), 29.8 (C-21), 40.0 (C-22), 28.0 (C-23), 16.6 (C-24), 16.2 (C-25), 16.0 (C-26), 14.5 (C-27), 18.0 (C-28), 109.3 (C-29), 19.3 (C-30), 167.6 (C-1'), 116.3 (C-2'), 144.5 (C-3'), 127.7 (C-1''), 115.5 (C-2''), 143.8 (C-3''), 146.2 (C-4''), 114.4 (C-5''), 122.3 (C-6'').

Antioxidant activity

The antioxidant activity (free radical scavenging activity) of the purified compounds on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method developed by Brand-Williams *et al*., 1995. In the experiment, 2.0 mg of each of the compounds **1-3** were dissolved in



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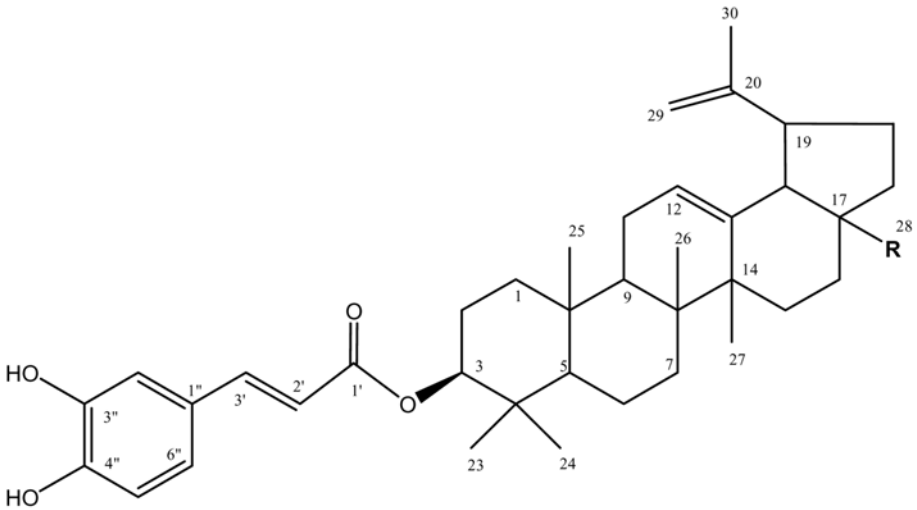

2 : R= -CH_2OH

3 : R= -CH_3

methanol. Solution of varying concentrations such as 500 μg/ml, 250 μg/ml, 125 μg/ml, 62.50 μg/ml, 31.25 μg/ml, 15.62 μg/ml, 7.8125 μg/ml, 3.91 μg/ml, 1.95 μg/ml and 0.98 μg/ml were obtained by serial dilution technique. 2 ml of a methanol solution of the extractive of each concentration was mixed with 3 ml of a DPPH-methanol solution (20 μg/ml) and was allowed to stand for 20 minutes for the reaction to occur. Then the absorbance was determined at 517 nm and from these values the corresponding percentage of inhibitions were calculated by using the following equation:

$$\% \text{ inhibition} = [\, 1- (ABS_{sample}/ABS_{control})] \times 100$$

Then % inhibitions were plotted against respective concentrations used and from the graph the IC_{50} was calculated. Here, *tert*-butyl-1-hydroxytoluene (BHT), a potential antioxidant, was used as positive control. The assay was conducted in triplicate and IC_{50} values were calculated as mean ± SD (n = 3).

RESULTS

Extensive chromatographic separation and purification of *n*-hexane and carbon tetrachloride soluble materials of the methanolic extract of the stem bark of *C. guianensis* provided three compounds (**1-3**). The structures of the isolated compounds were elucidated as β-amyrin (**1**), betulin-3β-caffeate **(2)** and lupeol-3β-caffeate (**3**) by extensive NMR and mass spectral analyses. The purified compounds **1-3** were subjected to antioxidant screening through free radical scavenging activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and by using *tert*-butyl-1-hydroxytoluene (BHT) as standard. The IC_{50} values of these compounds are shown in Table 1.

Table 1. IC_{50} values of compounds 1-3 and *tert*-butyl-1-hydroxytoluene

Samples	IC_{50} (μg/ml)*
BHT	19.0 ± 0.33
β-amyrin (**1**)	--
Betulin-3β-caffeate (**2**)	108.0 ± 1.11
Lupeol-3β-caffeate (**3**)	121.0 ± 1.25

*The values of IC_{50} are expressed as mean ± SD (n = 3); BHT: *tert*-butyl-1-hydroxytoluene (Std.)

DISCUSSION

The ESI mass spectrum of compound **1** exhibited the pseudo-molecular ion peak at *m/z* 427.4282 which was consistent with a molecular formula $C_{30}H_{50}O$ for this compound. Its ^{1}H NMR spectrum displayed eight methyl singlets at δ 1.12, 0.98, 0.96, 0.92, 0.86 (2 × Me), 0.82 and 0.78. The C-3 oxymethine proton resonance was seen at δ 3.23 (dd, *J* = 11.0, 5.0 Hz), while the olefinic proton triplet (*J* = 3.5 Hz) at δ 5.12 was attributed to H-12. This suggested the presence of a triterpenoid skeleton of the β-amyrin series. Comparison of the spectral data with published values confirmed the identity of compound **1** as β-amyrin (Thanakijcharoenpath and Theanphong, 2007; Ercil *et al*., 2004).

Compound **2** was obtained as amorphous mass. The electro-spray ionization ((ESI) mass spectrum of **2** displayed a pseudo-molecular ion peak at *m/z* 605.3871 suggesting a molecular formula $C_{39}H_{56}O_5$ for this compound. The ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of 2 displayed 39 carbon resonances, while DEPT and HSQC experiments indicated that 31 out of the 39 carbons had attached protons. This ^{13}C NMR spectral data was consistent with the molecular formula of this compound. The ^{1}H NMR spectrum showed signals at δ 7.09 (1H br. s), 6.85 d (1H, d, *J* = 8.0 Hz) and 6.98 (1H d, *J* = 8.0 Hz) together with a pair of *trans*-olefinic (*J* = 15.0 Hz) protons resonating at δ 6.24 and 7.54 indicated the presence of a *para*-caffeate moiety. The ^{1}H NMR spectrum also revealed a triterpene type carbon skeleton of the lupine series with two exomethylene proton resonances at δ 4.67 (1H, br, s, H_a-29) and 4.58 (1H, br, s H_b-29), which together with an allylic methyl at δ 1.68 (3H, br, s, H_3-30) confirmed an isoprenyl functionality. In addition, the ^{1}H NMR spectrum also displayed five tertiary methyl singlets at δ 0.86, 0.87, 0.90, 0.98 and 1.01 assignable to the methyl

groups at C-24, C-25, C-23, C-27 and C-26, respectively by comparison with the spectral data of lupeol, lupeol acetate and betulin-3β-caffeate (Aratanechemuge *et al*, 2003; Patra *et al*., 1988; Chen *et al*., 1999). In addition, the ^{1}H NMR spectrum exhibited an AB quartet with doublets (J = 10.8) centered at δ 3.81 and 3.35 for the non-equivalent *geminal* protons of a hydroxymethyl (-CH_2OH) group. The C-3 oxymethine proton was evident as a multiplet at δ 4.60, partially overlapped with one of the exomethylene protons. The down field nature of this signal could be explained by an esterification with the *para* caffeate moiety. The equatorial alpha (α) nature of the C-3 oxygenated substituent follows from the spin coupling pattern of the oxymethine proton, H-3. The occurrence of the H-3 resonance at δ 4.60 rather than at δ 3.20 as in lupeol suggested C-3 as the esterification site in compound **2**. The ^{13}C resonance of C-3 at δ 81.3 in compound **2** rather than at δ 79.0 in lupeol further confirmed that the *para*-caffeate moiety was linked to C-3 of the triterpene skeleton. In contrast, the chemical shift positions for the hydroxymethyl protons and its carbon (δ 60.7) remained almost unchanged as compared to those of betulin (Siddiqui *et al*., 1988; Mahanto *et al*., 1994). The location of the *trans para*-caffeate moiety at C-3 of the triterpene skeleton was also substantiated by both ^{1}H and ^{13}C NMR spectral data of compound **2** with those of betulin-3β-caffeate (Pan *et al*., 1994). From the above spectral data and by comparing these data with published values (Chen *et al*., 1999), compound **2** was identified as betulin-3β-caffeate. Although betulin-3β-caffeate has previously been reported from various plants but this is the first report of its occurrence from *C. guianensis*.

The electro-spray ionization (ESI) mass spectrum of compound **3** displayed a pseudo-molecular ion peak at *m/z* 589.4429 appropriate for a molecular formula $C_{39}H_{56}O_4$. The ^{13}C NMR spectrum (125 MHz, $CDCl_3$) showed 39 carbon resonances, consistent with the molecular formula of this compound. The ^{13}C and the ^{1}H NMR spectra of compound **3** were almost identical to those of compound **2**. Thus, it showed signals characteristic of a lupane-type triterpene and a *para*-caffeate moiety. However, the ^{1}H NMR spectrum of the compound displayed six tertiary methyl resonances instead of five observed in betulin-3β-caffeate. On the other hand, the hydroxymethyl group resonances observed in the ^{1}H and ^{13}C spectra of betulin-3β-caffeate at δ 3.35 and 3.81 (δc 60.7) were absent in the spectra of compound **3**. The absence of the hydroxymethyl group signals in NMR spectra of compound **3** and the presence of 6 tertiary methyl signals instead of 5 observed in betulin-3β-caffeate allowed the characterization of compound **3** as lupeol-3β-caffeate. The identity of compound **3** as lupeol-3β-caffeate was further confirmed by comparison of its NMR and MS data with literature values (Alvarenga and Ferro, 2000; Chumkaew *et al*., 2005). Although, lupeol-3β-caffeate has previously been reported from many plants but this is the first report of its occurrences in *C. guianensis*.

In case of screening for antioxidant activity (Table 1), compound **2** showed the highest antioxidant activity with IC_{50} value of 108.0 μg/ml. At the same time, compound **3** exhibited moderate antioxidant activity (IC_{50} = 121.0 μg/ml), whereas compound 1 displayed no free radical scavenging activity.

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