LUPANE TRITERPENOIDS FROM THE ROOTS OF *DIOSPYROS EHRETIOIDES* Wall. AND EVALUATION OF SOME BIOLOGICAL ACTIVITIES

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Abstract

In this research, the roots of *Diospyros ehretioides* from Tetma Village, Nyaung U Township, Mandalay Region were selected to investigate some chemical constituents and some biological activities such as acute toxicity and antimicrobial activities. Four known lupane triterpenoids, lupeol, betulin, betulinaldehyde, and betulinic acid were isolated from the roots of *Diospyros ehretioides* by column chromatography. The structures of these isolated compounds were identified by extensive spectroscopic analyses such as UV, IR, ^{1}H and ^{13}C NMR, DQF-COSY, HMQC, and HMBC spectra and comparison with the literature data. The acute toxicity of the ethanol extract of the roots evaluated by Organization for Economic Cooperation and Development (OECD) 425 guideline revealed no toxic effects and lethality at a dose of 5000 mg/kg (LD_{50}>5000 mg/kg). Moreover, the antimicrobial activity of crude extracts (ethanol, DCM and hexane) of *Diospyros ehretioides* root was evaluated against five pathogenic microorganisms by agar well diffusion method. Except for *E. faecalis*, all crude root extracts inhibited four microorganisms, with the inhibition zone diameters ranging from 10 to 15 mm, respectively.

Keywords: Lupane triterpenoids, *Diospyros ehretioides*, antimicrobial activities, acute toxicity

Introduction

Triterpenes comprise one of the most interesting groups of natural products due to their diverse structural features as well as biological and pharmacological activities (Hanson, 2003; Parmar *et al*., 2013). They are abundantly present in plants in the form of free aglycones and, more rarely, as glycoconjugates (Chappell, 1995). In many Asian countries, herbal products containing triterpenes are widely described to prevent or to treat a variety of diseases by traditional healers (Xu *et al*., 2004). They proved to have antibacterial, antiviral, antifungal, antioxidant and anti-inflammatory properties, as well as anticancer activity. Lupeol, the most common triterpenoid, has previously demonstrated to exhibit interesting therapeutic properties such as antimalarial, anti-inflammatory and antitumor activities (Alves *et al*., 1997).

Diospyros (family Ebenaceae) is a large genus of over 700 species of deciduous and evergreen trees and shrubs, which are distributed in both the hemispheres. However, the majority of the species are native to tropical and subtropical regions including Myanmar. *Diospyros ehretioides* grows abundantly in Cambodia, India, Thailand, and Myanmar (Chopra *et al*., 1965), where is known with the common names of Aukchinsa, Bok-pin, and Thipok (Kress *et al*., 2003). In herbal medicine a decoction of *Diospyros ehretioides* roots is used as an emetic and against diarrhea, whereas a bark decoction is used as an antidiuretic. In previous chemical studies, palmarumycins JC1 and JC2, isodiospyrin and isodiospyrol A were isolated from the fruits of *Diospyros ehretioides*. Palmarumycin JC2 and isodiospyrol A possess antimalarial, antifungal, antimycobacterial, and cytotoxic activities (Prajoubklang, 2005).

The isolation and identification of four lupane triterpenoids, lupeol (1), betulin (2), betulinaldehyde (3), and betulinic acid (4) from the ethanolic extract of the roots of *Diospyros ehretioides* are studied in this research.

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Materials and Methods

Plant Material

The roots of *Diospyros ehretioides* were collected in July 2012 from Tetma Village, Nyaung U Township, Mandalay Region, Myanmar and the species were identified by U Aung Kyaw Oo, Associate Professor (Rtd.), Department of Botany, Meiktila University. A voucher specimen of this plant (N-12) has been deposited at the Department of Chemistry, University of Mandalay.

General Experimental Procedure

Melting points were measured on Fisher-Johns melting apparatus. Optical rotation was measured with a Perkin Elmer 241 polarimeter, *c* in g/mL. Infrared spectra were recorded on NaCl disks on an FT-IR Perkin Elmer Paragon 100 PC spectrometer or KBr disks on a Shimadzu FT-IR spectrometer; ν in cm^{-1}. NMR experiments were performed on a Bruker AV 300 spectrometer, at 200 MHz (^1H) and 600 MHz (^1H) and 150 MHz (^{13}C) with TMS. NMR chemical shifts (δ) were reported in ppm and solvent peaks were used as internal standards. The coupling constant (*J*) values were reported in Hertz (Hz). The multiplicity of each carbon atom was determined by DEPT spectrum. DQF-COSY, HMQC, and HMBC spectra were recorded using standard pulse sequences. NMR spectra were recorded in $CDCl_3$, Sigma-Aldrich. ESIMS data were recorded on a Thermo TSQ mass spectrometer, by flow injection analysis (FIA), with an electron spray ionization source (ESI). For silica gel and reversed phase column chromatography, Merck Kieselgel 60 (40-63 μm) and Merck Li Chroprep RP-18 (25-40 μm) were employed, respectively; for direct phase and reversed phase TLC, (20 x 20 cm) silica gel 60 (GF_{254}, Merck) or RP-18 (F_{254S}, Merck), aluminium-supported plates were used. Compounds were visualized under UV light (254 and 366 nm) and, additionally, they were stained by exposure to a 0.5% solution of vanillin in H_2SO_4-EtOH (4:1), followed by gentle heating at 100 ˚C. Reagent grade solvents were purchased from Aldrich.

Extraction of *Diospyros ehretioides*

Dry powdered root of *Diospyros ehretioides* (1.0 kg) was percolated with 95% ethanol (3 L) for two weeks at room temperature. The filtrates were concentrated by a rotary evaporator to give 23.6 g of extract. The extract was partitioned between water and EtOAc. Each filtrate was evaporated to dryness under reduced pressure at 40 ˚C to yield EtOAc (4.0 g), and aqueous extracts (19.0 g). The ethyl acetate extract was further partitioned between *n*-hexane and acetonitrile. Each layer was evaporated to dryness under reduced pressure at 40 ˚C to yield residue A (1.8 g) and residue B (2.1 g) from the hexane and the acetonitrile fractions, respectively.

Isolation and Purification of Lupane Triterpenoids from the Hexane Fraction of *Diospyros ehretioides*

Residue A (1.8 g) was separated by column chromatography on silica gel 60. Elution with a *n*-hexane-EtOAc gradient gave 200 fractions, approximately 8 mL each. The fractions were combined on the basis of their TLC profiles, to give thirteen main fractions (fractions I-XIII). Fraction I (253.5 mg) was further chromatographed on a silica gel 60 column. Elution with 100% CH_2Cl_2 gave three combined fractions, I-A/C. The combined fraction, I-A (275.5 mg) displayed one main purple spoton TLC by applying vanillin spraying agent. This fraction was further crystallized from MeOH to yield 228.0 mg of lupeol (**1**) as colorless needles. Fraction IV (147.3 mg) was separated by column chromatography over a RP-18 column. Elution with 100% MeOH afforded five main fractions, IV-A/E. The fraction IV-B yielded 4.8 mg of betulinaldehyde (**3**) as colorless crystalline needles, which was detected on a TLC plate as a purple spot by the vanillin spraying reagent. Fraction VII (46.9 mg) was separated on a RP-18 column. Elution with

a MeOH-H_2O gradient yielded three main fractions, VII-A/C. The fraction VII-C (40.8 mg) was further purified by silica gel 60 column chromatography. Elution with a gradient of CH_2Cl_2-EtOAc gave 26.3 mg of betulin (**2**) as a colorless solid. Fraction XIII (94.2 mg) was separated on a RP-18 column. Elution with a gradient of MeOH-H_2O yielded 40 fractions of approximately 8 mL each, which were then combined according to their TLC profiles to give four main fractions, XIII-A/D. The fraction XIII-A (10.6 mg) was further purified on a silica gel 60 column eluted with a gradient of CH_2Cl_2-EtOAc to yield 6.0 mg of betulinic acid (**4**) as a colorless solid.

Some physicochemical properties such as melting points and optical rotation of these isolated compounds were determined. The structures of these isolated compounds were identified by using FT IR and modern NMR spectroscopic techniques such as ^{1}H NMR, ^{13}C NMR, and ESIMS Mass spectroscopies, and by comparing with the reported data. The NMR and Mass spectra of the isolated compounds were measured at Department of Pharmaceutical Science, Faculty of Pharmacy, Meijo University, Nagoya, Japan and Dipartimento di Chimica and CEMEC, Università di Pavia, Pavia, Italy.

Acute Toxicity Study on Albino Mice Model

The oral acute toxicity study of the ethanol extract of *Diospyros ehretioides* roots was carried out using the "Up-and-Down" method for testing in mice at single doses of 175, 550, 1750 and 5000 mg/kg in accordance with the OECD guideline no. 425 (OECD, 2008). It was determined at Biochemistry Research Division, Department of Medical Research (Pyin Oo Lwin Branch). Male albino mice were used for each dose level in the study. According to the test description, total number of 7 adult male albino mice, weighing (25-40 g) were selected and divided into five groups. Group (IV) contained three animals except groups (I-III). An animal was picked at a time, weighed and dosed with the equivalent volume of extract dissolved in distilled water. The extract was administered orally using feeding nozzle. Group (I), (II) and (III) mice were administered with extracts of sample 175 mg/kg, 550 mg/kg and 1750 mg/kg body weight per dose. Group (IV) mice were giving orally with extract of sample 5000 mg/kg body weight per dose. Group (V) mice performed as a control group. Each was then observed every 15 min in the first 4 h after dosing, every 30 min for 6 h and daily for 48 h according to the specifications of the OECD. The body weight of the mice was measured on day 1, 7, and 14. The mice were monitored for a total of 14 days for a possible long-term lethal outcome.

Preparation of Crude Extracts from Roots of *Diospyros ehretioides*

Dried powdered roots of *Diospyros ehretioides* (100 g) were percolated in 300 mL of ethanol for one week and filtered. This procedure was repeated for three times. Then the filtrate was concentrated to dryness by a vacuum rotatory evaporator to provide respective ethanol crude extract. Similarly, dichloromethane and hexane extracts of each dried roots powdered were prepared according to the above procedure.

Evaluation of Antimicrobial Activity by Agar Well Diffusion Method

The Agar-well diffusion method was used for evaluating antimicrobial activity of the ethanol, dichloromethane and hexane extract of *Diospyros ehretioides* roots. Gram negative bacteria (*Escherichia coli*), three-gram positive bacteria (*Staphylococcus aureus, Enterococcus faecalis* and *Bacillus cereus*), and one strain of fungus (*Candida albicans*) were used as test microorganisms and antibiotic, chloramphenicol was used as a standard. After overnight incubation at 27 ˚C, the diameters of inhibition zone including agar well were measured. In the antimicrobial test with the root extracts, the greater the inhibition zones for the test microorganisms, the better the antimicrobial activity of the extracts.

Results and Discussion

Structure Elucidation of Lupane Type Triterpenoid Compounds

Chromatographic separation of the hexane fraction from an ethanolic extract of the roots of *Diospyros ehretioides* afforded four lupane type triterpenoids (**1-4**) (Figure 1).

Compound **1** was obtained as colorless crystalline needles, mp 213-215˚C, $[\alpha]_{20}^{D}$ = +23.87 (*c* 0.009, CH_2Cl_2). It exhibited the molecular formula $C_{30}H_{50}O$ from the molecular ion peak at *m/z* 426. The IR spectrum showed the absorption bands for hydroxyl group at 3344 cm^{-1} and double bond at 1642 cm^{-1}. The ^{1}H NMR spectrum of compound **1** exhibited the characteristic signals of a lupane type triterpenoid, which included six methyl groups attached to quaternary carbons [δ 0.97 (Me-23), 0.79 (Me-24), 0.83 (Me-25), 1.03 (Me-26), 0.94 (Me-27), 0.80 (Me-28), respectively] and one vinyl methyl group at δ 1.68 (Me-30). The multiplet of one proton at δ 2.37, assignable to 19β-H, is characteristics of lupeol. The H-3 proton, geminal to an OH group, showed a double doublet at δ 3.19 while a pair of doublets at δ 4.69 and δ 4.57 (1H, each) was indicative of the olefinic protons H-29a and H-29b.

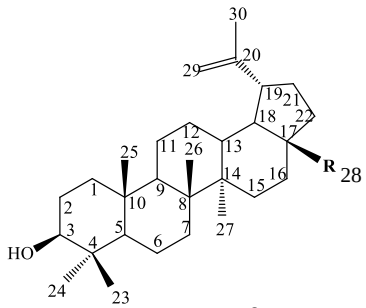
The structural assignment of compound **1** was further identified by its ^{13}C NMR and DEPT spectral data which showed 30 carbon signals as seven methyl groups at [δ_C 28.0 (C-23), δ_C 15.4 (C-24), δ_C 15.9 (C-25), δ_C 16.1 (C-26), δ_C 14.6 (C-27), δ_C 18 (C-28) and δ_C 19.8 (C-30)], a secondary hydroxyl bearing carbon at δ_C 79.0 (C-3) and an exomethylene carbon at δ_C 109.3 (C-29), sp^2 quaternary carbon at δ_C 150.9 (C-20), in addition to ten sp^3 methylene carbons, sp^3 five methine carbons and five quaternary carbons.

The shielding of C-23 (δ_C 28.0) methyl of compound **1** could be due to the influence of the adjacent C-3 hydroxyl group. The position of the hydroxyl group at C-3 was assigned by an HMBC spectrum in which the oxymethine proton at δ 3.19 (H-3) showed correlations with δ_C 38.7 (C-1), δ_C 38.8 (C-4), δ_C 27.4 (C-2) and δ_C 15.4 (C-24). The position of a methine proton at C-19 was determined from HMBC correlation of δ 2.37 (H-19) with δ_C 48.3 (C-18), δ_C 38.1 (C-13), δ_C 29.9 (C-21), δ_C 19.8 (C-30) and δ_C 150.9 (C-20). HMBC correlations of compound **1** is shown in Figure 2. Thus, on the basic of spectroscopic data and comparison with the previous report (Suryati *et al*., 2011), the structure of compound **1** was identified as lupeol. (Table 1)

Compound **2** was isolated as colorless solid, mp 248-252 ˚C, $[\alpha]_{20}^{D}$ = + 14.17 (*c* 0.002, CH_2Cl_2). The IR spectrum showed absorption bands of a hydroxyl group at 3381 cm^{-1} and a double bond at 1645 cm^{-1}. The molecular formula $C_{30}H_{50}O_2$ was determined from the peak at *m/z* 465.42 $[C_{30}H_{50}O_2+Na]^+$ in the ESIMS spectrum. As compound **1,** compound **2** was revealed as a purple spot on TLC plates sprayed by the sulpho-vanillin reagent. The ^{1}H NMR spectrum of compound **2** was very similar to that of compound **1**. It included a double of doublets resonating at δ 3.18, which was characteristic of αH-3, and two doublets of geminal protons at δ 4.58 and δ 4.69 attributable to 2H-29. These signals together with the methyl singlet at δ 1.68 suggested that compound **2** was a lupeol-type triterpenoid. AB system was observed at δ 3.79 and δ 3.33 (1H each), assigned to an oxymethylene group, replaced the signal of Me-28 (δ 0.80) in the ^{1}H NMR spectrum of lupeol (**1)**.

The ^{13}C NMR spectrum established compound **2** as a lupeol type triterpene derivative. The characteristics of sp^2 carbon comprising the double bond was observed at δ_C 109.7 (C-29). Oxygenated carbon shifts for C-3 and C-28 were observed at δ_C 78.9 and δ_C 60.6, respectively. The ^{13}C NMR and DEPT spectral data revealed that compound **2** with six sp^3 methyl carbons, totally thirty carbon atoms which is equivalent to the total number of carbon atoms in triterpenoid. The position of the hydroxyl group at C-3 was determined by an HMBC spectrum in which the carbinol methine proton at δ 3.18 (H-3) showed correlations with δ_C 27.4 (C-2) and δ_C 15.4 (C-24). The position of a methine proton at C-19 was determined from HMBC

correlations of δ 2.38 (H-19) with δ_C 47.8 (C-18), δ_C 29.8 (C-21), δ_C 109.7 (C-29) and δ_C 150.5 (C-20). Moreover, the oxymethylene protons signals at δ 3.33 and δ 3.79 (H-28) showed long-range correlations with δ_C 29.2 (C-16), δ_C 47.8 (C-17) and δ_C 33.9 (C-22). The long-range HMBC correlations of compound **2** is shown in Figure 3. Based on these data and comparison with the literature (Ayotollahi *et al*., 2011; Tijjani *et al*., 2012) compound **2** was assigned the known structure, 20(29)-lupene-3,28-diol, which is commonly known as the triterpenoid diol betulin. (Table 1)



(**1**) R = $-CH_3$ (**2**) R = $-CH_2OH$

(**3**) R = $-CHO$ (**4**) R = $-COOH$

Figure 1. Structures of lupane triterpenoids 1-4 isolated from *Diospyros ehretioides*

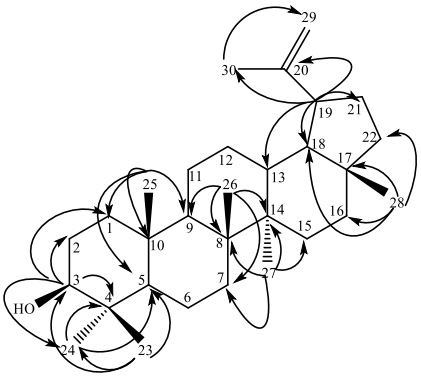


Figure 2. HMBC correlations of compound 1

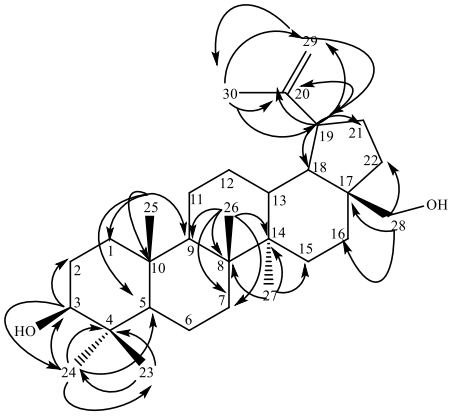


Figure 3. HMBC correlations of compound 2

Compound **3** was isolated as colorless crystalline needles from MeOH, mp. 186-189 ˚C, $[\alpha]_{20}^{D}$ = +17.49 (*c* 0.002, CH_2Cl_2). The mass spectrum exhibited an $[M+H]^+$ peak at m/z 441.42 corresponding to $C_{30}H_{48}O_2$. The IR spectrum showed the absorption at 3372 cm^{-1} (hydroxyl group), 1715 cm^{-1} (aldehyde carbonyl) and 1645 cm^{-1} (double bond). The ^1H NMR spectrum of compound **3** was very similar to those of **1**. It showed five methyl singlets at δ 0.96 (Me-23), 0.75 (Me-24), 0.82 (Me-25), 0.91 (Me-26), 0.97 (Me-27), respectively, one vinyl methyl at δ 1.69 (Me-30) and the signal of an aldehydic proton at δ 9.68 (H-28). The signal of the methine proton H-19 resonated at δ 2.85, and was downfield shifted with respect to the corresponding

signal of compound **1** (δ 2.37 ppm). This finding indicated the presence of a carbonyl group at C-28. The secondary carbinol at C-3 showed a multiplet at δ 3.17 while a pair of broad singlets at δ 4.75 and δ 4.63 was assigned to the olefinic protons H-29a and H-29b. These data indicated that compound **3** had the structure of a pentacyclic lupeol-type triterpenoid with an aldehyde group attached to C-17. The aldehydic proton was also confirmed by the FT IR spectrum. In FT IR spectrum, the aldehydic C-H stretching band was observed at 2704 cm^{-1}. Comparison of the spectroscopic data and physical data of compound **3** with the literature (Haque *et al*., 2013; Tung *et al*., 2010) confirmed its identity as with betulinaldehyde. (Table 2)

Compound **4** was isolated as a colorless solid by crystallization from MeOH, mp. 282-285 ˚C, $[\alpha]_{20}^{D}$ = + 13.33 (c 0.0006, CH_2Cl_2). It gave a red spot on a silica gel TLC plate sprayed by the sulpho-vanillin reagent. The IR spectrum showed absorption bands for hydroxyl and carboxylic groups at 3652 and 1688 cm^{-1}, respectively. The ESIMS spectrum displayed a $[M-H]^-$ negative peak at m/z 455.46, which indicated the molecular formula $C_{30}H_{48}O_3$. The ^1H NMR spectrum was typical of lupane-type compounds. In fact, it displayed signals attributable to an exomethylene group at δ 4.61 and δ 4.74 (1H each, broad singlets), which together with an allylic methyl at δ 1.69 indicated the presence of an isopropenyl unit. The double doublet at δ 3.19 could be assigned to αH-3 bound to an oxygenated carbon. In addition, a multiplet at δ 3.01 was assigned to βH-19 and five singlets of methyl groups resonated at δ 0.75 (Me-24), 0.82 (Me-25), 0.93 (Me-26), 0.96 (Me-23), and 0.97 (Me-27). The downfield-shift of the signal assigned to βH-19 (δ 3.01), compared with the corresponding proton of lupeol (**1**) (δ 2.37), indicated the presence of a β-COOH attached to C-17. Based on these data and comparison with the literature (Haque *et al*., 2013; Lee *et al*., 2005), compound **4** was identified as betulinic acid. (Table 2)

Physicochemical and spectroscopic data of compounds **1**-**4** are reported as below:

Lupeol (**1**)- Colorless crystalline needles; $[\alpha]_{20}^{D}$ = +23.87 (*c* 0.009, CH_2Cl_2); mp. 213-215 ˚C; ESIMS: $[M+Na]^+$ *m/z* 449 for $C_{30}H_{50}O$+Na; IR (KBr) ν = 3344.7, 3069.8, 2946.4, 2866.3, 1642.4, 1456.3, 1382.0, 1038.7, 882.5 cm^{-1}; the ^1H NMR and ^{13}C NMR spectral data are reported in Table 1.

Betulin (**2**)- Colorless solid; $[\alpha]_{20}^{D}$= +14.17 (*c* 0.002, CH_2Cl_2); mp. 248-252 ˚C; ESIMS: $[M+Na]^+$ *m/z* 465 for $C_{30}H_{50}O_2$+Na; IR (KBr) ν = 3381.3, 2936.7, 2870.2, 1645.3, 1541.2, 1457.3, 1030.9, 882.5 cm^{-1}; the ^1H NMR and ^{13}C NMR spectral data are reported in Table 1.

Betulinaldehyde (**3**)- Colorless crystalline needles; $[\alpha]_{20}^{D}$= +17.49 (*c* 0.002, CH_2Cl_2); mp. 186-189 ˚C; ESIMS: $[M+H]^+$ *m/z* 441.42 for $C_{30}H_{48}O_2$+H; IR (NaCl) ν = 3372.4, 2704.0, 1715.8, 1645.0 cm^{-1}; the ^1H NMR spectral data are reported in Table 2.

Betulinic acid (**4**)- Colorless solid; $[\alpha]_{20}^{D}$= +13.33 (*c* 0.0006, CH_2Cl_2); mp. 283-285 ˚C; ESIMS: $[M-H]^-$ *m/z* 455.46 for $C_{30}H_{48}O_3-H$; IR (KBr) ν = 3652.3, 2926.1, 2862.5, 1688.7, 1455.3, 1039.7, 892.2 cm^{-1}; the ^1H NMR spectral data are reported in Table 2.

Table 1. The Comparison of ^{13}C NMR Data and ^{1}H NMR Data of Compound 1, 2 and Lupeol and Betulin

Position	δ_C [ppm]		δ_H [ppm] (mult, J in Hz)		δ_C [ppm]		δ_H [ppm] (mult, J in Hz)	
	1[a]	**Lupeol[b]**	**1[a]**	**Lupeol[b]**	**2[a]**	**Betulin[b]**	**2[a]**	**Betulin[b]**
1	38.7	38.9	0.91, 1.67		38.7	38.9	0.91, 1.67	
2	27.4	24.6	1.68, 1.56	1.62, *m*	27.4	27.5	1.61, 1.57	
3	79.0	79.2	3.19, *dd* (11.5, 4.8)	3.18, *t*	78.9	79.2	3.18, *dd* (11.0, 5.3)	3.18, *dd* (11.2, 5.2)
4	38.8	39.0			38.9	38.8		
5	55.3	55.5	0.68	0.67, *t*	55.3	55.4	0.68	
6	18.3	18.5	1.36, 1.52		18.3	18.4	1.41, 1.53	
7	34.3	34.4	1.39		34.3	34.3	1.40	
8	40.9	41.0			40.9	41.0		
9	50.5	50.6	1.27	1.25, *t*	50.4	50.5	1.27	
10	37.2	37.3			37.2	37.4		
11	20.9	21.1	1.22, 1.41		20.8	20.9	1.19, 1.42	
12	25.2	25.3	1.07, 1.68		25.2	25.3	1.03, 1.68	
13	38.1	38.2	1.66		37.3	37.2	1.65	
14	42.4	43.0			42.7	42.8		
15	27.5	27.7	1.02, 1.59		27.1	27.1	1.05, 1.70	
16	35.6	35.8	1.37, 1.47		29.2	29.2	1.25	
17	43.0	43.2			47.8	47.9		
18	48.3	48.5	1.36	1.35, *dd*	47.8	47.9	1.58	
19	47.9	48.2	2.37, *m*	2.38, *m*	48.8	48.8	2.38	
20	150.9	151.2			150.5	150.6		
21	29.9	30.0	1.33, 1.92		29.8	29.8	1.42	
22	40.0	40.2	1.20, 1.38		33.9	34.1	1.03, 1.86	
23	28.0	28.2	0.97, *s*	0.96, *s*	27.9	28.1	0.97, *s*	0.96, *s*
24	15.4	15.6	0.79, *s*	0.75, *s*	15.4	15.4	0.77, *s*	0.75, *s*
25	15.9	16.2	0.83, *s*	0.82, *s*	16.1	16.2	0.83, *s*	0.80, *s*
26	16.1	16.3	1.03, *s*	1.02, *s*	15.9	16.1	1.03, *s*	0.97, *s*
27	14.6	14.7	0.94, *s*	0.95, *s*	14.8	14.8	0.98, *s*	0.99, *s*
28	18.0	18.1	0.80, *s*	0.78, *s*	60.6	60.6	3.33, *d* (10.8) 3.79, *d* (10.8)	3.33, *d* (11.0) 3.79, *d* (11.0)
29a	109.3	109.5	4.69, *d* (2.3)	4.68, *d*	109.7	109.8	4.69, *d* (2.1)	4.70, *d*
29b			4.57, *d* (2.3)	4.56, *d*			4.58, *d* (2.1)	4.58, *d*
30	19.8	19.5	1.68, *s*	1.67, *s*	19.1	19.2	1.68, *s*	1.67, *s*

[a]The ^{1}H NMR data were measured in $CDCl_3$ at 600 MHz and ^{13}C NMR at 150 MHz

[b]The ^{1}H NMR data were measured in $CDCl_3$ at 500 MHz and ^{13}C NMR at 125 MHz

Table 2. ^{1}H NMR Spectroscopic Data (δ in ppm, J in Hz) for Compounds 3 and 4, Betulinaldehyde and Betulinic Acid

Position	3	Betulinaldehyde*	4	Betulinic acid**
	δ_H (mult)[c]	δ_H (mult, J in Hz)[d]	δ_H (mult)[c]	δ_H (mult, J in Hz)[d]
3	3.17, dd (11.3, 4.7)	3.12, dd (11.2, 4.8)	3.19, dd	3.16, dd (10.8, 5.4)
19	2.85, m	2.80, m	3.01, dt (10.3, 4.5)	2.97, dt (10.4, 4.8)
23	0.96, s	0.84, s	0.96, s	0.80, s
24	0.75, s	0.68, s	0.75, s	0.73, s
25	0.82, s	0.75, s	0.82, s	0.91, s
26	0.91, s	0.90, s	0.93, s	0.94, s
27	0.97, s	0.89, s	0.97, s	0.95, s
28	9.68, s	9.60, s		
29a	4.75, brs	4.68, brs	4.74, brs	4.71, brs
29b	4.63, brs	4.56, brs	4.61, brs	4.58, brs
30	1.69, s	1.63, s	1.69, s	1.67, s

*(Tung *et al*., 2010) **(Lee *et al*., 2005)
[c]The ^{1}H NMR data were measured in $CDCl_3$ at 200 MHz
[d]The ^{1}H NMR data were measured in $CDCl_3$ at 400 MHz

Effect of Ethanol Extracts on Acute Toxicity

The acute toxicity test by the Up and Down method at an oral limit doses of 175, 550, 1750 and 5000 mg/kg of the ethanol extract of *Diospyros ehretioides* roots caused no death in the mice. No lethal effects were noted throughout the short and long-term observation periods (Table 3). No toxicity signs were observed in the mice throughout the 14 days study period. The acute toxicity study did not show any toxicity signs and symptoms at 175, 550, 1750 and 5000 mg/kg. No morbidity or mortality was observed in the treated groups at 175-5000 mg/kg doses during the acute toxicity study. Even with the doses up to 5000 mg/kg body weight administration, there is no lethality after 14 days. As a result, the oral doses of the extract could be greater than 5000 mg/kg body weight.

Table 3. Acute Toxicity of the Ethanol Extract of the *Diospyros ehretioides* Root Based on Daily Body Weight

Test	Groups	Dosage of Extract (mg/kg)	Sex	Marking	Body weight (g)			Mortality
					1st day	7th day	14th day	
1	I	175	Male	Back	30	30	27	Nil
2	II	550	Male	Head	37	35	34	Nil
3	III	1750	Male	L-leg	25	20	21	Nil
4	IV	5000	Male	Tail R-leg	34	32	33	Nil
5	IV	5000	Male	Head Back	30	29	27	Nil
6	IV	5000	Male	Head R-leg L-leg	31	30	31	Nil
7	V (control)	-	Male					Nil

Nil = no lethality of the albino mice

Antimicrobial Activity of Root Extracts

The roots of *Diospyros ehretioides* were extracted with ethanol, dichloromethane DCM and hexane respectively. The inhibitory effect of root extracts was tested against five pathogenic microorganisms by Agar well diffusion method. The result in different extents of inhibition is shown in Table 4 and Figure 4. Antimicrobial activity of the all crude extracts showed against four microorganisms: *Escheichia coli*, *Staphylococcus aureus*, *Bacillus cereus* and *Candida albicans* with the range of inhibition zone diameter between (10~15 mm). The DCM extract of *Diospyros ehretioides* exhibited low antimicrobial activity against all tested strains except *Enterococcus faecalis*. Ethanol extract of the sample responds medium activities on fungus stain, *Candia albicans*. The *Enterococcus faecalis* strain did not inhibit against the ethanol, DCM and hexane extracts of *Diospyros ehretioides*.

(a) **(b)** **(c)** **(d)** **(e)**

(a) *Escheichia coli* (b) *Enterococcus faecalis* (c) *Staphylococcus aureus*
(d) *Bacillus cereus* (e) *Candida albicans*

Figure 4. Inhibition Zone Diameters of the crude extracts of *Diospyros ehretioides* roots tested with five microorganisms

Table 4. Antimicrobial Activity of the Extracts of *Diospyros ehretioides* Root by Agar Well Diffusion Method

No	Test Microrganisms	Inhibition Zone Diameters (mm)			
		EtOH Extract	DCM Extract	Hexane Extract	Chloramphenicol
1	*E. coli*	12	10	10	26
2	*E. faecalis*	0	0	0	30
3	*S. aureus*	11	12	10	32
4	*B. cereus*	13	13	11	24
5	*C. albicans*	15	12	10	27

Agar well = 8 mm, 9mm ~ 14 mm = (+) low activity, 15 mm ~ 19 mm = (++) medium activity,
20 mm and above = (+++) high activity, Std. = Chloramphenicol

Conclusion

Medicinal plants used in folk medicine still represent an interesting source for the development of different products with various useful properties. In this chemical investigation of the constituents of *Diospyros ehretioides* roots, the lupane triterpenoids lupeol (**1**), betulin (**2**), betulinaldehyde (**3**), and betulinic acid (**4**) have been isolated and identified by modern NMR spectroscopic techniques. It is worth noting that with a content of 228 μg/g dry roots, the ethanolic extract of *Diospyros ehretioides* roots represents one of the richest known sources of the anticancer and anti-inflammatory triterpenoid lupeol (Saleem, 2009). In addition, betulinic acid (**4**), which is also present in the extract, has antiretroviral, antimalarial, and anti-inflammatory properties, as well as potential anticancer activity, as topoisomerase inhibitor. In the study of acute toxicity by OECD-425 guideline, no toxic sign and no death were recorded at the dose of 5000 mg/kg of ethanol extract. Therefore, the test sample showed free from acute

toxic effect up to the dose of 5000 mg/kg and can be considered relatively safe. The antimicrobial activities of the extracts determined by Agar well diffusion method on five pathogenic microorganisms. Ethanol extract, DCM extract and hexane extract respond low activities on four tested organisms such as *Escherichia coli*, *Staphylococcus aureus, Bacillus cereus* and *Candida albicans* and no activity on *Entrococcus faecalis.* Due to the content of bioactive compounds, the roots of *Diospyros ehretioides* have great potential for producing healthy products. Moreover, the use of the roots in herbal medicine appears to be supported by scientific evidence.

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