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# Cytotoxic lupane-type triterpenoids from *Acacia mellifera*

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**Abstract**

One new and eight previously described lupane-type metabolites were isolated for the first time from *Acacia mellifera* (Leguminosae). Based on spectral analyses, the structure of the new compound was elucidated as 28-hydroxy-3-oxo-lup-20-(29)-en-30-al (**1**), while the known compounds were identified as 3-oxo-lup-20-(29)-en-30-al (**2**), 3-hydroxy-lup-20-(29)-en-30-al (**3**), 28-hydroxy-lup-20-(29)-en-3-one (**4**), lupenone (**5**), lupeol (**6**), betulin (**7**), betulinic acid (**8**), and betulonic acid (**9**). Metabolites **2**, **3**, and **4** are reported for the first time in the Leguminosae family. The cytotoxicity of the isolated metabolites was evaluated on the NSCLC-N6 cell line, derived from a human non-small-cell bronchopulmonary carcinoma. Compounds **1** and **3** exhibited significant levels of activity.


*Keywords:* *Acacia mellifera*; Leguminosae; Isolation; Lupanes; 28-Hydroxy-3-oxo-lup-20-(29)-en-30-al

## 1. Introduction

The stem bark of *Acacia mellifera* (Leguminosae) is used in the traditional African ethnomedicine for the treatment of pneumonia, malaria, primary infection of syphilis, sterility and stomach-ache (Kokwaro, 1976). Chemical investigations on other *Acacia* species have led to the isolation of alkaloids (Johns et al., 1966), chalcone glycosides (Imperato, 1982), diterpenes (Foster and Jefferies, 1985), and flavonoids (Heerden et al., 1981). The isolation of the triterpenes, acacigenin B (Anjaneyulu et al., 1979), lupeol, lupenone, lupenyl palmitate and lupenyl cinnamate (Pereira et al., 1996) has also been reported.

In the course of our investigations towards the isolation of bioactive metabolites from terrestrial and marine organisms (Iliopoulou et al., 2003, Smyrniotopoulos et al., 2003) we recently studied the chemical composition of *A. mellifera* bark, traditionally used in African medicine. In the present study we report the isolation of the new lupane triterpene 28-hydroxy-3-oxo-lup-20-(29)-en-30-al (**1**) along with eight known triterpenoids. Among the isolated metabolites, 3-oxo-lup-20-(29)-en-30-al (**2**) (Wijeratne et al., 1981), 3-hydroxy-lup-20-(29)-en-30-al (**3**) (Kulshreshtha, 1979), and 28-hydroxy-lup-20-(29)-en-3-one (**4**) (Tinto et al., 1992) were isolated for the first time from the genus *Acacia*. Lupenone (**5**), lupeol (**6**) (Pereira et al., 1996), betulin (**7**) (Siddqui et al., 1998), betulinic acid (**8**) (Ikuta and Itokawa, 1988), and betulonic acid (**9**) (Akihisa et al., 2002) are reported for the first time as metabolites of *A. mellifera* (Fig. 1.). The known compounds were identified by comparison of their spectral features with published values (Mahato and Kundu, 1994; Wenkert et al., 1978; Amman et al., 1982; Hui and Li, 1976; Mclean et al., 1987; Burns et al., 2000). The elucidation of the structure of the new compound was accomplished by extensive analyses of its spectral data. In addition is presented the complete assignment of $^{1}H$ and $^{13}C$ NMR resonances, for metabolite **2**, since previous reports mention only selected signals.

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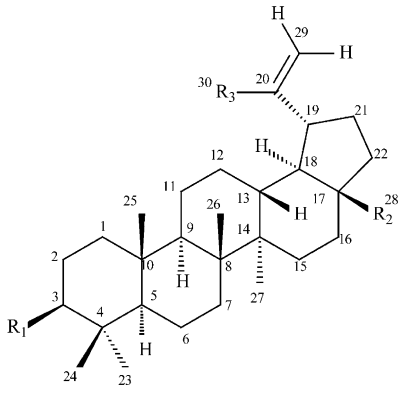

| Metabolite | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| **1** | = O | $CH_2OH$ | CHO |
| **2** | = O | $CH_3$ | CHO |
| **3** | β-OH | $CH_3$ | CHO |
| **4** | = O | $CH_2OH$ | $CH_3$ |
| **5** | = O | $CH_3$ | $CH_3$ |
| **6** | β-OH | $CH_3$ | $CH_3$ |
| **7** | β-OH | $CH_2OH$ | $CH_3$ |
| **8** | β-OH | COOH | $CH_3$ |
| **9** | = O | COOH | $CH_3$ |

Fig. 1. Isolated lupane triterpenes from *A. mellifera*.

## 2. Results and discussion

Compound **1,** isolated as a colourless gummy substance, showed strong absorption bands at 3625 and 1782 and 2866 $cm^{-1}$ in the IR spectrum indicative of the presence of hydroxyl and carbonyl groups respectively. Interestingly the $^{13}C$ NMR spectrum at 25 °C (Table 1) displayed signals for only twenty seven carbons which were distinguished as five methyls, eleven methylenes (nine aliphatic, one oxygenated and one vinylic), four methines (three aliphatic and one aldehydic) and seven quaternary carbons (four aliphatic, one vinylic and two carbonyls) with the aid of the DEPT (25 °C) experiments. The $^1H$ NMR spectrum of **1** displayed one aldehyde characteristic signal at $\delta_H$ 9.49 ppm (1H, *s*), two protons for a hydroxy methylene group at $\delta_H$ 3.36 and 3.78 ppm (1H each, *d*, $J = 10.6$ Hz), two deshielded exocyclic methylene protons at $\delta_H$ 5.91 and 6.27 ppm (1H each, *s*) and five tertiary methyl groups at $\delta_H$ 0.90, 0.95, 1.00, 1.04, and 1.05 ppm.

The assignment of the signals of the methyl groups and the remaining $^1H$ and $^{13}C$ signals was performed through analysis of the HSQC, HMBC and COSY experiments and the results were consistent with a lupane type triterpene (Amman et al., 1982; Reynolds et al., 1986). The FAB MS showed a molecular ion at *m/z* 454 suggesting, in combination with the above described data, a molecular formula of $C_{30}H_{46}O_3$.

The comparison of the chemical shifts of the A, B, and C rings of **1**, especially the resonances of the methyl groups, with those of lupenone (**5**) and 28-hydroxy-lup-20-(29)-en-3-one (**4**) showed that the oxidized methyl should be the C-28 and that the missing carbons should belong on the D ring or on the five-membered E ring of the molecule.

Confirmation of the oxidation on C-28 was provided by the strong $^3J_{CH}$ correlation of the hydroxymethylene protons with two methylenic carbons at $\delta_c$ 33.9 and 29.1 ppm whose upfield shifts are characteristic for the methylenes C-22 and C-16, respectively, of the lupane skeleton when Me-28 is oxidized to a hydroxymethylene, aldehyde or acid (Mahato et al., 1994) functionality. The aldehyde group at $\delta$ 9.49 was attached on C-30 as shown by the strong $^2J_{CH}$ correlation with C-20 (157.0 ppm) and the $^3J_{CH}$ correlations between the exocyclic methylene protons with C-30 (194.9 ppm).

The above data suggested that the two missing methines are the C-18 and C-19 and the missing methylene is the C-21 all spatially in the proximity of the isopropenal side chain. The invisibility of these carbon signals in the $^{13}C$ NMR spectrum at 25 °C suggest slow interconversion between side-chain conformations and the presence of a number of different rotamers. Experiments performed on similar triterpenes (with a hydroxyl group on C-3) at low temperatures (−30 °C) by Burns et al. (2000), showed two distinct peaks for each of C-18, C-19, C-20 and C-21 due to corresponding rotamers. However we were able to observe the missing carbons on the HMQC spectrum of metabolite **1** by raising the temperature to 38 °C.

The most obvious explanation for this phenomenon is that at elevated temperatures (38 °C) the energy barrier is overcomed allowing fast rotation around the C-19–C-20 bond thus leading to the collapse of the distinct peaks for the above mentioned carbons and the appearance of average signals for each of these carbons.

On the basis of the above mentioned observations, the structure of metabolite **1** was established as 28-hydroxy-3-oxo-lup-20-(29)-en-30-al.

The presence of the C-28 hydroxyl group in metabolite **1** exerts a through-bond rather than a through-space effect, since the most significant changes, between metabolites **1** and **2**, are observed in the chemical shifts of C-16, C-17 and C-22 and the corresponding protons leaving practically unaffected carbons C-13, C-15, C-19 and C-21.

The NOE experiments confirmed the proposed stereochemical configuration of the structure which is in agreement with the lupane biosynthesis (Caballero et al., 1984). The stereochemistry on the five membered ring was confirmed by the strong NOE effects between H-19/H-13, $H_a$-28/H-13, H-19 and $H_3$-27/H-18 (Table 2).

Metabolite **2**, previously reported from *Gymnosporia emarginata,* was also isolated from the non polar

Table 1
NMR spectral data for metabolites **1** and **2**[a,b,c]

| Position | **1** ($\delta_H$) | **1** ($\delta_C$) | **2** ($\delta_H$) | **2** ($\delta_C$) |
|---|---|---|---|---|
| 1 | α, 1.38 (*m*)<br>β, 1.85 (*ddd*, 4.4, 7.8, 13.3) | 39.6 | α, 1.36 (*m*)<br>β, 1.84 (*ddd*, 4.4, 7.5, 12.9) | 39.6 |
| 2 | α 2.36 (*ddd*,4.4,7.5, 16.0)<br>β, 2.46 (*ddd*, 7.5, 9.9, 16.0 ) | 34.1 | α, 2.35 (*ddd*, 4.4, 7.5, 15.7)<br>β, 2.46 (*ddd*, 7.5, 9.9, 15.7) | 34.1 |
| 3 | | 218.0 | | 218.3 |
| 4 | | 47.4 | | 47.4 |
| 5 | 1.31 (*m*) | 55.0 | 1.34 (*m*) | 54.9 |
| 6 | a, 1.49 (*m*)<br>b, 1.44 (*m*) | 19.6 | 2H, 1.42–1.53 (*m*) | 19.6 |
| 7 | 2H, 1.45 (*m*) | 33.5 | a, 1.37 (*m*)<br>b, 1.43 (*m*) | 33.5 |
| 8 | | 42.7[#] | | 42.7[d] |
| 9 | 1.33 (*m*) | 49.6 | 1.33 (*m*) | 49.6 |
| 10 | | 36.9 | | 36.8 |
| 11 | a, 1.35 (*m*)<br>b, 1.25 (*m*) | 21.4 | a, 1.35 (*m*)<br>b, 1.26 (*m*) | 21.4 |
| 12 | a, 0.91 (*m*)<br>b, 1.05 (*m*) | 27.6 | a, 0.93 (*m*)<br>b, 1.06 (*m*) | 27.6 |
| 13 | 1.66 (*ddd*, 12.3, 12.3, 3.8) | 37.1 | 1.68 (*m*) | 37.8 |
| 14 | | 40.8[d] | | 40.7[d] |
| 15 | α, 1.09 (*m*)<br>β, 1.71 (*ddd*, 13.6, 13.6, 4.4) | 26.9 | a, 1.02 (*m*)<br>b, 1.70 (*m*) | 27.3 |
| 16 | α, 1.26 (*m*)<br>β, 1.97 (*ddd*, 13.6, 4.4, 2.4) | 29.1 | a, 1.51 (*m*)<br>b, 1.45 (*m*) | 35.3 |
| 17 | | 48.0 | | 43.3 |
| 18 | 1.87 (*m*) | 52.3[c] | 1.67 (*m*) | 51.4[c] |
| 19 | 2.76 (*ddd*, 11.4, 11.3, 5.5) | 36.5[c] | 2.75 (*ddd*, 10.8, 10.8, 5.5) | 37.0[c] |
| 20 | | 157.0 | | 156.8 |
| 21 | α, 1.32 (*m*)<br>β, 2.17 (*dddd*, 11.3, 12.3, 9.9, 8.5) | 32.8[c] | a, 1.29 (*m*)<br>b, 2.17 (*m*) | 32.3[c] |
| 22 | α, 1.23 (*m*)<br>β, 1.91 (*dd*, 2.0, 8.5) | 33.9 | 1.37 (*m*) | 39.9 |
| 23 | 1.05 (*s*) | 26.6 | 1.05 (*s*) | 26.6 |
| 24 | 1.00 (*s*) | 21.1 | 1.00 (*s*) | 21.1 |
| 25 | 0.90 (*s*) | 15.9 | 0.91 (*s*) | 15.9 |
| 26 | 1.04 (*s*) | 15.8 | 1.04 (*s*) | 15.7 |
| 27 | 0.95 (*s*) | 14.6 | 0.92 (*s*) | 14.3 |
| 28 | 3.36 *d* (10.6), 3.78 *d* (10.6) | 60.2 | 0.82 (*s*) | 17.8 |
| 29 | 5.91 (*s*), 6.27 (*s*) | 133.2 | 5.89 (*s*), 6.27 (*s*) | 133.3 |
| 30 | 9.49 (*s*) | 194.9 | 9.49 (*s*) | 195.1 |

[a] $^1H$ (400 MHz) and $^{13}C$ (50.3 MHz) NMR spectra were recorded in $CDCl_3$ with TMS as internal standard, δ (ppm), (*J*) in Hz.
[b] Assignment of individual $CH_aH_b$ as α or β was based on the values of coupling constants and on NOE correlations.
[c] Signals determined from HMQC at 38 °C
[d] Shifts may be interchanged

fractions of the *A. mellifera* extract and was identified on the basis of its spectral characteristics. The original report on this compound did not provide $^{13}C$ NMR data and complete assignment of the $^1H$ NMR resonances. Following extensive 2D NMR experiments we report here the assignment of all signals.

Comparison between the metabolites bearing an aldehyde group (**1**, **2**, **3**) on the side-chain and those without the aldehyde moiety (**4**–**9**) showed that the through-space distance is clearly more important than the number of intervening bonds, since C-12, is affected more than C-13 and C-22, in accordance to the observations of Burns et al., 2000.

The different shift trends for C-12, C-18, C-19, C-21 and the corresponding protons observed between metabolites **1**–**9** are due to angularly dependent through-space effects of the conjugated carbonyl group such as the anisotropic magnetic susceptibility and/or electric field effect.

The antitumor activity of lupane derived triterpenoid compounds were first discovered over 20 years ago when extracts from the stem barks of various plants were tested for cytostatic activity using different in vivo cancer model systems (Ogura et al., 1977; Sanberg et al., 1987). This has led to betulinic acid, the best-known representative of the lupane-derived compounds with

Table 2
Important NOE correlations for (**1**)

| | |
|---|---|
| $H_a$-28 | H-13, $H_\beta$-15, H-19, $H_\beta$-21, $H_\beta$-22 |
| $H_b$-28 | H-19, $H_\beta$-21, $H_\beta$-22, $H_\beta$-15, $H_\beta$-16 |
| H-19 | $H_{a,b}$-28, $H_\beta$-13, $H_\beta$-12 |
| $H_\beta$-21 | $H_a$-28, $H_\beta$-19 |
| $H_\alpha$-2 | $H_3$-23 |
| $H_\beta$-2 | $H_3$-24, $H_3$-25 |
| $H_3$-25 | $H_\beta$-2, $H_\beta$-1, $H_\beta$-6 |
| $H_3$-27 | H-18 |

antiproliferative properties (Ryu et al., 1994). Many studies have suggested that topoisomerase I poisons induce cancer apoptosis (Tabata et al., 2001) and recently it was reported that betulinic acid is a potent inhibitor of eukaryotic topoisomerase I (Chowdhury et al., 2002). It is known that the cytotoxicity of isoprenoid carboxylic acid derivatives is often related to the presence of a free carboxyl group in the molecule. In a study on relationships between structure and activity of lupane triterpenes on the induction of B16 cell 2F2 cell differentiation and apoptosis, it was demonstrated that the keto function at C-3 enhanced their differentiation-inducing activities (Hata et al., 2003), and the carbonyl group at C-17 played an important role on the induction of melanoma cell apoptosis (Hata et al., 2002).

In the present study metabolites **1** and **3** showed significant cytotoxicity against NSCLC-N6 cell line, metabolites **6** and **4** were marginally active whereas metabolites **2**, **5** and **7** were inactive. The observed worth noting difference on the levels of activity (Table 3) and the structural similarities of the tested metabolites indicate that the presence of at least one hydroxyl group is important for expression of activity. Furthermore the position of the hydroxyl on C-3 is more important than on C-28, the presence of the conjugated carbonyl influences the activity very slightly and finally the presence of two hydroxyls (on C-3 and C-28) results in a reduction of activity. Even though the potencies of the assayed metabolites are two orders of magnitude lower than those expressed by navelbin on the same biological systems, the significantly lower toxicity of the tested metabolites remains a significant aspect.

Table 3
Cytotoxic activity of metabolites **1**–**7** ($CI_{50}$ in μg/ml)

| Metabolite | $CI_{50}$ |
|---|---|
| **1** | 15±0.06 |
| **2** | Inactive |
| **3** | 11±0.02 |
| **4** | 30±0.04 |
| **5** | Inactive |
| **6** | >30 |
| **7** | Inactive |

The isolated metabolites were probably derived from oxidosqualene, the precursor of most 3β-OH-triterpenoids through the action of oxidosqualene cyclases that can generate numerous tetracyclic triterpene alcohols since the tetracyclic cations frequently undergo rearrangement prior to neutralization by deproptonation or water addition. The all-chair dammarenyl cation can undergo D-ring expansion via C16 migration to yield the tetracyclic 6-6-6-6 baccharenyl cation. Baccharenyl cation via 18β E-ring cyclization yields lupyl cation, the precursor of lupeol and the rearranged lupanes.

Lupane-containing plants are systematically widespread within the angiosperms and are found predominantly among trees and bushes. Betulin (**7**) has been reported within the orders of Buxales, Dilleniales, Ebenales and Lamiales (Hayek et al., 1989). Lupenone (**5**) has been isolated from plants belonging to the Guttiferae, Rhamnaceae, Euphorbiaceae, Myrcinaceae, Leguninosae, Fagaceae and Asclepiadaceae families. Lupeol (**6**) has been found in plants of the Euphorbiaceae, Apocynaceae, Leguminosae, Sapindaceae and Ancistrocladaceae families. The higher oxidized derivatives exhibit a narrower distribution and metabolites **2**, **3** and **4** are reported for the first time in the Leguminosae family.

## 3. Experimental

### 3.1. General

Optical rotations were measured on a Perkin-Elmer model 341 polarimeter with a 10 cm cell. UV spectra were obtained in spectroscopic grade $C_6H_{14}$ on a Shimadzu UV-160A spectrophotometer. IR spectra were obtained using a Paragon 500 Perkin-Elmer spectrophotometer. NMR spectra were recorded using a Bruker AC 200 and a Bruker DRX 400 spectrometers. Chemical shifts are given in $\delta$ (ppm) scale using TMS as internal standard. The 2D experiments ($^1$H-$^1$H COSY, HMQC, HSQC, HMBC) were performed using standard Bruker microprograms. High Resolution Mass Spectra data were provided by the University of Notre Dame, Department of Chemistry and Biochemistry, Notre Dame, Indiana, USA. EIMS data were recorded on a Hewlett Packard 5973 Mass Selective Detector. Column chromatography was performed with Kieselgel 60 (Merck), HPLC was conducted on an Agilent 1100 series with refractive index detector, with Kromasil Sil 100, 5 um, 250×8 mm column. TLC were performed with Kieselgel 60 F254 (Merck aluminum support plates).

### 3.2. Plant material

The stem bark of *A. mellifera* was collected in January 2000, at Machakos, Kenya. The plant was identified by

Mr. Onesimus Mwangangi at the East African Herbarium-Museum and a voucher specimen is deposited at the collection of the same institute in Nairobi (voucher number ChM-1).

### 3.3. Extraction and isolation

Air-dried, powdered stem bark of *A. mellifera* (2.25 kg) were extracted with $CH_2Cl_2$ (100%) and MeOH (100%), filtered and separately reduced to dryness in vacuum. The $CH_2Cl_2$ extract was fractionated by gravity column chromatography on silica gel eluted by cyclohexane with increasing amounts of EtOAc (0–100% EtOAc), followed by EtOAc with increasing amounts of MeOH (0–100%) to afford sixty one fractions. These were further combined to fractions I- X on the basis of their TLC and $^1$H NMR profiles. Fraction III (8% EtOAc, 554 mg), and fraction V (20% EtOAc, 2.554 g) were further chromatographed successively by gravity column chromatography and later subjected to normal phase HPLC chromatography, using as mobile phase, mixtures of cyclohexane/EtOAc to yield compounds **1–9** in pure form.

### 3.4. Cytotoxicity evaluation

The NSCLC-N6 cell line (Roussakis et al., 1991), derived from a human non-small-cell broncho-pulmonary carcinoma (moderatly differentiated, rarely keratinizing, classified as T2N0M0) was used for all experiments. The cells were cultured in RPMI 1640 medium with 5% fetal calf serum, to which were added 100 IU penicillin.ml$^{-1}$, 100 μg streptomycin.ml$^{-1}$ and 2 mM glutamine, at 37 °C in an air/carbon dioxide (95:5, v/v) atmosphere. In these conditions, cell doubling time was 48 h. Cells used in all experiments never exceeded 35 passages.

Experiments were performed in 96 wells microtiter plates ($2\times10^5$ cells.ml$^{-1}$). Cell growth was estimated by a colorimetric assay based on the conversion of tetrazolium dye (MTT) to a blue formazan product by live mitochondria (Mossmann, 1983). Eight repeats were performed for each concentration. Control growth was estimated from 16 determinations. Optical density at 570 nm corresponding to solubilized formazan was read for each well on a Titertek Multiskan MKII.

### 3.5. 28-Hydroxy-3-oxo-lup-20-(29)-en-30-al

Colourless gummy substance, $[\alpha]_D^{20}$ +9.62 ($CHCl_3$; *c* 1.0); IR ($CHCl_3$), $\nu_{max}$ cm$^{-1}$: 3625, 2953, 2866, 1782, 1794, 1693,1460, 1378, 1250,1023; FAB-MS *m*/*z* (rel. int.%): $M^+$ = 454 (12), 423 (100), 367(12), 205 (36). HREIMS *m*/*z* 455.3523 (calcd for $C_{30}H_{47}O_4$, $[M+1]^+$, 454.34448).

### 3.6. 3-Oxo-lup-20-(29)-en-30-al

Colourless needles, $[\alpha]_D^{20}$ +22.3 ($CHCl_3$, *c* 1.0); IR ($CHCl_3$), $\nu_{max}$ cm$^{-1}$: 2950, 1692, 1451, 867; EIMS 70 eV, *m*/*z* (rel. int.%): ($M^+-15$) 423(62), 341(6), 409 (3), 219(30), 205 (100), 108 (67).

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