Triterpenoids from *Eucalyptus camaldulensis* DEHNH. Tissue Cultures

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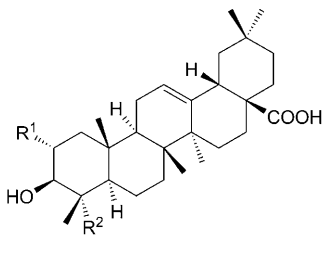
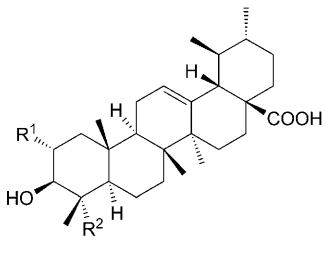
A chemical study on tissue cultures from leaves and flowers of *E. camaldulensis* DEHNH. afforded the new natural product (2α,3β)-2,3,23-trihydroxy-13,28-epoxyurs-11-en-28-one (dryobalanolide) together with the known pentacyclic triterpenoids: betulinic acid, oleanolic acid, ursolic acid, (2α,3β)-2,3,23-trihydroxyolean-12-en-28-oic acid (arjunolic acid), (2α,3β)-2,3,23-trihydroxyurs-12-en-28-oic acid (asiatic acid), (2α)-2-hydroxyursolic acid, (2α)-2-hydroxyoleanolic acid (maslinic acid), as well as β-sitosterol. The extracts and the isolated compounds were evaluated against eleven human pathogenic microorganisms, exhibiting a very interesting antibacterial spectrum of activities.

Introduction. – *Eucalyptus*, belonging to the family Myrtaceae, is one of the world's most important and widely planted genera. Among its main uses is the production of essential oils, which are used for medicinal and pharmaceutical purposes [1][2]. Most phytochemical reports are focused on *E. camaldulensis* leaves [3][4], while very little is known on the phytochemistry of calli and on the *in vitro* production of terpenoids from *Eucalyptus* sp. [5][6]. However, there are reports on the use of *Eucalyptus* species cell cultures for the biotransformation of several terpenoid metabolites [7][8]. This chemical study is, to the best of our knowledge, the first report on the triterpenoid constituents of calli induced from leaves and flowers of *E. camaldulensis* DEHNH. This paper describes the isolation and characterization of a new natural product and eight known triterpenoids. The evaluation of the antimicrobial activity of the isolates was also described herein.

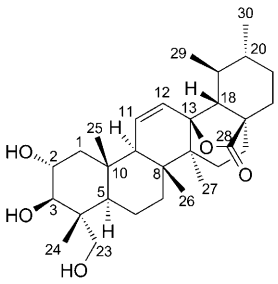
Results and Discussion. – The CH_2Cl_2 extract was shown to contain β-sitosterol (**1**), together with two classes of triterpenes, the oleananes and ursanes. So, betulinic acid (**2**), ursolic acid (**3**), oleanolic acid (**4**), (2α,3β)-2,3,23-trihydroxy-13,28-epoxyurs-11-en-28-one (**5**), 2α,3β,23-trihydroxyolean-12-en-28-oic acid (arjunolic acid) (**6**) and (2α,3β)-2,3,23-trihydroxyurs-12-en-28-oic acid (asiatic acid) (**7**) have been isolated and identified. From the methanolic extract of calli from the flowers were isolated again β-sitosterol together with (2α)-2-hydroxyursolic acid (**8**) and (2α)-2-hydroxyoleanolic acid (**9**). Compounds **3** and **4** were identified by direct comparison with authentic samples, while the other known compounds, **1** [9][10], **2** [11][12], **6** [5][13], **7** [5][14], **8** [12][15], and **9** [5], were identified by comparison with published spectral data.



	R^1	R^2
4	H	Me
9	OH	Me
6	OH	$HOCH_2$



	R^1	R^2
3	H	Me
8	OH	Me
7	OH	$HOCH_2$



5

Notably, compound **5** was a pentacyclic triterpenoid isolated for the first time as a natural product, but reported previously as a synthetic derivative [16]. The electron spray mass spectrum (ESI-MS) of compound **5** showed $[M+Na]^+$ and $[M+K]^+$ peaks at m/z 509 and 525, respectively, corresponding to the molecular formula $C_{30}H_{46}O_5$. The ^{1}H-NMR spectrum showed two olefinic signals at δ(H) 6.11 (d, $J=10.3$) and 5.64 (dd, $J=10.3$, 3.1), correlated with δ(C) 135.5 and 130.6 in the HMQC spectrum, and assigned to H–C(11) and H–C(12), respectively. Also, the presence of two CH signals at δ(H) 3.79 (ddd, $J=11.0, 9.5, 4.4$) and 3.40 (d, $J=9.5$) and two signals at δ(H) 3.55 (d, $J=11.2$) and 3.30 (d, $J=11.2$), which correspond to an oxygenated CH_2 group (HMQC), indicated the presence of a 2α,3β,23-trihydroxy structure in ring A. The presence of quaternary C-atom signals at δ(C) 92.3 (C(13)) and 179.5 (C(28)) indicated a lactone moiety between C(13) and C(28) [17]. These data suggested the presence of an 11-en-28,13β-olide structure. The assignment of the configuration of **5** was achieved by a NOESY experiment. The configuration of the substituents at positions C(2) and C(4) was confirmed by the interactions of Me(24) with Me(25) and H–C(2). The absence of interactions between H–C(3) and Me(24) indicated the β-configuration of the OH group at position 3. From the observed data, the structure of **5** was determined to be (2α,3β)-2,3,23-trihydroxy-13,28-epoxyurs-11-en-28-one.

To our knowledge, this is the first report on the chemical analysis of tissue cultures derived from *E. camaldulensis* DEHNH. Compounds **3**, **4**, **6**, **7**, and **8** have been isolated once before from cultured cells of *E. perinniana* [5]. A derivative (in the form of 3,23-*O*-isopropylidene) of compound **5** has been isolated from *Dryobalanops aromatica* resin [18]. Triterpenoids with an 11-en-28,13β-olide structure, like that of **5**, are rather rare. *Siddiqui* and co-workers [4] reported the identification of a derivative of that type, as a result of the transformation of (2α,3β,7β,11α)-2,3,7-trihydroxy-11-methoxyurs-12-en-28-oic acid, when left at room temperature for six months. However, *Sashida* and co-workers [19] have reported that calli possess the ability to produce such triterpenoids.

The extracts and the isolated compounds (**2**–**7**) were submitted to an antimicrobial assay (see *Table*). Metabolites **3** and **5**, as well as the mixture of **6** and **7** showed significant antibacterial activity mostly against *Staphylococcus aureus* and *S. epidermidis* (*Gram* positive bacteria) and interestingly against *E. coli* (*MIC*s of 20–80 μg/ml). Compounds **2**, **4**, and the mixture of **8** and **9** showed weak activities (*MIC*s of 125–

200 μg/ml). Furthermore, no activity was recorded on almost all tested metabolites and extracts against the tested pathogenic fungi. It appears from the biological assay results that the OH group at position C(23) makes an important contribution to the expression of activity. In addition, the noteworthy activity of the new natural triterpene **5** is in accordance with previously reported antimicrobial activities for triterpene of similar classes [20].

Experimental Part

General. Authentic samples of ursolic and oleanolic acids were purchased from *Extrasynthèse* (France). Column chromatography (CC): silica gel *60 H* (*Merck*, 0.015–0.040 mm). TLC: *Kieselgel 60* F_{254} precoated plates (*Merck*), visualization under UV light and by spraying with vanillin reagent. $[\alpha]_D$: *Perkin-Elmer 341* polarimeter. ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR (50 MHz, $CDCl_3$): *Brucker DRX400* and a *Brucker AC200* spectrometer, resp.; Me_4Si was used as an internal standard, δ in ppm, J in Hz. MS: *ZQ 2000 Waters* using a *Zspray* (ESI-MS).

Plant Material. Fresh immature flowers and leaves were collected from *E. camaldulensis* DEHNH. trees growing in the University Campus of the University of Athens in September 2003. All samples have been identified by Dr. *L. Koumpli-Sovantzi* (Lecturer of Dept. of Botany, University of Athens). A voucher specimen has been deposited with the Herbarium of the University of Athens (KS6187).

Initiation of Cultures from Immature Flowers and Leaves. Immature flowers and leaves were surface sterilized with 70% EtOH for 1.5 min and 10% common bleach for 4 min, and then rinsed three times with distilled sterile H_2O. The flowers and leaves were cut in half and then placed separately, under sterile conditions, in sterile *Petri* dishes containing MS supplemented with 8% agar, 2% sucrose, in the presence of 0.1 mg l^{-1} 2-benzylaminopurine (BA) and 0.01 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) [21]. The pH of the culture media was adjusted to 5.8. The explants were incubated at 25° in continuous darkness. Calli were initiated 6–8 d after the start of tissue culture.

Extraction of Calli and Isolation of Compounds. Two weeks after callus initiation, calli without the explant were transferred to *Petri* dishes and cultured under the same conditions. After two subcultures, 180 g of calli were collected and extracted 3 times with 200 ml of the solvents pentane, CH_2Cl_2, and MeOH. The CH_2Cl_2 extracts of flowers and leaves showed qualitative similarities, so they have been combined and concentrated under reduced pressure to yield a yellow-brownish residue (526.3 mg), which was chromatographed on a SiO_2 column eluted with cyclohexane/CH_2Cl_2 (100:0 → 0:100) and CH_2Cl_2/MeOH (99:1 → 70:30) step gradient, giving 36 fractions. *Fr. 10* and *13* eluted with CH_2Cl_2/MeOH (99:1) were pure β-sitosterol (**2**, 21.7 mg) and betulinic acid (**3**, 30.7 mg), resp., while *Fr. 34* eluted with CH_2Cl_2/MeOH (94:6) contained a mixture 7.5 mg of (2α,3β),2,3,23-trihydroxyolean-12-en-28-oic acid (**6**, arjunolic acid) and (2α,3β),2,3,23-trihydroxyurs-12-en-28-oic acid (**7**). *Fr. 17* (17.8 mg) eluted with CH_2Cl_2/MeOH (99:1) was subjected to prep. TLC ($CHCl_3$/MeOH 9.5:0.5) to afford ursolic acid (**3**, 3 mg) and oleanolic acid (**4**, 4.2 mg). From *Fr. 31* eluted with CH_2Cl_2/MeOH 96:4, the new triterpenoid (**5**, 5.8 mg) was obtained. The methanolic extract of flowers was concentrated under reduced pressure to yield a brownish residue (3.80 g) from which 2.07 g were chromatographed on a SiO_2 column (CH_2Cl_2/MeOH 100:0 → 0:100) giving 29 fractions. *Fr. 9* eluted with 100% CH_2Cl_2 contained β-sitosterol (15 mg). A part (23 mg) of *Fr. 19* (83.5 mg) eluted with CH_2Cl_2/MeOH 98:2 was subjected to prep. TLC (CH_2Cl_2/MeOH 93:7) to afford a mixture (13.7 mg) of (2α)-2-hydroxyursolic acid (**8**) and (2α)-2-hydroxyoleanolic acid (**9**).

Dryobalanolide (=*(2α,3β)-2,3,23-Trihydroxy-13,28-epoxyurs-11-en-28-one*; **5**). Amorphous solid. $[\alpha]_D = +22.9$ ($c = 0.24$, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): 0.71 (*s*, Me(24)); 0.85–1.08 (*m*, H_β–C(1), H–C(20), Me(25), Me(26), Me(29), Me(30)); 1.11–1.15 (*m*, H_β–C(7)); 1.20 (*s*, Me(27)); 1.28–1.36 (*m*, H–C(5), H_β–C(15), H_β–C(16)); 1.53–1.67 (*m*, H_α–C(6), H_β–C(6), H_α–C(7), H_α–C(15), H_α–C(21), H_β–C(21), H–C(18)); 1.69–1.75 (*m*, H_α–C(22), H_β–C(22)); 1.85–1.92 (*m*, H–C(19)); 2.15 (br. *s*, H–C(9)); 2.19 (*dd*, $J = 11.4$, 4.4, H_α–C(1)); 2.24–2.32 (*m*, H_α–C(16)); 3.30 (*d*, $J = 11.2$, H_β–C(23)); 3.40 (*d*, $J = 9.5$, H–C(3)); 3.55 (*d*, $J = 11.2$, H_α–C(23)); 3.79 (*ddd*, $J = 11.0$, 9.5, 4.4,

Table. *Antimicrobial Activity* MIC *Values* (μg/ml, $n=3$) *of the Assayed Extracts and Constituents.* The tested extracts were assayed at a concentration of 100 mg/ml, while the pure compounds at a concentration of 0.1 mg/ml each.

Tested samples	*S. aureus*	*S. epidermidis*	*P. aeruginosa*	*E. cloacae*	*K. pneumoniae*	*E. coli*	*C. albicans*	*C. tropicalis*	*C. glabrata*
CH_2Cl_2 Extract	160	150	120	n.a.[a])	n.a.	100	n.a.	n.a.	n.a.
MeOH Extract	>250	>250	n.a.	n.a.	n.a.	>250	n.a.	n.a.	n.a.
Betulinic acid (**2**)	125	130	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Ursolic acid (**3**)	30	40	150	180	200	80	500	350	300
Oleanolic acid (**4**)	175	205	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Dryobalanolide (**5**)	20	40	100	60	65	80	n.a.	n.a.	n.a.
Arjunolic acid (**6**) + asiatic acid (**7**)	20	30	120	70	90	40	n.a.	n.a.	n.a.
(2α)-2-Hydroxy ursolic acid (**8**) + (2α)-2-Hydroxy oleanolic acid (**9**)	190	185	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Netilmicin	4	4	8.8	8	8	10	n.t.[b])	n.t.	n.t.
5-Flucytocine	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	10	1	10.1

[a]) n.a.: not active. [b]) n.t.: not tested.

H–C(2)); 5.64 (*dd*, *J* = 10.3, 3.1, H–C(12)); 6.11 (*d*, *J* = 10.3, H–C(11)). ^{13}C-NMR (50 MHz, $CDCl_3$): 13.7 (C(24)); 16.6 (C(27); 17.8 (C(29)); 18.0 (C(30)); 18.9 (C(6)); 19.2 (C(25)); 19.4 (C(26)); 24.0 (C(16); 26.8 (C(15)); 30.3 (C(7)); 31.3 (C(21)); 32.9 (C(22)); 38.8 (C(19)); 40.8 (C(10)); 42.3 (C(20)); 42.7 (C(8)); 42.9 (C(14)); 43.7 (C(4)); 47.4 (C(1)); 47.5 (C(5)); 48.1 (C(17)); 54.1 (C(9)); 61.5 (C(18)); 65.6 (C(23)); 69.5 (C(2)); 77.5 (C(3)); 92.3 (C(13)); 130.6 (C(12)); 135.5 (C(11)); 179.5 (C(28)). ESI-MS: 509 ($[M + Na]^+$), 525 ($[M + K]^+$).

Antimicrobial Activity. All isolated compounds as well as the crude extracts (CH_2Cl_2, MeOH) of the plant were tested using the agar dilution technique [22] against the *Gram*-positive bacteria *Staphylococcus aureus* (ATCC 25923) and *S. epidermidis* (ATCC 12228), the *Gram*-negative *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047), *Klebsiella pneumoniae* (ATCC 13883), as well as against the three pathogenic fungi *Candida albicans* (ATCC10231), *C. tropicalis* (ATCC 13801), and *C. glabrata* (ATCC 28838). Standard antibiotics netilmicin and 5-flucytocine were used in order to control the sensitivity of the tested bacteria and fungi, resp.

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