A New Clerodane Diterpene and Other Constituents from *Ajuga chamaepitys* ssp. *laevigata*

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From *Ajuga chamepitys* ssp. *laevigata*, a new clerodane diterpene, ajugalaevigatic acid, has been isolated besides five known compounds, a diterpene, (13S)-15-hydroxylabd-8(17)-en-19-oic acid, a steroidal glucoside, 3-O-β-D-glucopyranosyl-stigmasta-5,25-diene, and triterpenes, α- and β-amyrin and ursolic acid. Their structural elucidation is based on NMR and MS spectroscopic analyses. For the new compound 2D NMR experiments were carried out. Ajugalaevigatic acid was tested against a panel of cytotoxic cell lines, and only found to be active against the A2780 human ovarian cancer cell line.

Key words: Ajuga chamepitys ssp. *laevigata,* Terpenoids, Biological Activity

Introduction

More than a hundred species and fifty subspecies and varieties of *Ajuga* plants are distributed over the world [1]. In Anatolia, thirteen *Ajuga* species occur with six species and one subspecies being endemic [2]. *Ajuga* species contain steroids, neoclerodane diterpenes, some iridods, polyphenolics and their glucosides [3 – 5]. *Ajuga* species have important medicinal and agrochemical interest [6] due to their clerodane diterpenes and ecdysteroids which have been reported as insect antifeedant and moulting hormone agents [7]. Ecdysteroids have also been reported to exhibit significant effects on induced hyperglycemia in experimental mammals, and more recently to potentiate the effects of insulin [8]. In Turkey, some *Ajuga* species have been used to treat inflammation and infectious diseases [9]. *Ajuga chamaepitys* (European Ground Pine, Yellow Bugle) grows throughout Europe as well as in Anatolia, and has afforded mainly neo-clerodanes and steroids [10 – 12]. The anthocyanin aglycon cyanidin was also isolated from *A. chamaepitys* [13]. However, the subspecies *laevigata* has not yet been investigated chemically. In our previous *Ajuga* study, we reported on the isolation and structure elucidation of two new steroids and terpenoids from *Ajuga relicta* [14], an endemic species to Turkey. We now report on the isolation and structural elucidaton of a new clerodane diterpene **(1)**, and the known compounds (13S)-15-hydroxylabd-8(17)-en-19-oic acid (**2)** [15], 3-O-β-D-glucopyranosyl-stigmasta-5,25-diene **(3)** [16], α- and β-amyrin **(4, 5)** [17], and ursolic acid **(6)** [17, 18] from *A. chamaepitys* ssp. *laevigata*. Compounds **1 – 3** were evaluated in a yeast based microtiter assay [19] for antifungal and cytotoxic potential, but none of them showed either selective DNA damaging or antifungal activity (Table 1). Also, compound **1** was investigated for cytotoxicity against a panel of cell lines [20] including the A2780 ovarian cancer cell line [21] against which it showed only weak activity (17.7 μg/ml).

Results and Discussion

The HRMS of compound **1** indicated the molecular formula $C_{22}H_{36}O_4$ (*m/z* 364.2615, calcd. 364.2614) showing five degrees of unsaturation, two of which were accounted for by a bicyclic ring system, the others by a double bond, an acetyl and an acid group. The presence of the acid followed from the IR spectrum with an absorption band at 1695 cm^{-1} which correlated with a large shoulder at 2600 – 3000 cm^{-1} and the ^{13}C NMR signal at $\delta = 179.76$. The acetyl group

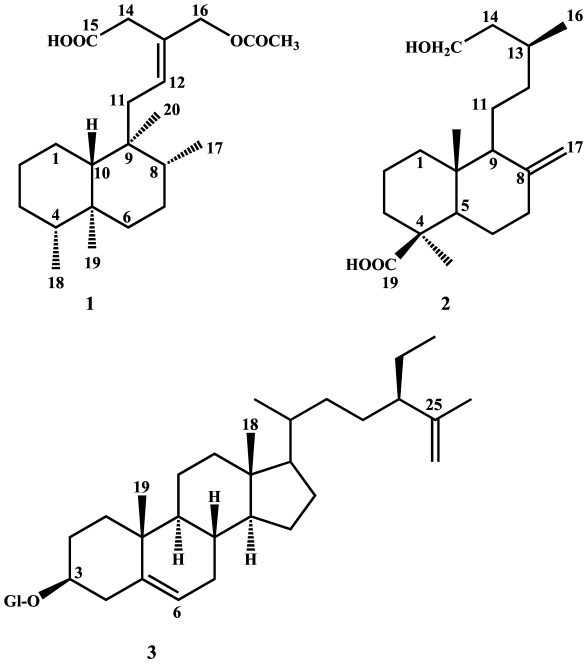
Table 1. ^{1}H and ^{13}C NMR data of compound **1**[a,b].

Position	^{13}C	^{1}H
1	17.21	1.81 ddd (3.2, 12.0, 11.5) and 0.89 m
2	23.95	1.61 m, 0.78 m
3	33.89	
4	30.41	
5	39.97	–
6	29.20	
7	35.04	
8	37.32	1.42 tq (3.0,7.0)
9	36.24	–
10	45.00	1.32 dd (2.0,11.5)
11	37.17	1.90 m, 1.33 m
12	129.20	5.65 t (3.2)
13	138.34	–
14	41.35	2.41 br dd (12.0,3.2), 2.12 dd (12.0,3.0)
15	179.76	–
16	66.66	4.58 brs
17	15.90	0.73 d (7.0)
18	19.90	0.98 d (7.0)
19	34.60	1.10 s
20	17.34	0.80 s
21	21.18	2.07 s
22	170.98	–

[a] δ, $CDCl_3$, J values in Hz (400 MHz for ^{1}H and 100 MHz for ^{13}C NMR); [b] assignments were made based on DEPT, HMQC and HMBC experiments.

Table 2. Microtiter assay results of compounds **1**, **2** and **3** (100 μg/ml).

Compound	YCp 50 Galactose	pRAD52 Galactose	pRAD52 Glucose
1	13.5	38.9	0
2	15.7	15.5	0
3	7.9	1.7	0



1 **2** **3**

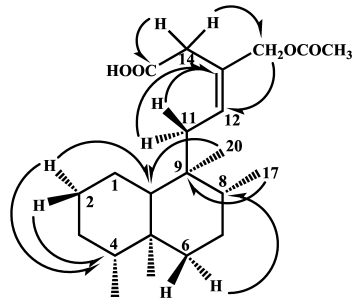


Fig. 1. HMBC correlations of compound **1**.

was observed by the IR bands at 1740 and 1260 cm^{-1} and the ^{13}C NMR signal at $\delta = 170.98$. The ^{1}H NMR spectrum of **1** exhibited two methyl singlet signals at $\delta = 0.77$ (3H, s, Me-20) and 1.10 (3H, s, Me-19), two methyl doublet signals at $\delta = 0.74$ and 0.98 with coupling constants of 7 Hz, which were assigned to Me-17 and Me-18, respectively, along with an acetyl methyl resonance at $\delta = 2.07$. There was a pair of oxymethylene protons at $\delta = 4.58$ as a broadened singlet. Also, an olefinic proton signal was observed at $\delta = 5.65$ as a triplet ($J = 3.5$ Hz). The ^{13}C NMR (DEPT) signals indicated the presence of five methyl, eight methylene, four methine and five quaternary carbon signals for 22 carbons of the molecule. The olefinic carbon signal was observed at $\delta = 129.20$ which correlated with the olefinic proton at $\delta = 5.65$ in the HMQC spectrum while the quaternary carbon of the olefinic bond was observed at $\delta = 138.34$. The location of the double bond between C-12 and C-13 as well as oxymethylene group clearly followed from the HMBC experiment observing correlation between the oxymethylene carbon (C-16) which appeared at $\delta = 66.66$ and the olefinic proton at $\delta = 5.65$ (H-12) as well as between C-16 and the acetyl proton at $\delta = 2.07$ thus assigning the double bond location at Δ^{12}, neighboured by the acetoxymethylene group. The other informative knowledge came from the observation of three bond correlations between C-14 ($\delta = 41.35$) and both the vinylic ($\delta = 5.65$) and acetoxymethylene protons ($\delta = 4.58$) in the HMBC experiment. Thus, the ^{1}H and ^{13}C NMR data revealed that the compound has a clerodane even a kolevane-like structure. This type of clerodanes are not common in Lamiaceae family, even in the plant kingdom. It is noteworthy that the iso-

lated diterpene differed from kolevane-like diterpenes [22, 23] by lacking of a vinylic Me at C-4. Instead, a double bond is placed between C-12 and C-13. The determination of the α-orientation of all four methyl groups could be achieved *via* NOESY experiments. A NOESY correlation was observed between Me-19 with Me-18 and Me-17, as well as between Me-17 with Me-20 and Me-19, and the NOESY correlation between H-10 and H-8 indicated their β-orientation. Z-Stereochemistry of the side chain was also determined based on NOESY observations. All the above spectral data (IR, ^{1}H NMR, COSY, ^{13}C NMR, HMQC, HMBC and mass) established the structure of compound **1** to be cleroda-12-en-16-acetoxy-15-oic acid [4(5)-dihydrokolevan-12-en-16-acetoxy-15-oic acid] which is named ajuleavigatic acid.

Compound **2** has been previously isolated from *Juniperus formosona* [15] and its structure was elucidated to be (13S)-15-hydroxylabd-8(17)-en-19-oic acid differing from its diastereomer imbricatolic acid [(13R)-15-hydroxylabd-8(17)-en-19-oic acid]. Compound **3** was identified as 3-O-β-D-glucopyranosyl-stigmasta-5,25-diene (24-ethyl-cholest-5,25-diene-3-O-β-D-glucoside), first isolated together with β-sitosterol glucoside from *Momordica charantia* and named charantin [24], and then from *M. foetida* as foetidin which is identical to charantin [25]. Hypoglycaemic activity of an equal mixture [26] of 3-O-β-D-glucopyranosyl-stigmasta-5,25-diene and β-sitosterol glucoside was shown. Antiinflammatory and antimicrobial activity of 3-O-β-D-glucopyranosyl-stigmasta-5,25-diene were also reported from *Ulva lactuca* [16].

Compound **1** was further evaluated for antimalarial potential [27], using a chloroquine-sensitive D6 strain of *Plasmodium falciparum*. The result was also negative. Compound **3** was furthermore tested against the A2780 cancer cell line where it showed slight activity ($IC_{50} = 32\ \mu$g/ml).

Conclusion

In this study, a new clerodane diterpene, with an uncommon structure for *Ajuga* species, even for Labiatae family, was isolated from acetone extract of *Ajuga chamaepitys* ssp. *laevigata* besides five known compounds. 3-O-β-D-glucopyranosyl-stigmasta-5,25-diene has been previously investigated for its hypoglycaemic [26], antimicrobial and anti-inflammatory activities [16] while α- and β-amyrin and ursolic acid have been investigated for their anti-inflammatory activity [28] and the latter compound was also known for antitumor-promotion, anti-hepatotoxic, anti-oxidant, and anti-viral effects [18, 28]. However, the new diterpene (**1**) showed no high activity against a panel of cell lines.

Experimental Section

The whole plant of *Ajuga chamaepitys* ssp. *laevigata* was collected from Southern Anatolia (Tarsus, Namrun Plato) in June 1999 and identified by Dr. Mecit Vural. A voucher specimen was deposited in the Herbarium of the Faculty of Pharmacy, Gazi University, Ankara, Turkey.

Dried and powdered plant material (850 g) was macerated twice with 2 l of acetone at room temperature for 4 days, then filtered, and 70 g of extract were obtained. The waxes of the plant were precipitated by treatment with 60% water in acetone. The remaining part was then extracted with ethyl acetate and 5 g of extract were obtained. This extract was subjected to a vacuum liquid chromatography column filled with 350 g Si-gel (Merck 9385). Elution was started with hexane (fractions 1 – 7) and added ethylacetate (fractions 8 – 16) gradually, then finally with methanol to collect 20 main fractions. Compounds **1** and **2** were obtained from fraction 8 by elution with hexane-CH_2Cl_2 (5:3). Compound **(2)** was purified by prep TLC eluting with hexane-ethyl acetate (70:30) while the new diterpene **(1)** eluting with hexane-ethyl acetate (65:35). Compound **3** was isolated during the ethylacetate (100%) elution and purified by prep. TLC eluting with CH_2Cl_2-methanol (8:2). Amyrins were separated from hexane-CH_2Cl_2 (5:4) solvent system, and ursolic acid from hexane-CH_2Cl_2 (1:9). 12 mg of **2**, 15 mg of **3**, 6 mg of **4**, 8 mg of **5**, and 11 mg of **6** were obtained, and identified by ^{1}H ^{13}C NMR, and mass spectrometric analyses and by comparison with authentic samples.

The spectra were recorded with the following instruments. IR: Perkin-Elmer 980 in $CHCl_3$; NMR: Varian 400 Unity, in $CDCl_3$ for compounds **1 – 3** and Bruker AC-200 L, 200 MHz and 50.32 MHz for ^{1}H and ^{13}C NMR, respectively for compounds **4 – 6**, and; MS: ZabSpec high resolution mass spectrometer; CC: Silica gel 60 was used for column chromatography and Kieselgel 60F_{254} (E. Merck) for preparative TLC with precoated plates.

Ajugalaevigatic acid [cleroda-12-en-16-acetoxy-15-oic acid = 4(5)-Dihydrokolevan-12-en-16-acetoxy-15-oic acid] (**1**) *(16 mg)*

$[\alpha]_D$: -52° ($c = 0.8$, MeOH). – IR (film): $\tilde{\nu} = 2600$ – 3000 and 1695 (COOH), 1740 and 1260 ($OCOCH_3$), 1640 (C=C), 840 cm^{-1}. – ^{1}H and ^{13}C NMR (400 and 100 MHz, respectively, $CDCl_3$, see Table 1). – MS (EI, 70 eV): *m/z* (%) = 364.2(5) $[M]^+$, 350.2(4)

$[M\text{-}CH_2]^+$, 304.2(51) $[M\text{-}HOAc]^+$, 289.1(48) $[304\text{-}Me]^+$, 270.1(18) $[289\text{-}H_2O]^+$, 223.1(17), 189.1(100), 173.1(18), 163.1(31), 147.1(39), 133.1(53), 119.0(62), 105.0(84), 95.0(65), 83.0(71), 69.0(71), 57.0(72). – MS (HREI, 70 eV): *m/z* (%) = $C_{22}H_{36}O_4$: calcd. 364.261360; found 364.261481.

(13S)-15-Hydroxylabda-8(17)-en-19-oic acid (**2**) *(12 mg)*

Colourless amorphous compound. $-[\alpha]_D$: $+16^\circ$ ($c = 0.9$, $CHCl_3$). – ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.60$ (s, 3H, Me-20), 0.90 (d, $J = 6.8$ Hz, 3H, Me-16), 1.21 (s, 3H, Me-18), 3.65 and 3.60 (dd, each 1H, $J = 3$ and 8 Hz, 15-$H^{1,2}$), 4.50 (brs, 1H, 17-H^1), 4.83 (brs, 1H, 17-H^2). – ^{13}C NMR (APT) (100 MHz, $CDCl_3$): 38.77 (C-1), 19.21 (C-2), 37.11 (C-3), 44.23 (C-4), 57.13 (C-5), 25.17 (C-6), 38.15 (C-7), 146.67 (C-8), 57.05 (C-9), 39.23 (C-10), 21.38 (C-11), 39.65 (C-12), 30.12 (C-13), 42.46 (C-14), 59.31 (C-15), 20.86 (C-16), 106.58 (C-17), 30.46 (C-18), 178.83 (C-19), 13.59(C-20). – MS (EI, 70 eV): *m/z* (%) = 322.1(5) $[M]^+$, 304.1(8) $[M\text{-}H_2O]^+$, 277(100) $[M\text{-}COOH]^+$, 261(5) $[M\text{-}COOH\text{-}OH]^+$, 223 [31], 207(15) [M-side chain]$^+$, 166(11), 149(23), 121(37), 95(22), 83(41), 69(77), 57(60). – MS (HREI, 70 eV): *m/z* (%) = $C_{20}H_{24}0_3$: calcd. 322.2525; found 322.2508.

3-O-β-D-glucopyranosyl-stigmasta-5,25-diene (**3**) *(15 mg)*

$[\alpha]_D$: -36° ($c = 0.5$, MeOH). – ^{1}H NMR (400 MHz, CD_3OD): $\delta = 5.32$ (t, 1H, $J = 3.5$ Hz, 6-H), 4.6 (brs, 1H, 26-H^1), 4.7 (br s, 1H, 26-H^2), 4.27 (d, $J = 7.5$ Hz, 1H, 1'-H anomeric proton) 3.69 (m, 1H, 3-H), 3.3 – 4.2 [m, 6 glucose H's, (2'-6')-H], 1.56 (s, 3H, 26-Me), 1.00 (s, 3H, 19-Me), 0.89 (d, $J = 6.5$ Hz, 3H, 21-Me), 0.79 (t, $J = 7.0$ Hz, 29-Me), 0.69 (s, 3H, 18-Me). – ^{13}C NMR (APT) (100 MHz, CD_3OD): $\delta = 147.53$ (C-25), 140.21 (C-5), 122.06 (C-6), 111.20 (C-26), 101.03 (C-1'), 79.09 (C-3), 76.34 (C-3'), 75.67 (C-5'), 73.48 (C-2'), 70.12 (C-4'), 61.82 (C-6'), 56.67 (C-14), 55.99 (C-17), 50.10 (C-9), 49.58 (C-24), 42.24 (C-13), 39.68 (C-4), 38.62 (C-12), 37.17 (C-1), 36.63 (C-10), 35.44 (C-20), 33.58 (C-22), 31.84 (C-2), 31.80 (C-8), 29.30 (C-16), 28.05 (C-23), 26.42 (C-28), 24.17 (C-15), 21.0 (C-11), 19.18 (C-19), 18.51 (C-21), 17.63 (C-26), 11.87 (C-29), 11.70 (C-18). – MS (EI, 70 eV): *m/z* (%) = 574(12) $[M]^+$, 412(65) [M-glucose]$^+$, 368(48), 301(32), 256(56), 233(25), 212(100), 199(38), 145(51).

Activity tests

Compounds **(1 – 3)** were evaluated in a yeast based microtiter assay [19] for antifungal and cytotoxic potential. Streptonigrin was used as positive control (Table 1). Compounds **1** and **3** were tested against KB (human epidermoid carcinoma in the mouth), LU1 (human lung carcinoma), Col 2 (human colon carcinoma), LNCaP (hormone dependent prostate carcinoma) and hTERT RPE1 (human telomerase reverse transcriptase) [20] and A2780 (mammalian ovarian cell line) [21].

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