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(-)-Desoxypodophyllotoxin and Diterpenoids from *Juniperus nana* Willd. Berries

Juniperus nana Willd. Kozalaklarındaki (-)-Dezoksipodofillotoksin ve Diterpenoitler

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Abstract

From the acidic fraction of the petroleum ether extract of *Juniperus nana* Willd. unripe berries, a lignan, (-)-desoxypodophyllotoxin (I) and diterpenoids, imbricatolic acid (II), isocupressic acid (III), isopimaric acid (IV), sandaracopimaric acid (V) in addition to a mixture of *trans*-communic acid (VI), myrceocommunic acid (VII) and *cis*-communic acid (VIII) were isolated. Their structures were elucidated by means of UV, IR, ^{1}H-NMR and ^{13}C-NMR (DEPT) spectroscopy. The acidic fraction showed strong cytotoxic activity according to Brine Shrimp bioassay (LD_{50} 0.16 μg/mL).

Keywords: *Juniperus nana*; cupressaceae; desoxypodophyllotoxin; diterpenoids; cytotoxicity.

Introduction

Juniperus nana Willd. [Syn. *Juniperus communis* var. *nana* (Willd.) Boiss., Cupressaceae] is a widespread and commonest low shrub, over 1100 m. in N. Anatolia, Turkey (Davis, 1965). The berries of this plant are used as antiseptic, diuretic, stomachic and for treatment of rheumatism in traditional medicine. The title plant can also be used in traditional medicine like *Juniperus communis* (Baytop, 1999). We also recorded that the berries of *Juniperus nana* were also used as an anticancer drug in home remedies in Turkey. The berries of *Juniperus communis* were used by native Americans to treat coldness (Bremness, 1994). Previous phytochemical studies on *Juniperus nana* leaves and berries resulted in the identification of several mono- and sesquiterpenes (Tanker *et al.*, 1975; Da Cunha *et al.*, 1989). The aim of this study is to determine the cytotoxic activity of the acidic and neutral fractions of the petroleum ether extract of the berries as well as the structure elucidation of the lipophilic compounds from the active fraction(s).

Plant Material

Juniperus nana Willd. (Cupressaceae) unripe berries were collected between Abant Lake-Mudurnu, Bolu, Turkey, in June 1995. A voucher specimen (HUEF 95-014) is deposited at the Herbarium of the Faculty of Pharmacy, Hacettepe University, Ankara, Turkey.

Extraction, Isolation and Purification

The unripe and gringed fresh berries (830 g) were exhaustively extracted with petroleum ether (40-60 °C, 3L) in a Soxhlet apparatus. The extract was treated with 5% Na_2CO_3 and extracted with petroleum ether (to give a neutral fraction). The aqueous phase was then acidified with 10% HCl. The acidic fraction was evaporated *in vacuo* to give a residue of 14.3 g.

The acidic fraction was chromatographed on a Si gel column (3 x 66 cm) with petroleum ether (40-60 °C)-EtOAc mixture (95:5 to 50:50) to afford 47 fractions (16 mL each). Similar fractions were then combined an rechromatographed either on Sephadex LH-20 by eluting with petroleum ether (40-60 °C)-chloroform -methanol (7:4:1) mixture to give **I** (24 mg) or preparative TLC [Silica gel HR 0.6 mm Merck] with petroleum ether(40-60°)-chloroform-methanol (7:4:1) and cyclohexane-EtOAc (8:2) mixtures to afford **II** (160 mg), **III** (80 mg), **IV** (52 mg), **V** (20 mg) as well as a mixture of **VI**, **VII** and **VIII** (1:1:1; 60 mg).

(-)-Desoxypodophyllotoxin (**I**). UV λ_{max}^{MeOH} nm: 290.5 nm; IR υ_{max}^{KBr} cm^{-1}: 2921,2850, 1769, 1589, 1505, 1485, 1239, 1127, 1038, ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 2.74 (m, 3H, H-7'a, H-8/8'), 3.06 (m, 1H, H-7'b), 3.75 (s, 6H, 10/12-OMe), 3.80 (s, 3H, 11-OMe), 3.93 (dd, 1H, *J*=10.3/8.6 Hz, H-9'a), 4.46 (dd, 1H, *J*=8.6/6.1 Hz, H-9'b), 4.61 (brs, 1H, H-7), 5.93 (d, 2H, *J*=1.3 Hz, 10'a), 5.96 (d, 1H, *J*=1.3 Hz, H-10'b) 6.35 (s, 2H, H-2/6), 6.52 (s, 1H, H-5'), 6.67 (s, 1H, H-2'). ^{13}C-NMR ($CDCl_3$, 200 MHz): δ 32.6 (C-8'), 33.0 (C-7'), 43.6 (C-7), 47.4 (C-8), 56.1 (C-10/12), 60.7 (C-11), 72.0 (C-9'), 101.1 (C-10'), 108.4 (C-2'/C-2/6), 110.4 (C-5'), 128.2(C-1'), 130.5 (C-6'), 136.2 (C-1/4), 146.8 (C-4'), 146.9 (C-3'), 152.4 (C-3/5), 174.9 (C-9).

Imbricatolic acid (**II**). IR υ_{max}^{KBr} cm^{-1}: 3384, 3080, 2933, 2847, 1694, 1645, 1057, 889; ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 3.66 (t, 2H, *J*= 7 Hz, H_2-15), 0.88 (d, 3H, *J*=6 Hz, H_3-16), 4.48 (s, 1H, H-17a), 4.82 (s, 1H, H-17b), 1.21 (s, 3H, H_3-18), 0.59 (s, 3H, H_3-20). ^{13}C-NMR ($CDCl_3$, 50.3 MHz) (Table 1).

Isocupressic acid (**III**). ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 5.38 (t, 1H, *J*= 7 Hz, H-14), 4.13 (d, 2H, *J*= 7 Hz, H_2-15), 1.65 (s, 3H, H_3-16). ^{13}C-NMR ($CDCl_3$, 50.3 MHz)(Table 1).

Isopimaric acid (**IV**). UV λ_{max}^{MeOH} nm: 230.6; IR υ_{max}^{KBr} cm^{-1}: 3080, 2935, 1694, 1470, 1274, 891; ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 5.31 (d, 1H, *J*= 4.4 Hz, H-7), 5.81 (dd, 1H, J_{AX}= 10.7 Hz, H-15), 4.86 (dd, 1H, J_{AB}= 10.7/1.4 Hz, H-16a), 4.93 (dd, 1H, J_{BX}= 17.5/1.4 Hz, H-16b), 0.90 (s, 3H, H_3-17), 1.24 (s, 3H, H_3-18), 0.85 (s, 3H, H_3-20). ^{13}C-NMR ($CDCl_3$, 50.3 MHz) (Table 1).

Sandaracopimaric acid (**V**). UV λ_{max}^{MeOH} nm: 231 nm; IR υ_{max}^{KBr} cm^{-1}: 2928, 1695, 1385, 1277, 910; ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 5.20 (brs, 1H, H-14), 5.77 (dd, 1H, *J*= 10.5/17.5 Hz, H-15), 4.89 (dd, 1H, *J*= 10.7/1.3 Hz, H-16a), 4.91 (dd, 1H, *J*= 17.5/1.3 Hz, H-16b), 1.02 (s, 3H, H_3-17), 1.18 (s, 3H, H_3-19), 0.84 (s, 3H, H_3-20). ^{13}C-NMR ($CDCl_3$, 50.3 MHz) (Table 1).

trans-communic acid (VI). ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 5.32 (t, 1H, H-12), 6.34 (dd, 1H, *J*= 17/10 Hz, H-14).
Myrceocommunic acid (VII). ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 6.34 (dd, 1H, *J*= 10.5/17.5 Hz, H-14), 4.94-5.25 (m, 4H, H_2-15/16), 4.57 (brs, 1H, H-17a), 4.88 (brs, 1H, H-17b), 1.24 (s, 3H, H_3-18), 0.59 (s, 3H, H_3-20).
cis-communic acid (VIII). ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 6.80 (dd, 2H, *J*= 16.5/10 Hz, H-15), 5.22 (brs, 1H, H-14).

Materials and Methods

UV spectra were obtained with a Shimadzu UV-160 A spectrophotometer. IR spectra were taken with a Perkin Elmer 1720X FTIR spectrometer. ^{1}H and ^{13}C-NMR (DEPT) spectra were measured in $CDCl_3$ with TMS as an internal standard using a Bruker WP 200 SY (200 MHz) spectrometer. TLC was carried out on pre-coated plates (Kieselgel 60 F_{254}, Merck). Spots were detected by UV (254 nm) and spraying with vanillin-sulphuric acid reagent (1 g vanillin, 100 mL, 95-98% sulphuric acid) followed by heating at 105 °C for 1-2 min. Column chromatography was carried out on silica gel (Kieselgel 60, 70-230 Mesh, Merck) and Sephadex LH-20 (Pharmacia). Cytotoxic activity was determinated by using *Artemisia salina* (Brine Shrimp) lethality (McLaughlin *et al.*, 1991).

Result and Discussion

The acidic fraction of the petroleum ether extract of the berries showed stronger cytotoxic activity (LD_{50} 0.16 μg/mL) than the neutral fraction (LD_{50} 13.82 μg/mL) according to the Brine Shrimp bioassay. Colchicine (Merck) was used as test compound for cytotoxic activity (LD_{50} 0.37 μg/mL). According to the results obtained from the cytotoxic activity tests, acidic fraction of the berries was studied and eight compounds (**I-VIII**) were isolated from this fraction.
IR spectrum of compound **I** showed absorbtion bands at 1769 cm^{-1} (γ-lactone) and 1589, 1505 cm^{-1} (aromatic rings). ^{1}H-NMR spectrum exhibited methoxyl signals arising from a 3,4,5-trimethoxy phenyl group at 3.75 ppm (2 x OMe) and 3.80 ppm (OMe) as well as doublets at 5.93 and 5.96 ppm (*J*=1.3 Hz) for methylendioxy group. The *trans*-configuration of the protons at C-8 and C-8' were deduced from the chemical shift of the signal appeared at 2.74 ppm as multiplet. Likewise, the *cis*-configuration of the substituents at C-7 and C-8 were deduced from the broad singlet at 4.61 ppm. DEPT spectrum showed 3 methylene group at 101.1 ppm for C-10', 72.0 ppm for C-9' and 33.0 ppm for C-7'. In addition, it also showed the presence of 3,4,5-trimethoxy phenyl moiety which also supported the ^{1}H-NMR spectroscopic data. This data indicated that **I** is (-)-desoxypodophyllotoxin, a phenyltetralin lignan (Dewick *et al.*, 1981; Ikedo *et al.*, 1998).
IR spectrum of compound **II** showed absorptions for hydroxyl (3384 cm^{-1}), vinylidenic double bound (3080, 1645, 889 cm^{-1}), and carboxyl (1694 cm^{-1}) functions. ^{1}H-NMR spectrum showed singlets at 1.21 and 0.59 ppm corresponding to the methyl groups placed at C-18 and C-20 and a doublet at 0.88 ppm (*J*= 6 Hz) assigned for H-16. Two broad singlets at 4.48 and 4.82 ppm were assignable for vinylidenic protons at C-17 and a triplet at 3.6 ppm was attributed for CH_2OH group. DEPT signals at 19.8, 29.0 and 12.7 ppm were assigned to the C-16, C-18 and C-20 methylene groups. In the ^{13}C-NMR spectrum of **II**, a signal at 183.1 ppm confirmed the axial disposition of the carboxyl group. Additionally, the

signals at 148.2 and 106.4 ppm showed the presence of a vinylic double bond at C-8(17). The ^{13}C-NMR resonance at 60.9 ppm was assigned for hydroxymethyl carbon function. These data showed that compound **II** was imbricatoloic acid (= 15-hydroxy-labd-8(17)-en-19-oic acid) (De Pascal Teresa *et al.*, 1974).

Table 1.^{13}C-NMR spectral data of compounds **II**, **III**, **IV** and **V**($CDCl_3$, 50.3 MHz, δ values).

C no.	II	III	IV	V
1	39.2	39.2	38.7	38.5
2	19.8	19.8	17.9	18.2
3	38.7	38.7	36.9	37.7
4	44.1	44.1	46.0	49.1
5	56.6	56.6	44.9	50.8
6	26.0	26.0	25.1	25.1
7	36.4	36.4	120.9	35.2
8	148.2	148.2	135.6	136.9
9	56.3	56.3	51.7	50.8
10	40.5	40.5	36.7	38.6
11	21.1	21.1	21.4	18.8
12	38.0	38.7	36.0	34.7
13	30.2	140.0	36.8	37.9
14	39.2	122.7	46.0	129.4
15	60.9	59.1	150.2	149.2
16	19.8	16.3	109.2	110.4
17	106.4	106.4	21.4	26.3
18	29.0	29.0	17.0	15.5
19	183.1	183.1	185.6	185.9
20	12.7	12.7	15.23	13.1

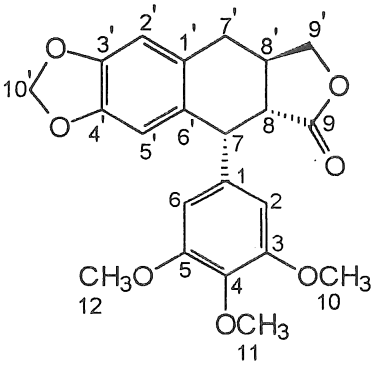
The NMR data of compound **III** showed that **III** had the same basic labdane skeleton with that of **II**. The difference in the ^{1}H-NMR spectrum of **III** displayed as the diagnostic signals for an AX_2 system appeared at 5.38 (1H, t, H-14) and 4.13 ppm (2H, d *J*= 7 Hz, H-15), which was characteristic for an allylic alcohol (= Me-C=CH-CH_2OH) group. The *E*-configuration of the Δ^{13} double bond was revealed from the chemical shifts of the methyl group (1.65 ppm in ^{1}H-NMR and 16.3 ppm in ^{13}C-NMR) at C-13 and the methylene group (38.7 ppm) at C-12 (San Feliciano *et al.*, 1988a). Thus, the structure of compound **III** was assigned as 15-hydroxy-labd-8(17)-13*E*-dien-19-oic acid (=isocupressic acid) (Barrero *et al.*, 1988; Fang *et al.*, 1989).

IR spectrum of compound **IV** showed absorbtion bonds of carboxyl (1694 cm^{-1}), vinyl (3080, 910 cm^{-1}) and double bound (1638 cm^{-1}) functions. The ^{1}H and ^{13}C NMR (Table 1) data of compound **IV** showed of the presence of a pimarene skeleton. In the ^{1}H-NMR spectrum the signals at 0.90, 1.24 and 0.85 ppm were assigned to the quaternary methyl groups at C-17, C-18 and C-20, respectively. An ABX system appeared at 4.86 (1H, dd, *J*= 1.4/17.5 Hz, H-16a), 4.93 (1H, dd, *J*= 1.4/10.7 Hz, H-16b) and 5.81 ppm (1H, dd, *J*=

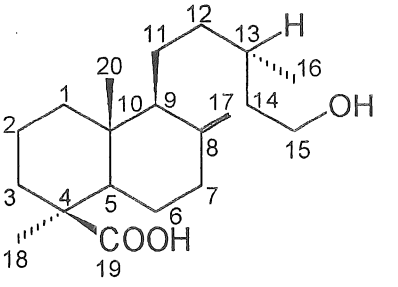
10.7/17.5 Hz, H-15) were attributed to the a vinyl group placed at C-13. In addition a downfield shifted doublet signal at 5.31 ppm (1H, *J*= 4.4 Hz) showed the presence of a double bound at C-7. This assumption was also supported by the ^{13}C-NMR signals at 120.9 (C-7) and 135.6 (C-8) ppm. ^{13}C-NMR resonance at 109.2 (C-16) and 150.2 (C-15) ppm supported the presence of a vinyl group at C-13 (36.8 ppm). Furthermore, the ^{13}C-NMR resonance appeared at 17.0 ppm showed the axial disposition at C-18 (Sakar *et al.*, 1994). The equatorial disposition the vinyl group at C-13 was proved by the carbon resonances at 21.4 (C-17) and 109.2 (C-16) ppm (San Feliciano *et al.*, 1988b). Consequently, the structure of compound IV was identified isopimaric acid.

Compound V showed almost similar ^{1}H and ^{13}C-NMR data with that of IV. However, in the DEPT spectrum of V, the signals at 136.9 and 129.4 ppm, which were readily attributed to C-8 and C-14, suggested the presence of a $\Delta^{8(14)}$ double bound instead of $\Delta^{7(14)}$ unsaturation. Therefore the structure of compound V was assigned to have a pimarene structure. A brief interpretation of the NMR data of compound V showed that V was sandaracopimaric acid (Sakar *et al.*, 1994).

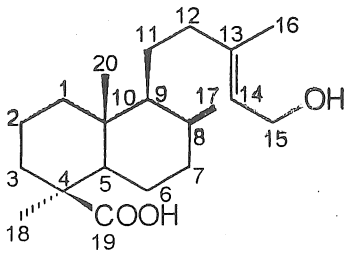
Although, compounds VI, VII and VIII were isolated as a mixture, but the interpretation of ^{1}H and ^{13}C-NMR spectra revealed the brief structure elucidation for each compound and showed that all compounds had a labdane skeleton. Therefore, ^{1}H-NMR signals at 6.80 (dd, *J*= 16.5/10.0 Hz, H-15) and 5.22 ppm (brs, H-14) were assigned for *cis*-communic acid (**VIII**) (Barrero *et al.*, 1987) where the signals at 6.34 (dd, *J*= 1.7/10.0 Hz, H-14) and 5.32 ppm (t, H-12) were attributed to *trans*-communic acid (**VI**) (Sakar, 1987). However, the signals at 6.34 ppm (1H, dd, *J*= 10.5/17.5 Hz, H-14) were assigned for myrceocommunic acid (**VII**) (Barrero *et al.*, 1987). On the other hand, the ^{13}C-NMR signals at 147.1 and 106.5 ppm were attributed to C-8 and C-17 of the exocyclic methylene group for myrceocommunic acid (**VII**), respectively. Likewise, the signals at 107.8 and 109.2 ppm were assigned to C-17 and C-15 of *trans*-communic acid (**VI**), respectively.



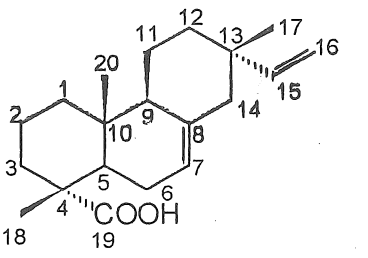
(-)-Desoxypodophyllotoxin (I)



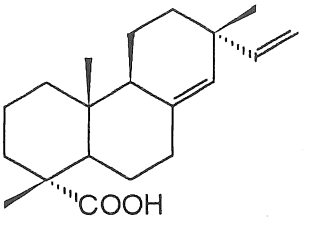
Imbricatolic acid (II)



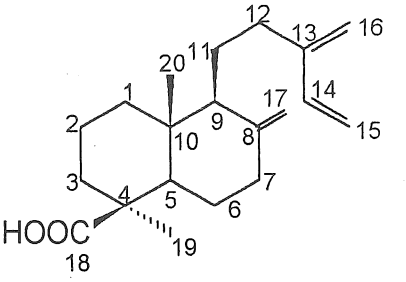
Isocupressic acid (III)



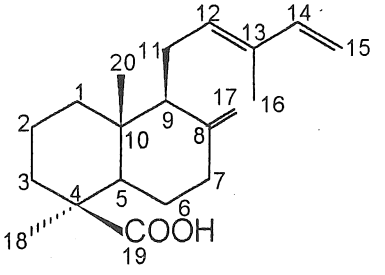
Isopimaric acid (IV)



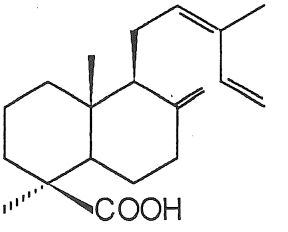
Sandarocopimaric (V)



Myrceocommunic acid (VII)



trans-communic acid (VI)



cis-communic acid (VIII)

Özet

Juniperus nana Willd.'in ham kozalaklarının petrol eterli ekstresinin asidik fraksiyonundan bir lignan, (-)-dezoksipodofilotoksin (I) ve diterpenoitler, imbrikatolik asit (II), izokupresik asit (III), izopimarik asit (IV), sandarakopimarik asit (V) ile bir karışım halinde *trans*-komunik asit (VI), mirseokomunik asit (VII) ve *cis*-komunik asit (VIII) izole edilmiştir. Bileşiklerin yapıları UV, IR, ^{1}H-NMR ve ^{13}C-NMR (DEPT) spektroskopik yöntemler yardımıyla tayin edilmiştir. Asidik fraksiyon "tuzlu su karidesi" yöntemine göre kuvvetli sitotoksik aktivite göstermiştir (LD_{50} 0,16 µg/mL).

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