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RESEARCH ARTICLES

CYTOTOXIC DITERPENOID CONSTITUENTS FROM *ANDROGRAPHIS PANICULATA* NEES. LEAVES

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ABSTRACT

In our search for antineoplastic constituents from Thai medicinal plants, the methanolic extract from the leaves of Andrographis paniculata *Nees. (Acanthaceae), known as "Fha Talai Joan", was found to show significant cytotoxicity against KB and P388 cells. Bioactivity guided chromatographic fractionation has led to the isolation of three diterpenoids. Interestingly, andrographolide, a major constituent, has been shown for the first time to have potent cytotoxic activity against KB cells cultures as well as P388 lymphocytic leukemia, whereas 14-deoxy-11,12-didehydroandrographolide and neoandrographide have shown no cytotoxic activity in the tumor cell lines. In addition, stigmasterol was isolated from the hexane extract of this plant. The structures of the isolated compounds were elucidated by spectroscopic data.*

INTRODUCTION

The leaves of *Andrographis paniculata* Nees. (family Acanthaceae), known as, "Fah Talai Joan" in Thai folklore, are used extensively for the treatment of various diseases, such as the bowels and liver, colic, undiagnosed fever, cholagogues and anthelminthic, etc[1-5]. This plant is well known as the source of diterpenoids, flavonoids, and steroids [2-15]. Several biological properties have been reported to possess anthelminthic, antibacterial, antihepatotoxic, and antitumor activity. Nakanishi et al.[5] reported that a methanolic extract had shown antitumor activity of Yoshida Sarcoma in rats.

As part of our search for antineoplastic constituents from Thai medicinal plants, the methanolic extract from the leaves of *A. paniculata* Nees. were found to show significant cytotoxicity against culture cells of the human epidermoid carcinoma of the nasopharynx (KB) and P388 lymphocytic leukemia. Bioactivity guided chromatographic fraction led to

the isolation of three diterpenoids. Andrographolide, a major constituent has been shown to have potent cytotoxic activity against KB and P388 cell cultures whereas 14-deoxy-11,12-didehydroandrographolide and neoandrographolide have shown no cytotoxic activity in the tumor cell lines. In addition, stigmasterol was also isolated from n-hexane extract of this plant. The cytotoxicity of diterpenoids and one sterol from *A. paniculata* Nees. in KB and P388 test systems are now discussed and the isolation and structure elucidation of these constituents from this plant are reported.

MATERIALS AND METHODS

General experiment procedures

Melting points were determined by a Buchi 512 melting point apparatus and are uncorrected. UV absorption spectra were determined on a double beam spectrophotometer, Hitachi 220A. IR absorption spectra were determined by a Shimadzu 440 instrument using KBr disc. ^{1}HNMR spectra were recorded at 400 MHz with a Bruker WM 400 spectrometer in $CDCl_3$/ $(CD_3)_2SO$ with TMS as internal standard. Mass spectra were obtained on Jeol DX-300/JMA 2000 operating at 70 eV. Plates for thin layer chromatography (TLC) were prepared from silica gel 60 GF_{254} (Merck) and were activated by drying at 100°C for 2 hours. Silica gel 70-230 mesh (Merck) was used for column chromatography. Acetate derivatives were prepared by the standard procedure (acetic anhydride/ pyridine at room temperature). Visualization was obtained under UV light and spraying with Kedde reagent which was prepared according to the method of Stahl.

Plant Materials

The leaves of *A. paniculata* Nees. were collected from Chantaburi Province, Thailand in 1988. A voucher specimen of this species was deposited at the Herbarium of the Natural Products Research Section, Research Division, National Cancer Institute, Thailand.

Extraction and Isolation

The dried coarsely powder leaves (1kg) of *A. paniculata* Nees. were extracted in a Soxhlet apparatus with n-hexane. Concentration of the extract under reduced pressure gave a brown viscous residue (36.2 gm). The defatted marc was further exhaustively perculated with methanol. The combined methanol extracts were concentrated *in vacuo*, and then treated with charcoal (3x70 gm); on cooling in refrigerator for 24 hours, a pale green precipitate (25.3 gm) formed, which was filtered off. Concentration of the filtrate under reduce pressure, afforded a brownish residue (147 gm), this showed significant cytotoxic activity against KB and P388 tumor cells *in vitro*.

Isolation and purification of diterpenoid

A portion of the residue (77 gm) chromatographed on a column of silica gel (200 gm), eluted with chloroform and ethanol in order of increasing polarity. Fractions of 50 ml were collected and then combined (t.l.c.) to six fractions (A-F). Fractions (B-E) which showed significant cytotoxic activity against KB and P388 tumor cells *in vitro*, were further purified by repeated column chromatography. Fraction B was rechromatographed on silica gel; elution

with ether two compounds were isolated. The earlier fractions on recrystallization from ether afforded compound 1 (2.32 gm) as colourless needles and the later fractions gave, after recrystallization from methanol, colourless needles of an unidentified diterpenoid (0.15 gm). Fraction c was rechromatographed on a column of silica gel, eluting with chloroform:ethanol (14:1); recrystallization from methanol gave compound 2 (17.5 gm) as colourless plates. Fraction D was rechromatographed on silica gel, eluting with chloroform:ethanol (9:1); recrystallization from methanol afforded compound 2 (2.06 gm) and compound 3 (1.25 gm) as colourless needles. Fraction E and the unidentified diterpenoid are being investigated.

Compound 1: 14-deoxy-11,12-didehydroandrographolide; colourless needles from ether, m.p. 190°-191° (lit. 203°-204°[10]); $C_{20}H_{28}O_4$; M.W. 332; UV λ_{max}^{MeOH} nm: 248 IR υ_{max}^{KBr} cm^{-1}: 3450, 3000, 2950, 1742, 1645, 1460, 1100, 1060, and 900; ^{1}HNMR δ: 7.17 (H, s , 14-H), 6.87 (H, m, 11-H), 6.11 (H, dd, 12-H), 4.82 (H, s, 15-H), 4.7 and 4.53 (2H, s, 17-H), 3.35 and 3.48 (2H, dd, 19-H), 2.81 and 2.57 (2H, brd, OH), 1.26 and 0.81 (6H, s, 2xCH3); MS m/z (% rel. int.): 332 (M^+, 24),176(34), 131(41), 121(100), 119(70), 107(46), 105(52), 93(50), 91(60), 79(40), 77(30), 69(29), 55(40).

Compound 2: andrographolide; colourless plates from MeOH, m.p. 228°-230° (lit. 228°[6], 227.5°[7], 230°-231°[10]); $C_{20}H_{30}O_5$; M.W. 350, UV λ_{max}^{MeOH} nm: 241; IR υ_{max}^{KBr} cm^{-1}: 3400-3000, 2910, 2850, 1725, 1644 and 900; ^{1}HMNR δ: 6.8 (1H, t, 12-H), 4.94 (1H, brt, 14-H), 4.86 and 4.64 (each 1H, s, 17-H), 4.15 and 4.41 (each 1H, dd, 15-H), 3.48 (1H, m, 3β-H), 3.25 (1H, t, 19-H), 0.70 and 1.12 (each 3H, s, 2Me); MS m/z (% rel. int.): 350 (M^+, 17), 332(80), 314(68), 302(98), 285(70), 274(99), 257(64), 213(6), 199(8), 187(25), 181(11), 173(23), 159(27), 147(38), 133(38), 117(88), 119(100), 105(72), 91(84), 79(76), 67(53), 55(88)[10,11,18].

Compound 3: neoandrographolide: colourless needles from MeOH; m.p. 169°-170° (lit. 167°-168°[11] ,159°-160°[18]), $C_{26}H_{40}O_8$, UV λ_{max}^{KBr} nm: 240 nm IR υ_{max}^{KBr} cm^{-1}: 3400, 2940, 1744, 1675, 1600, 1381, 1200, and 900; ^{1}HNMR δ: 0.85 and 1.2 (each 3H, s, 2Me), 3.17-4.1 (H, m, 19-H), 7.12 (1H, m, 14-H), 4.9 and 4.7 (each 1H, 2xs, 17-H); MS m/z (% rel. int.): 287(100), 273(26), 231(11), 217(32), 205(75), 191(64), 175(27), 153(50), 121(54), 119(34), 109(59), 95(60), 93(51), 79(38), 69(53), 55(59) [11,12,18].

Acetylation of compound 3: Compound 3 (50 mg) was acetylated with acetic anhydride (1ml) and pyridine (1.5 ml.). The reaction was stirred overnight at room temperature. Water (50 ml) was added, resulting in precipitation of acetyl derivative. The mixture were extracted with chloroform and evaporated to dryness *in vacuo*. Recrystallization from methanol afforded neoandrographolide tetraacetate (compound 4, 0.035 gm) as colourless needles, m.p. 155°-157° (lit. 156°-157°)[10]. ^{1}HNMR δ: 0.66 and 0.92 (each 3H, s, 2Me), 2.00, 2.02, 2.07 (3H, 6H, and 3H, respectively, 4xoAC), 3.17 and 3.96 (2H, d, AB system, J=9.5 Hz, 2x19-H), 4.7 and 4.8 (each H, 2xs, 17-H) [10,11,18].

Isolation and purification of sterol

Crude n-hexane extract (33 gm) was chromatographed on a column of silica gel ; the fraction eluted with n-hexane:chloroform (1:1) was recrystallized from n-hexane to afford

stigmasterol (compound 5, 0.32 gm) as colourless needles, m.p. 135°-136°; the compound gave a deep green colour with the Liebermann-Burchard test for sterol and had spectra identical with those of stigmasterol. UV λ_{max}^{MeOH} nm: 244; IR υ_{max}^{KBr} cm^{-1} : 3400(OH) , 3000, 2900, 1630(C=O), 1480, 1375, 1060, 1010, 960 and 780 (trans C=O). ^{1}HNMR δ: 0.7, 0.8, 0.9 and 1.2 (each 3H, s, CH3), 3.52 (1H, brm, 3α-OH), 5.02(1H, dd, 23-H(=CH-CH), 5.15(1H, dd, 22-H(-CH-CH=)), MS m/z (% rel. int.): 412 (M^+, 65), 397(14), 394(100, M^+-H_2O), 382(21), 300(28), 273(18), 271(18), 255(M^+-H_2O-$C_{10}H_9$), 253(17), 213(25), 159(41), 147(37), 145(46), 107(46), 83(70), 81(70), 55(77).

Bioassay of cytotoxic activity against KB and P388 tumor cell cultures.

Cell cultures:

KB cells, a stable monolayer cell line from a human epidermoid carcinoma, were maintained on Eagle's minimum essential medium (EMEM, GIBCO Laboratory, U.S.A.) supplement with 10% fetal calf serum (GIBCO). For P388 cells, a stable suspension cell line from a mouse lymphoid leukemia were cultivated in RPMI-1640 medium (GIBCO) supplemented with 10% fetal calf serum (GIBCO).

Cytotoxicity Assay:

KB cell culture:

KB stock cells were diluted with complete medium to an approximate count of $4x10^4$ cells/ml. On day 0, three ml of these cells were added simultaneously with 1 ml of each concentration of the test solution into culture flasks. The treated cells were incubated at 37°C in 5% CO_2 for 4 days. The amount of protein in cultured cells was analysed according to the modified method of Oyama and Eagle[20]. The results were expressed as the mean effect dose of the drug which inhibits growth to 50% of control growth. The ED_{50} values were estimated from a semi-log plot of the drug concentrations (μg/ml) vs. the percent of viable cells.

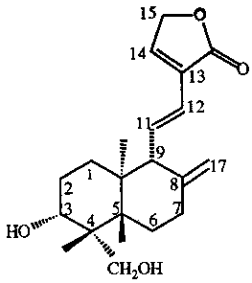
P388 cell culture:

On day 0, the cells were diluted to approximately 6x104 cells/ml in complete medium. The test solution was added to cell suspensions at various concentrations from 100 μg/ml to 20 μg/ml. The treated cells were incubated at 37°C in 5% CO_2 for 3 days. On day 3, viable cells were counted by means of a haemacytometer according to the method of Tryplan blue dye exclusion stainning. The results were evaluated by the ED_{50} values.

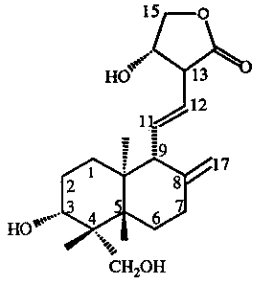
Tests were done in duplicate. 0.5% DMSO in NSS was used for the negative control. For the positive control, 5-fluorouracil (ABIC, Israel) at 1 and 0.05 μg/ml concentration was applied in the treatment of KB and P388 tumor cell cultures, respectively.

RESULTS AND DISCUSSION

The methanolic extract from the leaves of *A. paniculata* Nees. showed significant cytotoxic activity against the growth of the human epidermoid carcinoma of the nasopharynx (KB) as well as the P388 lymphocytic leukemia *in vitro* as shown in Table 1. Silica gel



(1)



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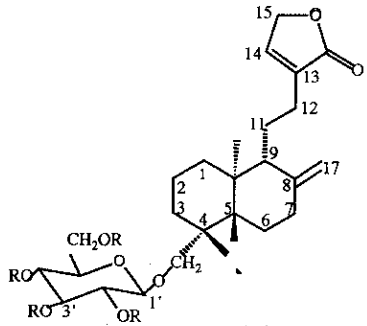
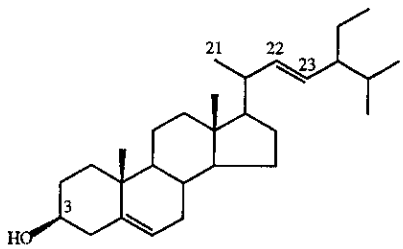


(3) R=H
(4) R=Ac



(5)

column chromatography of the active fractions (B-E) was guided by bioassay and led to isolate one unidentified minor diterpenoid and three known diterpenoids, namely, 14-deoxy-11,12-didehydroandrographolide (1), andrographolide (2), and neoandrographolide (3). In addition, stigmasterol (5) was also isolated from the n-hexane extract of this plant.

Compound 1 was isolated as colourless needles. The molecular formula was determined as $C_{20}H_{28}O_4$ by MS. The UV spectrum showed λ_{max}^{MeOH} 248 nm., suggesting α, β-unsaturated lactone ring. The IR spectrum revealed the presence of hydroxy group, carbonyl group and exocyclic methylene group at 3450, 1742 and 900 cm^{-1}, respectively. The NMR spectrum showed signals of the olefinic β-proton (H-14) at δ 7.17; a trans-disubstituted double bond was indicated at δ 6.87. Two broad resonances at δ 2.81 and 2.57 revealed one primary and one secondary hydroxy group. The signals at δ 1.26 and 0.81 showed the presence of two tertiary methyl groups. The mass spectrum revealed the molecular ion and base peak m/z at 332 and 121, respectively.

The comparision of the spectral data (UV, IR, NMR and MS) suggested that compound 1 was 14-deoxy-11,12-didehydroandrographolide.[10]

Compound 2 was isolated as colourless plates, having the molecular formula as $C_{20}H_{30}O_5$. The UV spectrum showed λ_{max}^{MeOH} 241 nm, indicating the α, β-unsaturated lactone system. The IR spectrum showed the hydroxy group at 3400-3000 cm^{-1}, carbonyl group at 1725 cm^{-1} and exocyclic methylene group at 900 cm^{-1}. The ^{1}HMNR spectrum showed a triplet signal centred around δ 6.8, which was typical of an olefinic proton in an

α, β-unsaturated lactone system (C-12H). The C-14H resonated as a broad triplet centred at δ 4.94. Signals from the two C-15H protons appeared as doublets at δ 4.15 and 4.41. The 3β-H attached to the OH group resonated as a triplet at δ 3.48. The two tertiary methyl groups showed signals at δ 0.72 and 1.22. The mass spectrum showed the molecular ion and base peak at m/z 350 and 119, respectively.

On the basis of these spectral data, compound 2 was identified as andrographolide.

Compound 3 was isolated as colourless needles, m.p. 169°-170°. The molecular formula was determined as $C_{26}H_{40}O_8$ by the mass spectrum. The UV spectrum showed λ_{max}^{MeOH} 240 nm, suggesting an α,β-unsaturated lactone ring. The IR spectrum revealed the presence of OH groups (3400 cm^{-1}), a carbonyl group (1744 cm^{-1}), and an exocyclic methylene group (909 cm^{-1}). The ^{1}HNMR spectrum showed signals of the olefinic β-proton of the α,β-unsaturated lactone system resonating at δ 7.12. The C-17H resonated as two singlets at δ 4.9 and 4.7. Two tertiary methyl groups showed signals at δ 0.85 and 1.2. The mass spectrum showed the base peak at m/z 287.

Acetylation of compound 3 with acetic anhydride and pyridine afforded the tetraacetate ($C_{34}H_{48}O_{12}$, compound 4), m.p. 155°-157°. The ^{1}HNMR spectrum showed the presence of four acetates at δ 2.00(3H), 2.02(6H) and 2.07(3H), and two tertiary methyl groups (δ 0.67, 0.92). A narrow multiplet at δ 7.12 assigned to the olefinic β-proton of the α,β-unsaturated lactone system. The small coupling constant of this proton suggested that the double bond was endocyclic.

By the comparision of these spectral data of compound 3 and its acetate derivative, the compound 3 was identified as neoandrographolide[9, 10, 11, 19]

TABLE 1. Cytotoxic activity against KB and P388 tumor cell lines of *A. paniculata* Nees.

Compounds	ED_{50} (μg/ml)	
	KB	**P388**
crude MeOH extract	5.3	3.1
andrographolide (2)	1.5	1.0
14-deoxy-11,12-didehydro-andrographolide (1)	20	9.2
neoandrographolide (3)	>25	>40
tetraacetate neoandrographolide (4)	>25	>40
stigmasterol (5)	>25	>25

For significant activity of the crude extract and pure compounds, ED_{50} <30 μg/ml and <4 μg/ml respectively are required[21].

The cytotoxic activity of the isolated compounds against the growth of the human epidermoid carcinoma of nasopharynx (KB) and P388 lymphocytic leukemia cells *in vitro* are summarized in Table 1. The results demonstrate that andrographolide (2), a major diterpenoid , showed significant cytotoxic activity against KB and P388 tumor cells *in vitro* at ED_{50} values 1.5 and 1.0 μg/ml, respectively whereas 14-deoxy-11,12-didehydro-andrographolide (1), neoandrographolide (3), tetraacetate neoandrographolide (4) and stigmasterol (5) did not show cytotoxic activity against the same bioassays (ED50 >4 μg/ml).

From these preliminary results, andrographolide as a major active constituent has shown ED_{50} values superior to those recommended by the protocol of National Cancer Institute of U.S.A.[21]; no previous study has reported this compound as being active in these bioassays. Thus, more assays with this product should be performed in order to confirm the *in vivo* antitumor activity. Furthermore, the isolation and identification of the other diterpenoid constituents in the active fraction are under investigation. Also, although 14-deoxy-11,12-didehydroandrographolide (1), neoandrographolide (3), tetraacetate neoandrographolide (4) and stigmasterol (5) did not show cytotoxic activity against KB and P388 tumor cell cultures in this study, the compounds should be tested for antineoplastic activity in other bioassay models.

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