Chemical and Biological Investigation of *Araucaria heterophylla* Salisb. Resin

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Three labdane diterpenes, namely labda-8(17),14-diene, 13-epicupressic acid, and 13-*O*-acetyl-13-epicupressic acid, were isolated from the resin collected from stem exudates of *Araucaria heterophylla* Salisb. (Araucariaceae). The isolated compounds were identified using different spectroscopic methods (^{1}H NMR, ^{13}C NMR, HMQC, HMBC and COSY). The resin extract showed antiulcerogenic activity against ethanol-induced stomach ulcers in Sprauge Dawely rats using ranitidine as standard. In addition, the resin and the isolated compounds showed variable cytotoxic activities against breast (MCF7) and colon (HCT116) cancer cell lines.

Key words: Araucaria heterophylla, Labdane Diterpenes, Antiulcerogenic and Cytotoxic Activities

Introduction

Araucariaceae (Coniferae) is a small family; it comprises two genera, *Araucaria* and *Agathis*, and 38 species of trees (Trease and Evans, 1978). *Araucaria heterophylla* Salisb. Franco (syn. *A. excelsa*) is a popular columnar tree used as Christmas tree (Schans *et al.*, 2004). Diterpenes (Caputo and Mangoni, 1974; Caputo *et al.*, 1974a, b; Cox *et al.*, 2007) and lignans (Ohashi *et al.*, 1992; Fonseca *et al.*, 1978, 1979) were isolated from resin exudates of different *Araucaria* species including *A. heterophylla* Salisb. (Caputo *et al.*, 1972). The resins of this genus were reported to possess cytotoxic and gastroprotective activities (Lee and Cheng, 2001; Schmeda-Hirschmann *et al.*, 2005a, b). In the present study, the resin exudates from stems of *A. heterophylla* Salisb. growing in Egypt as ornamental plant were subjected to chemical and biological investigations.

Material and Methods

Plant material

The resin exudates from the stems of *A. heterophylla* Salisb. were collected from El-Muntaza Palace Garden, Alexandria, Egypt in August 2007. The plant was identified by Mrs. T. Labib, taxonomist of Orman Botanical Garden, Giza, Egypt. A specimen was deposited in the herbarium of Orman Botanical Garden, Giza, Egypt.

General

^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), and 2D NMR spectra were measured on a JHA-LAA 400 WB-FT Jeol spectrophotometer. TLC was performed on precoated silica gel plates using the solvent system *n*-hexane/EtOAc (4:1); the chromatograms were visualized by spraying with anisaldehyde/sulfuric acid spray reagent followed by heating at 110 ºC for 5 min.

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Extraction and fractionation

The resin of *A. heterophylla* Salisb. (20 g) was extracted with $CHCl_3$, then with $CHCl_3$/MeOH (1:1, 3 × 100 ml for each solvent). The combined extracts were evaporated under reduced pressure (≤ 60 °C) to give 15 g of a sticky yellow residue.

The residue (10 g) was chromatographed on a silica gel G 60 column (7 × 20 cm, VLC) eluted with *n*-hexane, *n*-hexane/$CHCl_3$ and $CHCl_3$/MeOH mixtures. Fractions of 200 ml each were collected and monitored by TLC. Similar fractions were pooled together. Fractions 8–10 (2.8 g), eluted with *n*-hexane/$CHCl_3$ 3:7 to 1:9, showed three major spots. The combined fractions were rechromatographed on successive silica gel columns using *n*-hexane/ethyl acetate mixtures to afford compounds **1** (12 mg), **2** (20 mg), and **3** (50 mg). The three compounds were isolated as yellow resins.

Antiulcerogenic effect

Sprauge Dawely rats (150–200 g body wt.) were kept under standard hygienic conditions before use. They were fed and watered *ad libitum*. Rats were randomly divided into 4 equal groups of 5 rats each. Animals were starved for 48 h before use to ensure an empty stomach and were kept in cages with raised floors of wide wire mesh to prevent corporophagy (Garg *et al.*, 1993). To prevent excessive dehydration during the fasting period, rats were supplied with 0.2% sucrose (BHD) which was removed 1 h before the experiment (Glavin and Mikhaeil, 1976). The control group was given an equal volume of distilled water instead of the resin extract, but received ethanol in the same dose and route (orally) as the other groups. In addition, the second group was given ranitidine as a standard in a dose of 100 mg/kg body wt. orally by a stomach tube. The third and fourth groups were orally given the resin extract in doses of 100 and 200 mg/kg body wt., respectively. On the first day, rats of the third and fourth groups were orally given two doses of the resin extract with 6 h apart; a third dose was given on the second day 1.5 h before oral administration of ethanol (Merck), 50% (v/v in distilled water), in a dose of 10 ml/kg. 1 h after ethanol administration, all rats were euthanized by an overdose of chloroform, their abdomens were opened and their stomachs rapidly removed, opened along their greater curvature and gently raised under running water. Lesions in the glandular part of the stomach were measured under an illuminated magnifying microscope (10×). Long lesions were counted and measured along their greater length. Petechial lesions were taken as 1 mm ulcer (Cho and Ogle, 1978). The sum of the total length of long ulcers and petechial lesions in each group of rats was divided by its number to calculate the ulcer index (in mm). The curative ratio was determined by the following formula:

curative ratio = (control ulcer index – test ulcer index)/(control ulcer index) · 100.

Statistical analysis was carried out by using one-way ANOVA followed by Dubcan test and SPSS Version 14.0. The difference of means at $P < 0.05$ is considered significant (Snedecor and Cochran, 1969).

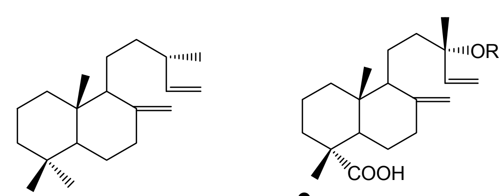
Cytotoxic activity

The resin extract of *A. heterophylla* Salisb. as well as the isolated compounds were tested *in vitro* for their cytotoxic activity using the sulphorhodamine B assay (Skehan *et al.*, 1990) against colon (HCT116) and breast (MCF7) human cancer cell lines. The results are shown in Table III.

Results and Discussion

Three labdane diterpenes, **1**–**3** (Fig. 1), were isolated from the resin of *A. heterophylla* Salisb. by chromatographic fractionation on series of silica gel columns. ^{1}H NMR and ^{13}C NMR spectra showed a labdane diterpene skeleton with two double bonds at C-8(17) and C-14 (Wang *et al.*, 2008).

The ^{1}H NMR spectrum of **1** (Table I) showed three methyl singlets at δ_H 0.64 (CH_3-18), 1.22 (CH_3-19), and 1.23 (CH_3-20). A methyl doublet



1

2 **R= COCH$_3$**

3 **R= H**

Fig. 1. Chemical structures of the compounds isolated from *A. heterophylla*.

Table I. [1]H NMR spectral data of compounds **1**–**3** (δ_H in ppm).

H	1	2	3
14	5.85 (1H, *m*)	5.88 (1H, *dd, J* = 17.2, 10.8 Hz)	5.81 (1H, *dd, J* = 17.2, 10.8 Hz)
15α	5.10 (1H, *d*, *J* = 14 Hz)	5.02 (1H, *d*, *J* = 10.8 Hz)	4.98 (1H, *d*, *J* = 10.8 Hz)
β	4.93 (1H, *d*, *J* = 14 Hz)	5.03 (1H, *d*, *J* = 10.8 Hz)	5.13 (1H, *d*, *J* = 10.8 Hz)
16	0.82 (3H, *d*, *J* = 8 Hz)	1.45 (3H, *s*)	1.22 (3H, *s*)
17α	4.55 (1H, *s*)	4.43 (1H, br *s*)	4.48 (1H, *s*)
β	4.83 (1H, *s*)	4.76 (1H, br *s*)	4.79 (1H, *s*)
18	0.64 (3H, *s*)	0.52 (3H, *s*)	0.54 (3H, *s*)
19	1.22 (3H, *s*)	1.16 (3H, *s*)	1.18 (3H, *s*)
20	1.23 (3H, *s*)	–	–
$COC\underline{H}_3$	–	1.94 (3H, *s*)	–

at δ_H 0.82 (3H, J = 8 Hz) was assigned to CH_3-16. Two doublets at δ_H 4.93 and 5.10 (each 1H, J = 14 Hz) were assigned to the two olefinic protons at C-15, while a multiplet at δ_H 5.85 (1H, *m*) was assigned to the olefinic proton at C-14. Inspection of the ^{13}C NMR spectrum of **1** (Table II) indicated the assignment of signals at δ_C 111.9 and 146.6 to C-15 and C-14, respectively. The two exomethylene protons (H-17) appeared at δ_H 4.55 (Hα) and 4.83 (Hβ), corresponding to a carbon signal at δ_C 107.4 (C-17), while the signal at 150.0 was assigned to C-8. Compound **1** was identified as labda-8(17)14-diene by comparison of its ^{1}H and ^{13}C NMR spectral data with those of closely related labdane diterpenes (Su *et al.*, 1994; Wang *et al.*, 2008). This compound was only identified in the essential oil of *Cistus monspeliensis* by GC-MS (Angelopoulou *et al.*, 2002) and for the first time in the family Araucariaceae.

The ^{1}H NMR spectrum of **3** showed three methyl singlets at δ_H 1.22, 0.54 and 1.18 assigned to C-16, C-18 and C-19, respectively. The two singlets resonating at δ_H 4.48 and 4.79 (each 1H) were assigned to α- and β-exomethylene protons at C-17 and confirmed by HMQC relation to the olefinic carbon signal at δ_C 106.6 (C-17) and the presence of a quaternary carbon signal at δ_C 147.8 (C-8). The two doublets at δ_H 4.98 and 5.13 (each 1H, *d*, J = 10.8 Hz) were assigned to the two olefinic protons at C-15, while the doublet of doublet at δ_H 5.81 (1H, J = 17.2 and 10.8 Hz) was assigned to the olefinic proton at C-14. This was further confirmed by the correlation to olefinic carbon signals at δ_C 111.6 (C-15) and 144.9 (C-14) in the HMQC spectrum. The presence of a tertiary OH group at C-13 was indicated by the quaternary signal at δ_C 73.7, which showed a long-range correlation to the downfield shifted proton of CH_3-16 at δ_H 1.22. A quaternary carbon signal at δ_C 183.3 was assigned to COOH (C-19) at C-4. Complete assignments of proton and carbon signals were confirmed by the analysis of ^{1}H-^{1}H COSY, HMQC and HMQC spectra. From the previous data and by comparison with reported spectral data (Caputo *et al.*, 1974b; Su *et al.*, 1994; Wang *et al.*, 2008), compound **3** was identified as 13-epicupressic acid. This is the first report on the isolation of 13-epicupressic acid from *A. heterophylla* Salisb.

Spectral data of **2** were heavily similar to those of **3**, except for the presence of an additional downfield shifted methyl signal at δ_H 1.94 (3H, *s*, $OCOC\underline{H}_3$) and a quaternary carbon signal at

Table II. ^{13}C NMR spectral data of compounds **1**–**3** (δ_C in ppm).

C	1	2	3
1	39.0	38.9	39.0
2	18.8	19.8	19.8
3	42.5	37.8	37.9
4	32.9	44.1	44.1
5	54.8	56.3	56.3
6	23.4	26.0	25.9
7	37.6	38.6	38.7
8	150.0	147.8	147.8
9	57.1	56.5	56.5
10	40.8	39.2	40.6
11	20.9	17.5	17.8
12	30.0	40.6	41.3
13	39.8	83.3	73.7
14	146.6	141.8	144.9
15	111.9	113.0	111.6
16	19.2	23.4	28.9
17	107.4	106.4	106.6
18	28.4	28.9	27.4
19	27.4	184.0	183.3
20	13.4	12.7	12.9
$CO\underline{C}H_3$	–	21.0	–
$\underline{C}OCH_3$	–	171.1	–

δ_C 171.1, both indicating the acylation of the OH group at C-13 with an acetyl moiety. Acylation of OH at C-13 was further confirmed by the downfield shift of C-13 (δ_C 83.3) relative to the same carbon atom in **3** (δ_C 73.7). The HMBC spectrum showed a long correlation between the signal at δ_C 83.3 (C-13) and proton signals at δ_H 5.88 (H-14) and 1.45 (H-16), which confirmed the presence of an acetyl group at C-13. Thus compound **2** was identified as 13-*O*-acetyl-13-epicupressic acid, a new natural product.

The resin extract of *A. heterophylla* Salisb. showed dose-dependent antiulcerogenic activity against ethanol-induced stomach ulcers in Sprauge Dawely rats. A curative ratio of 55.87 compared to 61.64 for ranitidine at the same dose (100 mg/kg body wt.) was recorded, the curative ratio of the resin extract raised to 63.26 at a dose of 200 mg/kg body wt. The activity of the resin extract as a gastroprotective may be attributed to its diterpene constituents. In this concern several terpenes or their derivatives have been shown to possess gastroprotective activity in different models of induced gastric lesions in animals (Wada *et al.*, 1985; Giorando *et al.*, 1990; Lewis and Hanson, 1991; Farina *et al.*, 1998; Mastuda *et al.*, 1998). The gastroprotective activity of the diterpenes and their derivatives have been explained by mechanisms that include stimulation of prostaglandin synthesis, increase of mucus production, and suppression of gastric acid secretion (Hiruma-Lima *et al.*, 1999, 2002).

Table III. Results of the *in vitro* cytotoxicity test of the resin extract and isolated compounds of *A. heterophylla* Salisb.

Compound	IC_{50} [μg/ml][a]	
	MCF7[b]	HCT116[b]
Doxorubicin®	0.7	0.69
Resin extract	0.54	0.94
1	3.88	4.59
2	2.33	8.04
3	9.77	–

[a] IC_{50}, the concentration that causes 50% death of the cancer cells.

[b] MCF7, breast cancer cell line; HCT116, colon cancer cell line; –, no effect.

The resin extract of *A. heterophylla* Salisb. exhibited also a strong cytotoxic activity against breast (MCF7) and colon (HCT116) cancer cell lines; the recorded IC_{50} values were 0.54 and 0.94 μg/ml, respectively. The isolated compounds **1**–**3** were subjected to an *in vitro* cytotoxicity test. Only compounds **1** and **2** showed moderate cytotoxic activity against both tested cell lines with IC_{50} values lower than that of the resin extract (IC_{50} 2.33–8.04 μg/ml), see Table III. Compound **3** showed only weak activity against the breast (MCF7) cancer cell line (IC_{50} 9.77 μg/ml). It is worth to mention that the *in vitro* cytotoxic effect of the resin extract was comparable to that of the reference drug Doxorubicin®. This effect may be a synergism of the effect of the diterpene content of the resin.

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