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Isolation and identification of cytotoxic compounds from Bay leaf (*Laurus nobilis*)

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Abstract

Bay leaf belongs to the family Lauraceae, and it is one of the most popular culinary spices in all Western countries. Bay leaf has been used as herbal medicine and has pharmacological activity which includes anti-bacterial, anti-fungal, anti-diabetes and anti-inflammatory effects. The goal of this study was to identify compounds from Bay leaf (*Laurus nobilis*), which are responsible for inducing apoptosis using bioassay-directed isolation. The isolation of active compounds was carried out in three steps: multiple extractions, fractionation using column chromatography and purification using semi-preparative HPLC. The structure of separated compounds was determined on the basis of ^{1}H, ^{13}C nuclear magnetic resonance data, atmospheric pressure chemical ionization mass spectrometry data, and electron ionization mass spectrometry. Six compounds were identified; all of them are sesquiterpene lactones.

Keywords: Bay leaf; *Laurus nobilis*; HPLC; Mass spectrometry; Sesquiterpene lactones

1. Introduction

Human epidemiology and animal studies have indicated that cancer risk may be modified by dietary components. The naturally occurring, non-nutritive chemical components of fruits, vegetables, grains, nuts, tea and seeds, which are commonly referred as phytochemicals or active compounds, may prevent or reduce the risk of cancer.

Bay leaf (*Laurus nobilis*) belongs to the family Lauraceae, and is one of the most widely used culinary spices in all Western countries. Bay leaf traditionally has been used as herbal medicine to treat rheumatism, earaches, indigestion, sprains, and to promote perspiration (Heinerman, 1983). Recent research revealed that it can be used in treating diabetes and preventing migraine (Duke, 1997).

Sesquiterpene lactones identified in Bay leaf were found to have different pharmacological properties including inhibitory effects on NO production (anti-inflammatory) (Matsuda et al., 2000), inhibitory effects on alcohol absorption (Yoshikawa et al., 2000), and enhancement of liver glutathione S-transferase (GST) activity (Wada, Ueda, Sawada, Amemiya, & Haga, 1997). In this study, compounds from Bay leaf which are cytotoxic and induce apoptosis were identified from Bay leaf using a bioassay directed isolation. The isolation of active compounds was carried out in three steps: multiple extractions, fractionation using column chromatography and purification using semi-preparative HPLC. The structure of the separated compounds was determined on the basis of ^{1}H, ^{13}C nuclear magnetic

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resonance (NMR) data, atmospheric pressure chemical ionization mass spectrometry (ApCI/MS) data, and electron ionization mass spectrometry (EI/MS) data.

2. Materials and methods

2.1. Plant material

Dried Bay leaf (origin Turkey) used in this study was donated by the A.M. Todd Company (Montgomeryville, PA).

2.2. General procedures

NMR analysis were carried out on a Varian AM-600 NMR spectrometer (Sugar Land, TX) with TMS as internal standard, ^{1}H (600 MHz), and ^{13}C (150 MHz) spectra were obtained. The EI-MS analysis was performed on a Varian 3400 GC coupled to a Finnigan MAT 8230 high-resolution mass spectrometer (San Jose, CA); A Finnigan MAT SS300 data system was used to process the data. LC/MS was performed on a Varian 9012 HPLC pump coupled to a Micromass Platform II mass analyzer (Micromass Co., MA); Mass Lynx NT v.3.0 was used as the data system, and mass spectra were obtained using atmospheric pressure chemical ionization (APCI) in the positive ion mode. The source temperature was set at 150 °C and the probe temperature was set at 450 °C, the sample cone voltage was 15 V, the corona discharge was 3.0 kV. Analytical HPLC analysis was performed on a Varian 5500 Liquid Chromatograph pump coupled to a Varian 9065 Polychrome diode array detector (Sugar Land, TX). Semi-preparative purification was performed on a Varian 9012 HPLC pump coupled to an ABI 783A Programmable Absorbance UV detector (Ramsey, NJ). Selecto Scientific silica gel (100–200 and 200–425 mesh) was used for column chromatography. All fractions were screened on Whatman silica gel thin-layer chromatography (TLC) plates (AL SIL G/UV, 250 μm thickness) with compounds visualized by spraying with 5% (v/v) H_2SO_4 in ethanol solution. The column packing and TLC plates were purchased from Fisher Scientific (Springfield, NJ). All solvents used for extraction and isolation was of HPLC grade and purchased form Fisher Scientific.

2.3. Extraction of Bay leaf

The powdered Bay leaf (500 g) was extracted with methanol (4×1000 ml) and the methanol was concentrated under vacuum using a rotary evaporator. The residue from the methanol extract was then first extracted with hexane, followed by ethyl acetate and thirdly with *n*-butanol. The dried residues of the three extracts were subjected to bioassay. The initial results showed that the crude hexane and ethyl acetate fractions were positive in the bioassays. The level of cytotoxicity was tested on a WI38VA cell line by the MTT-assay, with a LD50 of 10 μg/ml.

2.4. Column chromatography of leaf extracts

The scheme for the column chromatography of Bay leaf extract is shown in Fig. 1. The dried hexane extract was chromatographed on a silica gel column (200–425 mesh). Elution was performed using a solvent mixture of hexane/chloroform with increasing amount of chloroform (8:2, 7:3, 6:4). Successive fractions were collected and dried under vacuum using a rotary evaporator. The fraction with hexane/chloroform 6:4 was tested for its biological activity. The result was positive.

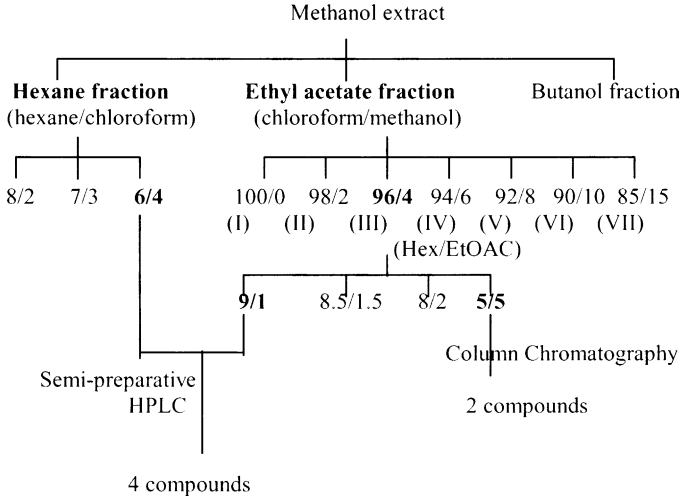

Fig. 1. Separation scheme (the active fractions are bolded).

The dried ethyl acetate extract was chromatographed on a silica gel column (100–200 μm particle size); elution was carried out using a mixture of chloroform/methanol with increasing amount of methanol [100:0 (I), 98:2 (II), 96:4 (III), 94:6 (IV), 92:8 (V), 90:10 (VI), 85:15 (VII)]. Successive fractions were collected and dried under vacuum using rotary evaporator. Bioassays were carried out on all seven fractions, and fraction III eluted with chloroform/methanol 96:4, was determined to have the strongest biological activity, having an LD50 of 10 μg/ml. The LD50 of the rest of the fractions was greater than 50 μg/ml.

The biological active fraction that had been extracted with ethyl acetate and chromatographed with chloroform/methanol 96:4, was further chromatographed using a mixture of hexane/ethyl acetate with increasing amount of ethyl acetate (9:1, 8.5:1.5, 8:2, 5:5). The fractions 9:1 and 5:5 were determined to have the strongest cytotoxic activity (10 μg/ml). Fraction 5:5 was further purified by column chromatography, and two compounds were separated. Ethyl acetate fraction III, subfraction hexane/ethyl acetate 9:1 and the active hexane fraction were further purified by semi-preparative HPLC.

2.5. Semi-preparative HPLC of Bay leaf fractions

The biological active hexane fraction (hexane/chloroform 6:4) and ethyl acetate fraction III, subfraction hexane/ethyl acetate 9:1 were purified by semi-preparative HPLC on a Zorbax Rx-sil normal phase column (25 cm × 9.4 mm, 5 μm). Compounds were eluted by an isocratic solvent mixture containing hexane/ethyl acetate 92:8, the flow rate was 3 ml/min, the wavelength monitored was 254 nm. Four pure compounds were separated.

2.6. Analytical HPLC of Bay leaf fractions

The purity of the four compounds were confirmed by analytical HPLC using a diode array detector. The analytical HPLC was run on a Supelco discovery C18 reverse phase column, 25 cm × 4.6 mm, 5 μm. The mobile phase consisted of two solvents, water (0.05% formic acid) and acetonitrile. The run started with 100% water and increased to 100% acetonitrile in 35 min, the flow rate was 1 ml/min.

2.7. Spectral identification of compounds

10-epigazaniolide: APCI-MS, *m/z* 231 $[M + 1]^+$; ^1H NMR ($CDCl_3$, 600 MHz) δ 5.27 (1H, d, $J = 9.0$ Hz, H-1), 5.81 (1H, dd, $J = 5.4$, 9.6 Hz, H-2), 5.70 (1H, d, $J = 4.8$ Hz, H-3), 1.90 (1H, br d, $J = 10.8$ Hz, H-5), 3.89 (1H, t, $J = 11.4$ Hz, H-6), 2.33 (1H, m, H-7), 2.01 (2H, m, H-8), 1.43 (1H, m, H-8), 1.75 (1H, m, H-9), 1.43 (1H, m, H-9), 6.03 (1H, d, $J = 3.0$, H-13), 5.36 (1H, d, $J = 3.0$, H-13), 1.0 (3H, br s, H-14), 1.94 (3H, br s, H-15); EI-MS, *m/z*: 230 $[M]^+$(100), 215(86), 197(41), 169(37), 143(27), 132(41), 119(60), 105(77), 91(42) [Identical with that reported by Marco, Sanz-Cervera, Garcia-Lliso, and Batlle, 1997].

Gazaniolide: APCI-MS, *m/z* 231 $[M + 1]^+$, ^1H NMR ($CDCl_3$, 600 MHz) δ 5.52 (1H, d, $J = 9.6$ Hz, H-1), 5.79 (1H, dd, $J = 5.4$, 9 Hz, H-2), 5.69 (1H, m, H-3), 2.65 (1H, br d, $J = 11.4$ Hz, H-5), 4.00 (1H, t, $J = 10.8$ Hz, H-6), 2.51 (1H, m, H-7), 2.03 (1H, m, H-8), 1.64 (1H, m, H-8), 6.05 (1H, d, $J = 3.0$ Hz, H-13), 5.38 (1H, d, $J = 3.0$ Hz, H-13), 0.89 (3H, s, H-14), 1.98 (3H, s, H-15); ^{13}C NMR ($CDCl_3$) δ 120.7 (d, C-1), 137.3 (d, C-2), 123.2 (d, C-3), 136.5 (s, C-4), 51.2 (d, C-5), 81.6 (d, C-6), 51.2 (d, C-7), 21.8 (t, C-8), 37.0 (t, C-9), 37.9 (d, C-10), 139.3 (s, C-11), 171.0 (s, C-12), 117.0 (t, C-13), 15.8 (q, C-14), 22.4 (q, C-15); EI-MS, *m/z*: 230 $[M]^+$(88), 215(43), 197(29), 169(28), 143(30), 132(31), 119(61), 105 (100), 91(43) [Identical with that reported by Bohlmann and Zdero, 1979, and Vasquez et al., 1990].

Spirafolide: APCI-MS, *m/z* 247 $[M + 1]^+$, ^1H NMR ($CDCl_3$, 600 MHz) δ 4.60 (1H, d, $J = 7.2$ Hz, H-1), 6.14 (1H, d, $J = 7.2$ Hz, H-2), 6.38 (1H, br s, H-3), 2.81 (1H, d, $J = 7.2$ Hz, H-5), 4.23 (1H, dd, $J = 7.2$, 12 Hz, H-6), 3.07 (1H, m, H-7), 2.09 (1H, m, H-8), 1.56 (1H, m, H-8), 1.80 (1H, m, H-9), 1.46 (1H, m, H-9), 6.10 (1H, d, $J = 3$, H-13), 5.39 (1H, d, $J = 3$, H-13), 1.19 (3H, s, H-14), 1.72 (3H, s, H-15); ^{13}C NMR ($CDCl_3$) δ 119.0 (d, C-1), 142.3 (d, C-2), 141.5 (d, C-3), 112.7 (s, C-4), 49.7 (d, C-5), 81.4 (d, C-6), 39.5 (d, C-7), 22.1 (t, C-8), 33.9 (t, C-9), 39.1 (s, C-10), 140.4 (s, C-11), 171.0 (s, C-12), 118.5 (t, C-13), 30.9 (q, C-14), 23.1 (q, C-15); EI-MS: 246 $[M]^+$(37), 231(6), 164(51), 135(31), 123(100), 109(47), 95(62) [Identical with that reported by Hashemi-Nejad, Jakupovic, and Castro, 1990].

Costunolide: APCI-MS, *m/z* 233 $[M + 1]^+$, ^1H NMR ($CDCl_3$, 600 MHz) δ 4.81 (1H, m, H-1), 2.0–2.32 (1H, m, H-2), 1.62–1.72 (1H, m, H-2), 2.0–2.32 (1H, m, H-3), 2.43 (1H, m, H-3), 4.72 (1H, d, $J = 9.6$ Hz, H-5), 4.55 (1H, t, $J = 8.4$ Hz, H-6), 2.55 (1H, t, $J = 7.8$ Hz, H-7), 2.0–2.32 (2H, m, H-8), 2.0–2.32 (2H, m, H-9), 6.25 (1H, d, $J = 3.6$ Hz, H-13), 5.50 (1H, d, $J = 3.6$ Hz, H-13), 1.40 (3H, s, H-14), 1.68 (3H, s, H-15); EI-MS: 232 $[M]^+$(10), 217(18), 149(23), 121(40), 109(50), 93(37), 81(100) [Identical with the reference Ming, Mayer, Zimmermann, and Ruecker, 1989].

Reynosin: APCI-MS, *m/z* 249 $[M + 1]^+$, ^1H NMR ($CDCl_3$, 600 MHz) δ 3.50 (1H, dd, $J = 4.2$, 11.4 Hz, H-1), 2.15 (1H, br d, $J = 11.4$ Hz, H-5), 4.00 (1H, t, $J = 10.8$ Hz, H-6), 6.06 (1H, d, $J = 3$ Hz, H-13), 5.39 (1H, d, $J = 3$ Hz, H-13), 0.78 (3H, s, H-14), 4.83 (1H, s, H-15), 4.96 (1H, s, H-15); ^{13}C NMR ($CDCl_3$) 878.4 (d, C-1), 31.5 (t, C-2), 33.7 (t, C-3), 142.7 (s, C-4), 53.2

(d, C-5), 79.8 (d, C-6), 49.8 (d, C-7), 21.6 (t, C-8), 35.9 (t, C-9), 43.2 (s, C-10), 139.4 (s, C-11), 170.9 (s, C-12), 117.3 (t, C-13), 11.8 (q, C-14), 110.8 (t, C-15); EI-MS: 248 $[M]^+$(6), 230(98), 163(62), 145(25), 119(28), 105(41), 91(70), 79(57), 67(40), 53(100), 41(79) [Identical with the reference Yoshioka, Renold, Fischer, Higo, and Mabry, 1970].

Santamarine: APCI-MS, *m/z* 249 $[M + 1]^+$, ^{1}H NMR ($CDCl_3$, 600 MHz) δ 3.65 (1H, dd, $J = 6.6$, 10.2 Hz, H-1), 1.96 (1H, m, H-2), 2.38 (1H, m, H-2), 5.33 (1H, br s, H-3), 2.32 (1H, br d, $J = 11.4$ Hz, H-5), 3.92 (1H, t, $J = 11.4$ Hz, H-6), 2.47 (1H, m, H-7), 2.05 (1H, m, H-8), 1.64 (1H, m, H-8), 1.64 (1H, m, H-9), 2.05 (1H, m, H-9), 6.05 (1H, d, $J = 3$ Hz, H-13), 5.39 (1H, d, $J = 3$ Hz, H-13), 0.86 (3H, s, H-14), 1.82 (3H, s, H-15); ^{13}C NMR ($CDCl_3$) 675.5 (d, C-1), 33.0 (t, C-2), 121.5 (d, C-3), 133.7 (s, C-4), 51.4 (d, C-5), 81.7 (d, C-6), 51.3 (d, C-7), 21.4 (t, C-8), 34.5 (t, C-9), 41.1 (s, C-10), 139.2 (s, C-11), 171.0 (s, C-12), 117.0 (t, C-13), 11.3 (q, C-14), 23.6 (q, C-15); EI-MS: $[M]^+$ 248(77), 230(18), 175(15), 163(22), 152(61), 119(30), 107(80), 91(72), 79(52), 67(42), 53(88), 41(100) [Identical with the reference Glasl et al., 1995].

3. Results and discussion

Our screening results showed that the hexane and ethyl acetate extracts of Bay leaf exhibited potent biological activity in inducing apoptosis. Further fractionation of these two extracts showed that the fraction Hexane/chloroform 6:4 of hexane extract and fraction chloroform/methanol 96:4 of ethyl acetate extract (EtIII) had the strongest biological activity. The biological active fraction of hexane extract was further subjected to semi-preparative HPLC; the HPLC chromatogram is presented in Fig. 2.

All peaks were collected and analyzed by Analytical HPLC using a photo diode array detector. Peaks 1–4 indicated pure compounds. Further investigation by ApCI/MS, EI/MS, ^{1}H NMR and ^{13}C NMR identified four compounds corresponding to peaks 1–4. They are all sesquiterpene lactones, and compound 1 and 2 were identified from Bay leaf for the first time. These four compounds are 10-epigazaniolid, gazaniolid, spirafolide and costunolid. Their structures are shown in Fig. 3.

The fraction EtIII of ethyl acetate extract which showed strong bioactivity was further fractionated into four fractions. Two of them were biologically active; they are fractions with hexane/ethyl acetate 9:1 and 5:5. The fraction 9:1 was further purified by semi-preparative HPLC as described before, the HPLC chromatogram was shown in Fig. 4.

The retention times of these four peaks were the same as peaks 1–4 in Fig. 2. Analytical HPLC analysis showed the same results as the four compounds of hexane fraction. ApcI/MS and EI/MS further confirmed that the four compounds obtained here were identical as the four compounds identified before.

The other active fraction from fraction EtIII, the fraction 5:5 (hexane:ethyl acetate) was further purified

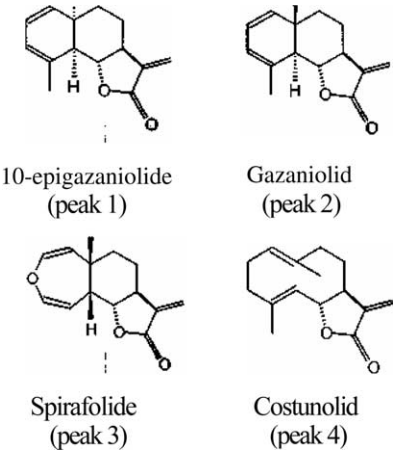

Fig. 3. Structure of compounds isolated from the active hexane fraction.

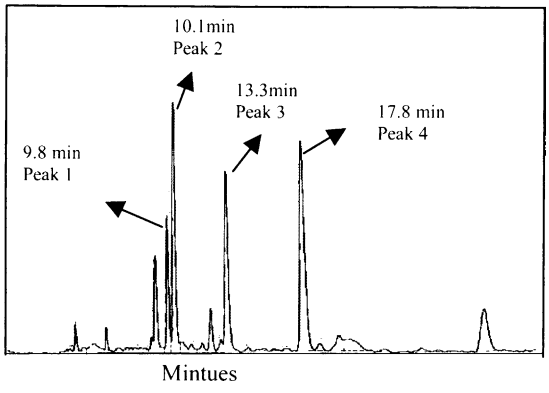

Fig. 2. Semi-preparative HPLC for the active hexane fraction (Rx-sil normal phase column).

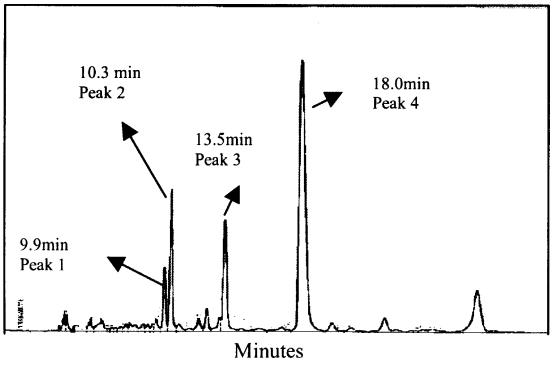

Fig. 4. Normal phase semi-preparative HPLC for the active fraction EtIII, subfraction 9:1 (hexane:ethylactate).

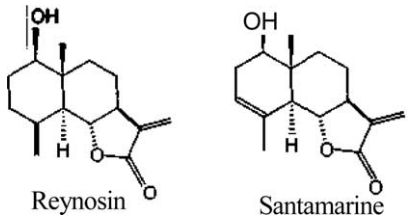

Fig. 5. Structure of compounds isolated from active fraction EtIII.

by column chromatography combined with thin layer chromatography, two pure compounds were separated. They are identified by ApCI/MS, EI/MS, ^{1}H and ^{13}C NMR. The two compounds were identified as reynosin and santamarine. Their chemical structures are shown in Fig. 5.

Gazaniolide and 10-epigazaniolide are now reported for the first time in Bay leaf. 10-epigazaniolide was first identified in *Artemisia lucentica* (compositae) (Marco et al., 1997). Gazaniolide was first identified in *Gazania krebsiana* (Bohlmann & Zdero, 1979), later it was also found in *Rudbeckia* species (Vasquez et al., 1990). Reynosin, santamarine, spirafolide and costunolide were found in Bay leaf in previous studies (Hashemi-Nejad et al., 1990; Matsuda et al., 2000; Vasquez et al., 1990), with costunolide as the most abundant. Santamarine was first identified in *Chrysanthemum parthenium* (De Vivar & Jimenez, 1965), and reynosin was first identified in *Ambrosia confertiflora* (Yoshioka et al., 1970), later they were found in many other plants. Reynosin and santamarine have been previously reported to exhibit cytotoxic activity against the human lung carcinoma cell Line GLC4 and the colorectal cancer cell line COLO 320 ($IC_{50} = 124$ mM) (Goren, Woerdenbag, & Bozok-Johansson, 1996), and studies also found that they have inhibitory effects on alcohol absorption, and NO production (Matsuda et al., 2000; Yoshikawa et al., 2000). Spirafolide was also identified in *Spiracantha cornifolia* (Hashemi-Nejad et al., 1990). Spirafolide also has been reported as having an anti-inflammatory effect (Matsuda et al., 2000) and also has the ability to inhibit alcohol absorption (Yoshikawa et al., 2000). Costunolide is the most studied seaquiterpene lactone, and it is found in numerous species belonging to many genera of the compositae, and a known source is costus root oil, obtained from *Saussurea lappa*. This latter compound has also been reported to enhance liver glutathione S-transferase activity (Wada et al., 1997), and work as a phase II enzyme-inducer responsible for the detoxification of xenobiotics. A recent study also found that costunolide induces apoptosis by decreasing the anti-apoptotic protein Bcl-2 (Babish, Howell, & Pacioretty, 2002).

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