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Identification of cytotoxic sesquiterpenes from *Laurus nobilis* L.

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Abstract

A new sesquiterpene, lauroxepine and six known sesquiterpene lactones, were obtained through bioactivity-directed isolation from a methanol extract of the fruits of *Laurus nobilis*. The hexane-soluble part of the methanol extract yielded lauroxepine, costunolide and gazaniolide, while the dichloromethane-soluble part of the methanol extract afforded costunolide and four other sesquiterpene lactones including santamarine, reynosin, 11,13-dehydrosantonin and spirafolide. The new sesquiterpene lauroxepine and spirafolide have a rare molecular structure carrying an oxepine ring. Structures of the compounds were determined through 1D and 2D NMR and mass (EI-MS) techniques. The extracts were investigated for both ovarian cytotoxic activity and DNA damaging properties against three yeasts. Among the three tested extracts prepared from flowers, leaves and fruits of *L. nobilis*, the most cytotoxic active extract against ovarian cancer cell line was found to be the fruit extract with 98% inhibition. Among all tested extracts, only the fruit extract showed marginal inhibition (63.2%) against one DNA repair-deficient yeast strain (pRAD52 Gal). Six known sesquiterpene lactones were found to be highly cytotoxic against the A2780 ovarian cancer cell line, however, lauroxepine was not found to be active in A2780.

Keywords: *Laurus nobilis*; Sesquiterpene lactones; Spirafolide; Lauroxepine; Ovarian cytotoxic activity (A2780); Antifungal activity

1. Introduction

The Lauraceae has 32 genera and about 2000–2500 species. A member of the family *Laurus nobilis* L. (Lauraceae), mythologically Apollo's Laurel is a plant native to the southern Mediterranean region and is widely cultivated mainly in Europe and the USA as an ornamental plant (Garg, Siddiqui, & Agarwal, 1992). The Bay Laurel, *L. nobilis*, is the only European representative of the family (William, 1989). It is grown commercially for its aromatic leaves in Turkey, Algeria, Morocco, Portugal, Spain, Italy, France and Mexico. In Turkey, the essential oil of *L. nobilis* is produced from leaves, and a modest amount is exported. Alkaloids, volatile oils and fixed oils occur in many species. The oil (seed oil) of *L. nobilis* is obtained from fruits of the plant by pressing or boiling in water and is used locally and also exported (Başer, 1997). Both the volatile and the seed oils are used for cosmetic, food and medicinal purposes.

The biological activities and phytochemistry of *L. nobilis* have previously been extensively investigated. In the first century, Dioscorides named this plant "Daphne" in his immortal book "Materia Medica", and recorded that its leaves and fruits soothe the stomach; they have been reported to possess aromatic, stimulant and narcotic properties (Buttery et al., 1974). The anti-convulsive and anti-epileptic activities of *L. nobilis* extracts have also been confirmed (Sayyah, Valizadeh, & Kamalinejad, 2002). The leaves of *L. nobilis* are traditionally used orally to treat the

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symptoms of gastrointestinal problems, such as epigastric bloating, digestion, eructations, and flatulence. Recent studies on this plant have shown that the leaves increase the secretion of gastric fluids and treat digestive disorders such as flatulent colic. The major sesquiterpene lactone of the plant, costunolide, and its α-methylene-γ-butyrolactone moiety were reported to be essential for this activity (Matsuda, Shimoda, Ninomiya, & Yoshikawa, 2002). The antioxidant activity of the leaves of *L. nobilis* has been investigated (Simic, Kundakovic, & Kovacevic, 2003), and isoquercitrin was found to be the compound responsible for its alkyl radical scavenging activity (Kang et al., 2002). Dehydrocostus lactone, zaluzanin D and (1*R*,4*S*)-1-hydroperoxy-*p*-menth-2-en-8-ol acetate isolated from the methanol extract of *L. nobilis* leaves have been reported to show trypanocidal activity (Uchiyama et al., 2002). The sesquiterpenes costunolide and zaluzanin D isolated from *L. nobilis* displayed strong growth inhibitory effects against human promyelotic leukemia (HL-60) cells (Hibasami et al., 2003). The antinociceptive, analgesic and anti-inflammatory activity of the leaf essential oil of *L. nobilis* were further investigated (Sayyah, Saroukhani, Peirovi, & Kamalinejad, 2003). It is known that contact with *L. nobilis* could be a cause of allergic reactions (Simic et al., 2003).

In Turkish folk medicine, *L. nobilis* leaves are used as antiseptic and for the treatment of stomach-ache, while its fruits act as an antimicrobial, anti-hemorrhoidal, antirheumatic, diuretic, and as an antidote to snake bites (Baytop, 1984). Preliminary brine shrimp toxicity tests and a few studies related to its cytotoxic properties were carried out on a leaf extract of Turkish *L. nobilis* (Kivçak & Mert, 2002). Its gastroprotective effects on ethanol induced ulcerogenesis have also been studied (Gürbüz, Ustün, Yeşilada, Sezik, & Akyürek, 2002). In the latter study, the authors reported that *L. nobilis* was found to be one of the five folk medicines which were chosen effective plant remedies for the treatment of stomach-ache, and it was included in the Data Bank of Turkish Folk Remedies (TÜHİB).

There are two reported studies on flavonoids of *L. nobilis* (Fiorini, David, Fouraste, & Vercauteren, 1998 and Kang et al., 2002). One of them was carried out on a commercial *L. nobilis* purchased in Turkey. In this study, the plant extract was found to have the most potent alkylperoxy radical scavenging activity among 120 herbs and edible plants screened (Kang et al., 2002). In addition, its unique flavonoid constituent, isoquercetin, was present as the active principle. It has also been analysed for alkaloids (Pech & Bruneton, 1982).

Although several isolation and biological activity studies have been carried out on the leaves of *L. nobilis,* there has been very little work on its fruits. In one study, three new fatty acid esters were isolated from an Indian commercial sample (Garg et al., 1992). In another study (Appendino, Tagliapietra, Nano, & Cisero, 1992), again on a commercial sample of the drug, the guaianolides eremanthin, dehydrocostuslactone, zaluzanin D and the germacronolide costunolide were isolated besides a new sesquiterpene alcohol 12-acetoxy germacra-1(10),5-dien-4,11-diol.

2. Materials and methods

2.1. Plant material

L. nobilis L. (Lauraceae) was collected from Balikesir (Marmara region of Turkey) in the garden of the Faculty of Education in July 2000, and identified by Prof. Dr. Gülendam Tümen. A voucher specimen was deposited in the special Herbarium [T. Dirmenci, 1237B].

2.2. Spectral measurements

IR spectra were obtained on a Perkin–Elmer 983 instrument, Tetra Tecnologic Systems, İstanbul, Turkey. ^{1}H and ^{13}C NMR spectra in $CDCl_3$ were recorded on Varian Unity 400 (Inova, New York, NY, USA) and JEOL Eclipse 500 (Inova, New York, NY, USA) instruments, respectively (only for spirafolide and lauroxepine) at Virginia Polytechnic Institute and State University, VA, USA and MS were measured on a VG ZabSpec instrument (VG Analytical, Manchester, UK) at TÜBİTAK, Marmara Research Center, Gebze, Turkey.

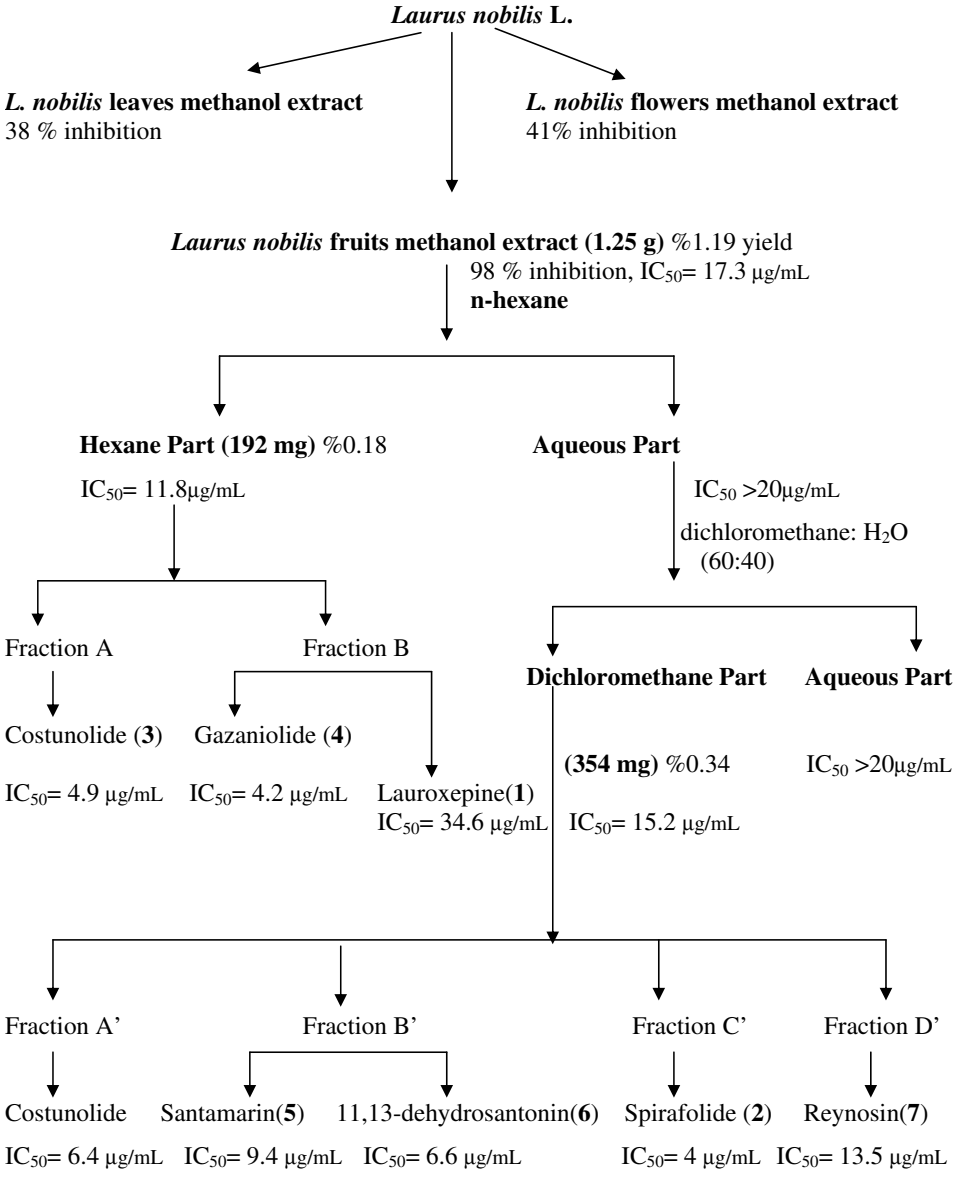
2.3. Plant extraction

The leaves, flowers and fruits (each 100 g) were separetely extracted with MeOH at 25 °C for 24 h. For this purpose, 105 g fruits of *L. nobilis* L. were extracted with MeOH for 24 h at room temperature. Evaporation of the solvent under vacuum yielded 1.25 g crude MeOH extract, which showed highest activity with an IC_{50} value of 17.3 μg/mL in the A2780 bioassay.

2.4. Bioassay-guided fractionation process

The constituents of a cultivated Turkish collection of *L. nobilis* L. from Balikesir were analyzed. The work was carried out by the bioassay-guided fractionation and isolation approach. The crude methanol extracts of fruits, leaves and flowers of *L. nobilis* were prepared separately and tested for cytotoxicity against the A2780 human ovarian cancer cell line. Since the fruit extract showed the highest activity with 98% inhibition it was selected for investigation of chemical constituents. The methanol extract of fruits was dissolved in 60% MeOH and re-extracted (partitioned) with hexane (3 × 100 mL) and dichloromethane (3 × 100 mL), successively (Scheme 1). The hexane extract (192 mg) and CH_2Cl_2 extract (354 mg) were tested against the A2780 ovarian cell lines and were found to be highly cytotoxic and warranted further investigation with IC_{50} values of 11.8 μg/mL and 15.2 μg/mL, respectively.

The hexane extract was subjected to column chromatography on silica-gel (12 g) eluting with hexane (500 mL), a



Scheme 1. Bioactivity-guided isolation scheme of the sesquiterpenes from *Laurus nobilis* extracts.

gradient of dichloromethane and acetone (10, 25, 50, 75, and 100%, each 500 mL), and finally MeOH (Scheme 1). Similar fractions were combined, and further separated by column chromatography on silica gel to yield 12 main fractions. The most active fraction (Fr. A) ($IC_{50} = 3.4$ μg/mL) afforded costunolide **3** (15 mg, $IC_{50} = 4.9$ μg/mL) (Hibasami et al., 2003). The following active fraction (Fr. B) ($IC_{50} = 5.8$ μg/mL) of the hexane extract yielded gazaniolide **4** (14 mg, $IC_{50} = 4.2$ μg/mL) (Vasquez et al., 1990), and lauroxepine **1** (3.5 mg, $IC_{50} = 34.6$ μg/mL).

The dichloromethane extract (354 mg) was subjected to column chromatography on silica gel (20 g) eluting with hexane (300 mL), a gradient of CH_2Cl_2 and acetone (10, 25, 50, 75, and 100%, each 500 mL) and finally MeOH (Scheme 1). Similar fractions were combined, and further separated by silica gel column chromatography to yield nine main fractions. The first fraction (Fr. A′) ($IC_{50} = 11.2$ μg/mL) was found to contain costunolide (17 mg, $IC_{50} = 6.4$ μg/mL), and the following fraction (Fr. B′) ($IC_{50} = 6.4$ μg/mL) afforded santamarin **5** (3 mg, $IC_{50} = 9.4$ μg/mL) (Abegaz, 1991) and 11,13-dehydrosantonin **6** (3.5 mg, $IC_{50} = 6.6$ μg/mL) (Iida, Wakuri, Mineka, Nishitani, & Yamakawa, 1993). The next fraction (Fr. C′) ($IC_{50} = 12$ μg/mL) afforded spirafolide **2** (2.1 mg, $IC_{50} = 4$ μg/mL) (Matsuda et al., 2000 and Ulubelen et al., 1985 and Hashemi-Nejad, Jakupovic, & Castro, 1990). Reynosin **7** (4 mg, $IC_{50} = 13.5$ μg/mL) (Fang et al., 2005) was obtained from the last and least active fraction (Fr. D′) ($IC_{50} = 19.9$ μg/mL). Santamarin, reynosin, 11,13-dehydrosantonin and spirafolide were isolated from the dichloromethane fraction, while a new sesquiterpene, named lauroxepine, and gazaniolide were isolated from the hexane fraction. Costunolide was the only sesquiterpene lactone that was found in both hexane and dichloromethane extracts (Fig. 1).

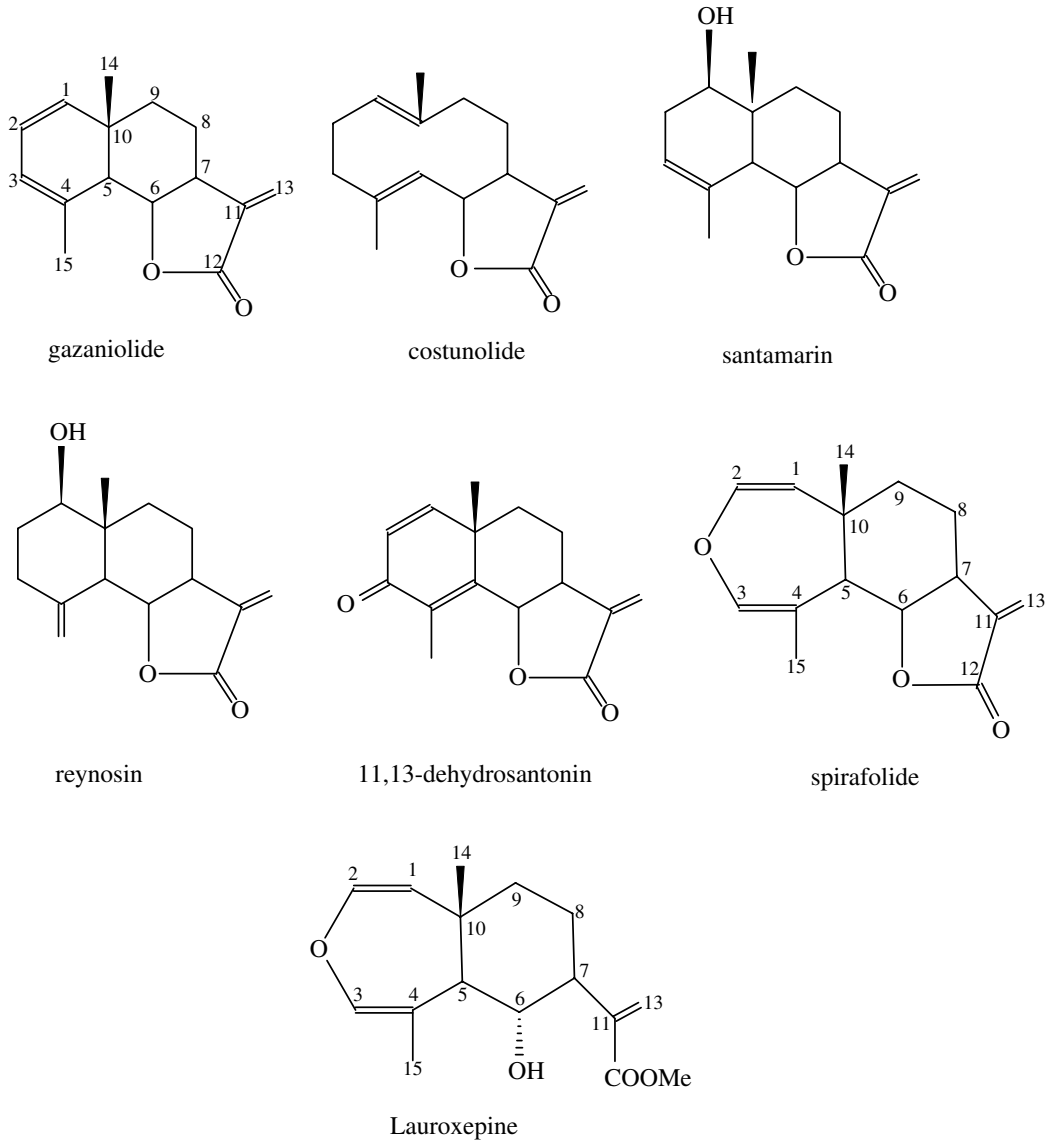


Fig. 1. Structures of the isolated sesquiterpenes from *Laurus nobilis* fruits methanol extract.

All the isolated sesquiterpene lactones showed IC_{50} values between 4 and 13.5 μg/mL, however, the new compound showed an IC_{50} value to be 34.6 μg/mL. It should be considered that the lack of lactone moiety caused a decrease in the potential cytotoxic activity of the compound. NMR data of lauroxepine and spirafolide are given in Table 1.

2.5. Cytotoxicity assay

The extracts and isolated sesquiterpene lactones were evaluated for their cytotoxic activity against A2780 human ovarian cancer cell lines (Gonzalez, Darias, Alonso, Boada, & Feria, 1978). This was carried out according to (Schwikkard et al., 2000) and Actinomycin D was used as a positive control. Cytotoxicity was determined against A2780 human ovarian cancer cells (Laboratory of Cellular and Molecular Biology, National Cancer Institute, Washington DC, USA), using a a microtiter plate assay. The plates were seeded with cells and the compounds [dissolved in dimethylsulphoxide: H_2O (1:1, v/v)] were added to the cells at specific concentrations. The plates were incubated at 37 °C and 5% CO_2 for 48 h. Then Alamar Blue (Biosource International) was added to the cells and the plates were incubated for 3 h. During this time the Alamar Blue was taken up by the live cells and reduced. The reduced form of Alamar Blue was stable and fluorescent. The fluorescence of each well in the plate was measured. The fluorescence is directly proportional to the percent inhibition of the growth of the cells. The IC_{50} value was determined by plotting the data on a dose response curve of percent inhibition versus concentration. The IC_{50} value is defined as the concentration of sample necessary to produce 50% inhibition of the growth of the cells. The smaller the IC_{50} value the more active the compound.

Table 1
^{1}H and ^{13}C NMR data of lauroxepine and spirafolide (in $CDCL_3$, 500 MHz, *J* values in Hz

C	^{1}H NMR		^{13}C NMR		COSY	HMBC	
	1	2	1	2	1 and 2	1	2
1	4.78 d (7.5)	4.62 d (7.2)	115.5	112.1	H-2	C-5, C-10, C-9	C-5, C-9, C-2, C-14
2	6.14 d (7.5)	6.16 d (7.2)	142.2	142.3	H-3	C-1, C-3, C-9	C-10,C-3, C-4
3	6.36 brs	6.39 brs	139.2	141.4	H-15	C-15,C-4, C-5, C-2	C-15, C-5,C-1,C-2
4	–	–	113.6	118.8	–		
5	2.08 d (6.9)	2.81 d(7)	52.1	49.2	H-6	C-15, C-1, C-9, C-11	C-6, C-7, C-9
6	4.07d (6.4)	4.22 dd(7.2;10.8)	69.7	81.2	H-5, H-7		C-5,C-7,C-8, C-11
7	3.18 m	3.12 m	41.2	39.6	H-8		C-5
8	1.45 m/2.66 m	1.70 m/2.11 m	19.9	22.3	H8a, H8b	C-7,C-6	C-6, C-7, C-9
9	1.30 m/2.15 m	1.82 m/1.46 m	36.7	34.2	H9a, H9b		C-5, C-7, C-8
10	–	–	36.5	39.1	–		
11	–	–	124.1	138.7	–		
12	–	–	168.3	167.6	–		
13	6.21 d (3.5)	6.11 d (3.2)	113.5	118.6	H-7	C-7	C-12
	5.21 d (3.4)	5.41 d (3.6)					
14	1.10 s	1.21 s	32.4	30.1	–	C-10, C-1, C-5	C-5
15	1.70 s	1.73 s	21.5	23.5	H-3	C-3, C-4, C-5	C-10
–OMe	3.76 s		53.2			C-12	

2.6. Yeast microtiter assay

The yeast based dose response microtiter assay, was carried out according to McBrien et al. (1995). The bioactivity of the samples was evaluated throughout the fractionation using RS321N, pRAD52 and RS321NYCp50 genetically engineered *Saccharomyces cerevisae* yeast strains. Growth inhibition was determined using a microplate assay in which the RS321NpRAD52 strain was seeded individually in minimal media (Difco) plus glucose and galactose (respectively), and RS321NYCp50 was seeded in minimal media plus galactose. Samples were dissolved in 10% DMSO and transferred to the seeded microtiter wells at a 1:10 dilution, for a final testing concentration of 100 μg/mL. Microtiter plates were incubated at 28 °C for 48–72 h, or until an optimum optical density of 0.15–0.25 was reached. Growth inhibition was elucidated using a linear regression analysis of the dose response scheme, and activity was reported in terms of an IC_{50} value, which is the concentration in μg/mL necessary to produce 50% cell inhibition. Streptonigrin at 0.001 μg/mL and etoposide at 20 μg/mL were both used as positive controls for the RS321NpRAD52 and RS321NYCp50 strains.

3. Results and discussion

In the present study, costunolide was the major sesquiterpene lactone identified in *L. nobilis* and was isolated from both the hexane and chloroform extracts of the fruits. It has also been isolated by other researchers from the leaves of *L. nobilis* (Matsuda et al., 2002; Hibasami et al., 2003; Appendino et al., 1992; Matsuda et al., 2000; Fang et al., 2005; Yoshikawa et al., 2000). Reynosin and 11,13-dehydrosantonin were isolated from only Turkish *L. nobilis* in the present study for the first time, although their isomers have recently been reported (Fang et al., 2005). Reynosin and santamarine were reported as being formed by cyclization of 1(10)-epoxycostunolide during work-up procedures (Vasquez et al., 1990), but the latter compound was not isolated from the extract.

Spirafolide was first isolated from *Spiracantha cornifolia* (Hashemi-Nejad et al., 1990). In a later study (Matsuda et al., 2000), it was found in the leaves of *L. nobilis* together with 13 other sesquiterpenes, seven of which showed high inhibitory effect on nitric oxide production in lipopolysaccharide stimulated macrophages. This activity was related to the α-methylene-γ-lactone moiety of the sesquiterpene lactones, which costunolide and dehydrocostus lactone showed the highest activity.

The ^{13}C NMR and 2D NMR data of spirafolide have not previously been reported and is presented in Table 1. In the ^{1}H NMR spectrum of lauroxepine, the presence of oxepine ring followed by the characteristic doublet signals at δ 4.78 and 6.14 with *J* couplings of 7.5 Hz together with a singlet signal at δ 6.36 remained spirafolide which was also isolated in this study. While the olefinic methyl signal was resonated at the same field with that of spirafolide (1.73 ppm, s), the other methyl signal (H-14) was shifted downfield. Chemical shift differences for H-13 methylene protons are considered to be a differentiation or absence of the lactone moiety. The IR spectrum revealed that no lactone group was present giving only a carbonyl signal at 1723 cm^{-1}, indicative of either an ester carbonyl or an isolated keto group rather than a five membered lactone carbonyl as observed at 1760 cm^{-1} in spirafolide. The main difference was the presence of a signal at δ 3.76 (s) attributed to a methoxy group. HMBC correlations between methoxy proton signal at δ 3.76 (s) and carbonyl signal in the ^{13}C NMR (δ 168) exhibited its presence as a carboxymethyl moiety, and no HMBC correlation was observed with the methoxy signal (Table 1). The observa-

tion of an oxygenated methine doublet signal at δ 4.07 with a J value of 6.4 Hz, instead of a doublet of doublet (7.2 and 10.8 Hz) at δ 4.22 as observed in spirafolide, was verified that C-6 proton should be next to a hydroxyl group rather than being a lactone proton. Its J value as 6.4 Hz attributed to α-orientation of this hydroxyl group at C-6 (Jakupovic et al., 1992; Yoshioka, Mabry, & Timmermann, 1973). EI-MS of lauroxepine gave a molecular ion peak at m/z (rel. int.) 278.2 [M^+] (calcd for $C_{16}H_{22}O_4$:278) which was consistent with the structure.[13]C NMR and 2D NMR data of lauroxepine are presented in Table 1. Lauroxepine may be considered as an artefact which can form through spirafolide.

Although some studies of cytotoxicity and antitumor activity have been carried out on *L. nobilis* extracts and its isolated constituents, this is the first study on the non-volatile constituents of a sample, collected from a tree growing in Balikesir, Marmara region of Turkey. The sesquiterpenes costunolide and zaluzanin D, obtained in one of the previous studies by Hibasami et al. (2003), are considered to be responsible for the observed antitumor activity, showing strong growth inhibitory effect against human promyelotic leukemia (HL-60) cells and apoptosis. It has been known that sesquiterpenes and their α-methylene-γ-butyrolactone moiety are essential for cytotoxicity and antitumor activity (McBrien et al., 1995). Our results also support this finding. Interestingly, costunolide and its α-methylene-γ-butyrolactone moiety were found to be also responsible for the gastroprotective effect of *L. nobilis* by Matsuda et al. (2002) Furthermore, Yoshikawa et al. (2000) showed that the α-methylene-γ-butyrolactone moiety of the isolated sesquiterpenes from *L. nobilis* is needed to inhibit blood ethanol elevation (Gonzalez et al., 1978). In a recent study, the trypanocidal activity of several sesquiterpene lactones from *L. nobilis* was related to covalent bond formation of the same α,β-unsaturated γ-lactone moiety with nucleophiles (Uchiyama et al., 2002). Thus, sesquiterpene-containing plants might be an important source for the development of new therapeutics. In this study, among isolated sesquiterpenes, spirafolide having a rare skeleton carrying oxepine ring showed the highest ovarian cytotoxic activity, which was higher than that of gazaniolide and the well known costunolide.

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