Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 2647–2652

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

PREVENTIVE EFFECT OF SESQUITERPENES FROM BAY LEAF ON BLOOD ETHANOL ELEVATION IN ETHANOL-LOADED RAT: STRUCTURE REQUIREMENT AND SUPPRESSION OF GASTRIC EMPTYING

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Received 24 June 1999; accepted 3 August 1999

Abstract: The methanolic extract from the leaves of *Laurus nobilis* (bay leaf, laurel) potently inhibited the elevation of blood ethanol level in ethanol-loaded rat. Through bioassay-guided separation, costunolide, dehydrocostus lactone, and santamarine were isolated as the active constituents and the α-methylene-γ-butyrolactone structure was found to be essential for the preventive effect on ethanol absorption. In addition, the retardation of gastric emptying seemed to be partially involved in the preventive effects.

Long-term consumption of alcohol in large quantities induces a number of disorders in addition to hematopathy, gastrointestinal disorder, chronic pancreatitis, peripheral nerve disorder, myocardosis, hypertentonia and hematopoiesis. Not only organ disorders but also mental disorders in alcoholism have serious social effects, and many patients with alcoholic disorders is increasing in many countries. Cyanamide and disulfilam, aldehyde dehydrogenase inhibitors, are used clinically for the treatment of chronic alcoholism. However, it is known that dehydrogenase inhibitors force alcoholics to quit drinking based on the fear of unpleasant reaction elicited after ethanol intake, but these medicine are also reported to show many strong side effects. Recently, we found many active saponins from natural medicines that prevent increases in blood alcohol levels:[1] for example, elatosides[2] from the bark of *Aralia elata*, sapindosides[1] from the pericarps of *Sapindus mukurossi*, escins[3] from the seeds of *Aesculus hippocastanum*, camelliasaponins[4] from the seeds of *Camellia japonica*, senegins and senegasaponins[5] from the root of *Polygala senega* var. *latifolia*, and momordins[6] from the fruit of *Kochia scoparia*. Furthermore, we have characterized new dihydroflavonols with inhibitory effect on alcohol-induced muscular relaxation and hepatoprotective activity from the fruit of *Hovenia dulcis*, which has been used for treatment of alcohol intoxication in Chinese traditional medicine.[7]

In our continuing search for inhibitors of blood alcohol elevation from natural resources, we found a potent preventive activity in the methanolic extract of the leaves of Lauraceae plant *Laurus nobilis* (bay leaf, laurel). *L. nobilis* is widely cultivated in Mediterranean and the leaves are used as a herb in cooking for soup or stew. The leaves of *L. nobilis* are also used as a herbal medicine for excitant, stomachic, and digestin in Europe. As the pharmacological properties of *L. nobilis*, it has been reported to have antiulcer (seed)[8] and antidiabetic (leaf)[9] effects, and to enhance in liver glutathione *S*-transferase (GST) activity[10]. Here, we found that the methanolic extract from the leaves of *L. nobilis* has a preventive effect on increases in blood ethanol level. In this paper, we describe the characterization of the active sesquiterpenes from the leaves of *L. nobilis*, the structure-requirement for the activity, and the action property.

0960-894X/99/$ - see front matter
PII: S0960-894X(99)00442-4

Dried leaves of *L. nobilis* (harvested in Turkey) were extracted with methanol at room temperature, and solvent was evaporated *in vacuo* at less than 40°C. Methanolic extract (yield; 20.0%) was partitioned into ethyl acetate and water, and each soluble fraction was evaporated as described above. The sesquiterpenes shown in Chart. 1 were isolated and purified from the ethyl acetate soluble fraction. α-Methylene-γ-butyrolactone (**5**) was purchased from Sigma (USA). Male Wistar rats weighing 110—140 g were fasted for 20—22 h prior to experiments, but were given with water *ad libitum*. Test samples were suspended in 5% acacia solution and given orally at 5 mL/kg in each experiment. For ethanol- or glucose-loading, test samples were administered orally to fasted rats. Thirty min later, 20%(v/v) ethanol (5 mL/kg) or 20% glucose (5 mL/kg) was given orally. Blood samples were collected from the infraorbital venous plexus at 0.5, 1 and 2 h after administration of ethanol or glucose. To determine the blood ethanol level, blood was immediately mixed with 10 volumes of ice-cold 0.33N perchloric acid and centrifuged (4°C, 3000 r.p.m., 10 min). Ethanol level in the supernatant was assessed by an enzymatic method (F-kit® ethanol, Boehringer Mannheim, Germany). For serum glucose determination, blood was centrifuged (4°C, 3000 r.p.m., 10 min) and glucose level in the supernatant was assessed by an enzymatic method (Glucose C-II test Wako®, Wako Pure Chemical, Japan). For examination of gastric emptying, a mixture of 20% ethanol and 0.05% phenol red was given orally (0.6 mL/rat) to rats 30 min after administration of test sample. The stomach was removed 30 min later and was homogenized with 50 mL of 0.1N NaOH. Then, 0.5 mL of 20% trichloroacetic acid was added to 5 mL of homogenate, and the solution was centrifuged (room temperature, 2500 r.p.m., 20 min). The supernatant (4 mL) was mixed with the same volume of 0.5N NaOH, and the amount of phenol red was determined from the absorbance at 560 nm. Gastric emptying (%) was calculated as shown below.

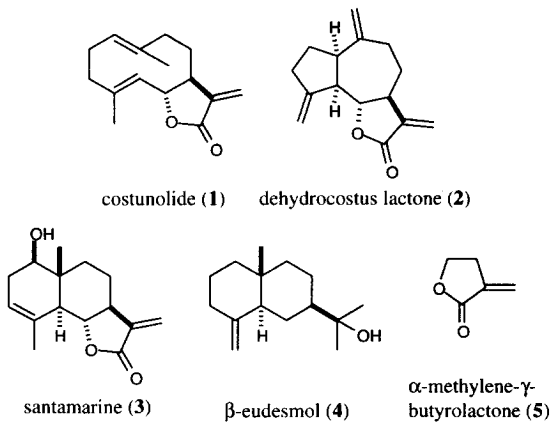

Chart 1. Sesquiterpenes (**1**, **2**, **3**, **4**) from *Laurus nobilis* and α-Methylene-γ-butyrolactone (**5**)

$$\text{gastric emptying (\%)} = (PR_0 - PR_S) / PR_0 \times 100$$

PR_0: amount of phenol red given orally

PR_S: amount of phenol red remaining in the stomach

Table 1 shows the effects of the methanolic extract and its fractions from the leaves of *L. nobilis* on increases in blood ethanol level in ethanol (0.8 g/kg)-loaded rats. The methanolic extract inhibited the increase in blood ethanol level from 125 to 500 mg/kg in a dose-dependent manner. The ethyl acetate-soluble fraction

Table 1. Effects of Methanolic Extract from the Leaves of *Laurus nobilis* and its Fractions on Blood Ethanol Level in Ethanol-loaded Rats

Treatment	Dose (mg/kg)	n	Blood ethanol (mg/mL) 0.5 h	1h	2h
Control	—	5	0.64±0.04	0.59±0.03	0.28±0.04
Methanolic extract	125	5	0.15±0.04 **	0.34±0.10 **	0.32±0.03
	250	5	0.05±0.02 **	0.05±0.02 **	0.11±0.03 **
	500	5	0.01±0.01 **	0.01±0.01 **	0.03±0.02 **
Control	—	5	0.88±0.03	0.64±0.02	0.32±0.02
AcOEt-soluble fraction	125	5	0.12±0.02 **	0.24±0.06 **	0.10±0.05 **
	250	5	0.05±0.01 **	0.08±0.01 **	0.11±0.04 **
Water-soluble fraction	125	5	0.90±0.04	0.65±0.03	0.34±0.02
	250	5	0.87±0.03	0.64±0.04	0.36±0.04

Ethanol (0.8 g/kg) was given orally 30 min after oral administration of each sample. Blood samples were collected from the infraorbital venous plexus at 0.5, 1 and 2 h after administration of ethanol. Values are means with S.E.M. Asterisks denote significant differences from control **: $p<0.01$.

also potently inhibited blood ethanol elevation, but no suppressive effect was observed in the water-soluble fraction. Fig. 1 shows the effects of sesquiterpenes isolated from the ethyl acetate-soluble fraction. The sesquiterpenes with an α-methylene-γ-butyrolactone moiety such as costunolide (**1**), dehydrocostus lactone (**2**) and santamarine (**3**) potently prevented blood ethanol elevation at 25 and 50 mg/kg. The yields of these compounds from *L. nobilis* were 0.18, 0.01, and 0.04%, respectively. β-Eudesmol (**4**, yield: 0.003%), which lacks a lactone ring, showed no effect. These results revealed that sesquiterpenes with an α-methylene-γ-butyrolactone moiety such as **1**, **2** and **3** are active constituents of the leaves of *L. nobilis*. α-Methylene-γ-butyrolactone (**5**) completely inhibited blood ethanol elevation at a dose of 25 mg/kg. The results of this study suggested that the active site of sesquiterpenes is the α-methylene-γ-butyrolactone moiety. The pharmacological activity of this moiety is very strong, and its properties have been well studied; *i. e.* it has been shown to have a vasoactive effect,[11] prevent NO and TNF-α production,[12,13] and to be cytotoxic against cytotoxic T lymphocytes.[14] Some of these reports indicated that the α-methylene-γ-butyrolactone moiety is required for expression of the activity. The results of the present study were in good agreement with the structure-activity relationship of these compounds.

We examined the effects of the methanolic extract and costunolide (**1**) from the leaves of *L. nobilis* on serum glucose elevation to investigate their specificity against ethanol absorption. Table 2 shows the effects of the methanolic extract and costunolide (**1**), an active constituent in the ethanol-loading test, on serum glucose elevation in glucose (1 g/kg)-loaded rats. High dose (500 mg/kg) of the methanolic extract slightly inhibited (34.5%) serum glucose elevation 30 min after glucose loading, although a significant increase in serum glucose level was observed in the group given 500 mg/kg extract at 2 h after glucose loading. On the other hand, **1** showed no effect on blood glucose level at 50 mg/kg. The results suggested that the preventive effect of **1** on the elevation of blood ethanol level is specific. The inhibitory effect on serum glucose elevation at a high dose

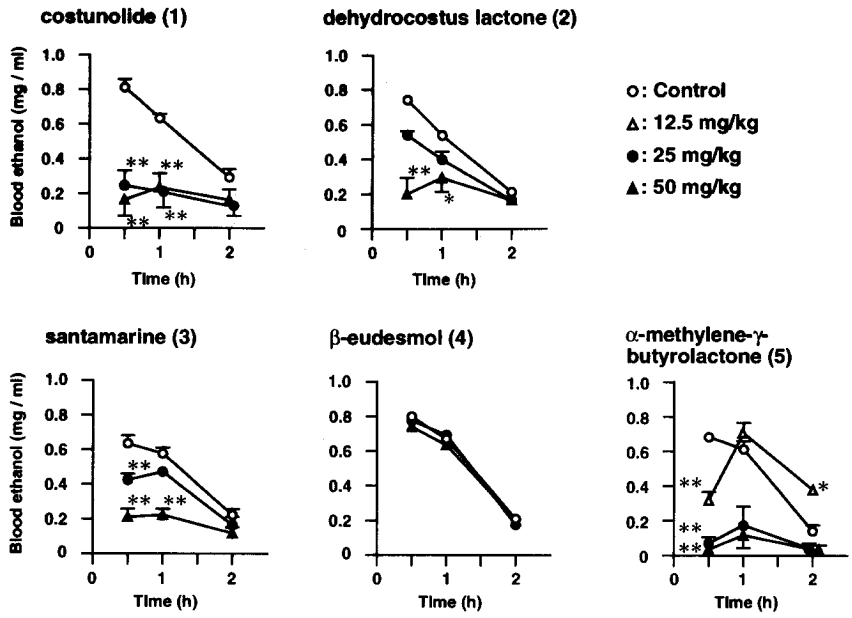

Fig. 1. Effects of Sesquiterpenes from the Leaves of *Laurus nobilis* (**1**—**4**) and α-Methylene-γ-butyrolactone (**5**) on Blood Ethanol Level in Ethanol-loaded Rats
Values are means with S.E.M. (n=5). Asterisks denote significant differences from control *: $p<0.05$, **: $p<0.01$, respectively.

of methanolic extract seemed to be due to inhibition of glucose absorption or to be due to nonspecific retardation of gastric emptying.

To clarify the mechanism responsible for the preventive effect of active sesquiterpenes on the increase in blood ethanol level, we examined their effects on gastric emptying in rats given a test food containing 20% ethanol. Table 3 shows the effects of the methanolic extract and costunolide (**1**) from the leaves of *L. nobilis* on gastric emptying in ethanol (20%, 5 mL/kg)-loaded rats. The methanolic extract significantly delayed gastric emptying at 250 and 500 mg/kg. Costunolide (**1**) and α-methylene-γ-butyrolactone (**5**) also inhibited gastric emptying at 25 and 12.5 mg/kg, respectively. Marked stagnation of gastric juice was observed in rats given the methanolic extract and **5** (data not shown). These results suggested that sesquiterpenes with an α-methylene-γ-butyrolactone ring suppress gastric emptying and delay ethanol absorption from the intestinal tract. This suppressive effect of sesquiterpenes on gastric emptying appeared to participate in the preventive effect of the *L. nobilis* on elevation of blood ethanol level.

In conclusion, we found that the methanolic extract of *Laurus nobilis* exhibited a preventive effect on blood ethanol elevation. Through bioassay-guided separation, active sesquiterpene lactones, costunolide (**1**),

Table 2. Effects of Methanolic Extract from the Leaves of *Laurus nobilis* and Costunolide (**1**) on Serum Glucose Level in Glucose-loaded Rats

Treatment	Dose (mg/kg)	n	Serum glucose (mg/dl) 0.5 h	1h	2h
Normal (Glucose unloaded)	—	5	80.0± 5.2 **	77.7±4.8 **	78.5±5.6 **
Control	—	5	181.5± 4.9	140.7±1.5	107.2±3.9
Methanolic extract	250	5	167.9± 4.8	148.8±4.9	115.9±2.8
	500	5	146.5±10.4 **	140.5±8.9	125.8±5.3 *
Costunolide (**1**)	25	5	178.6±10.3	151.0±7.6	116.6±4.3
	50	5	171.2± 3.8	150.3±1.3	111.9±5.4

Glucose (1g/kg) was given orally 30 min after oral administration of each sample. Blood samples were collected from the infraorbital venous plexus at 0.5, 1 and 2 h after the administration of glucose. Values are means with S.E.M. Asterisks denote significant differences from control,*: $p<0.05$, **: $p<0.01$.

Table 3. Effects of Methanolic Extract from the Leaves of *Laurus nobilis*, Costunolide (**1**) and α-Methylene-γ-butyrolactone (**5**) on Gastric Emptying in Ethanol-loaded Rats

Treatment	Dose (mg/kg)	n	Gastric emptying (%) at 30 min	Inhibition (%)
Control	—	5	83.3±2.0	—
Methanolic extract	250	4	44.7±2.0 **	46.3
	500	4	39.1±1.5 **	53.1
Costunolide (**1**)	25	5	62.1±6.6 **	25.5
	50	5	48.2±5.7 **	42.1
Control	—	5	78.3±5.0	—
α-Methylene-γ-butyrolactone (**5**)	12.5	5	47.9±4.7 **	38.8
	25	5	33.5±3.2 **	57.2
	50	5	22.2±0.9 **	71.6

Test mixture consisting of 20% ethanol and 0.05% phenol red was given orally (0.6 mL/body) to rats. Thirty min later, the stomach was removed and the amount of phenol red was determined. Test compounds were given orally 30 min before administration of test mixture. Values are means with S.E.M. Asterisks denote significant differences from control, **: $p<0.01$.

dehydrocostus lactone (**2**) and santamarine (**3**) were isolated and the α-methylene-γ-butyrolactone structure was found to be essential for the activity. The delay of gastric emptying was presumed to partially involve in their inhibitory effects.

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