Isolation of the Antiulcer Compound in Essential Oil from the Leaves of *Cryptomeria japonica*

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Essential oil from the leaves of Tateyamasugi (*Cryptomeria japonica*) exhibited strong inhibitory activity on ulceration induced by HCl/ethanol, HCl/aspirin, water-immersion stress and pylorus-ligation. We separated the antiulcer compounds from cedar essential oil by use of distillation and chromatography. As a result, terpinen-4-ol, a monoterpene, and elemol, a sesquiterpene, were isolated as active compounds. The antiulcer activity of the former was more potent than that of the latter. Terpinen-4-ol was a mixture of optical isomers and each possessed potent antiulcer activity. Secretion of gastric juice and output of acid and pepsin activity were lowered by terpinen-4-ol.

These results suggest that terpinen-4-ol isolated from cedar essential oil could be a valuable antiulcer agent.

Key words terpinen-4-ol; antiulcer activity; *Cryptomeria japonica*; essential oil

It has been reported that various parts of *Cryptomeria japonica* have been available as herbal medicine in Japan.²⁾ Cedar woods have been generally used as building materials, but the leaves are usually discarded. We postulated that the leaves might include bioactive compounds, since cedar leaves contain essential oils, flavones and so on.²⁾ Misawa and Kizawa³⁾ reported that volatile oil from cedar leaf displays the antitussive effects in guinea pigs, and Boyd and Sheppard⁴⁾ showed that cedar leaf oil increased the output of fluid in the respiratory tract.

In the present study, we report that essential oil from the leaves of *Cryptomeria japonica* inhibited the formation of gastric mucosal lesions in rats, and that several terpenes were isolated from this essential oil as antiulcer compounds.

MATERIALS AND METHODS

Preparation of Essential Oil from the Leaves of *Cryptomeria japonica* Needle leaves were collected from Tateyamasugi (*Cryptomeria japonica*) and cut into small pieces. The pieces were put into a tank filled with hot water (4 l/kg of leaves) in a steam distillation apparatus and distilled for 3 h. Cedar leaf oil was obtained as the upper phase in the distilled fluid and dehydrated by adding sodium sulfate. Overall yield was about 0.5%.

Distillation of Cedar Leaf Oil Cedar leaf oil (600 g) was put into a three-necked flask (1 l) fitted with a Widmar dissertation column (30 cm) and distilled into three fractions, monoterpene hydrocarbons fraction (Fr. 1), monoterpene alcohol and sesquiterpene hydrocarbons fraction (Fr. 2), and sesquiterpene alcohol and diterpene fraction (Fr. 3, Chart 1). Separation of each fraction was confirmed by GC using a fused-silica capillary column (HP-5, 30 m×0.25 mm).

Separation of Fraction 3 by Centrifugal Partition Chromatography (CPC) Fraction 3 was separated by CPC using hexane–acetonitrile (50:50, descending). Separation was carried out on a CPC model LLL (Sanki Engineering) at

20 °C monitoring at 210 nm. Finally, Fr. 3 was separated into seven polar components (Fr. A to G) and one nonpolar fraction (Fr. H, Chart 1).

Separation of Nonpolar Compounds (Fr. H) by High Performance Liquid Chromatography (HPLC) The nonpolar fraction (Fr. H) was separated by reversed-phase HPLC (RP-HPLC). RP-HPLC was carried out using a Waters 600 system equipped with a YMC octadecyl silica column (ODS, 20 mm i.d.×250 mm) and monitored at 210 nm. As a result, Fr. H was separated into four fractions (Fr. H-1 to H-4, Chart 1).

Separation of Fr. C by HPLC Fraction C containing antiulcer compounds was separated by RP-HPLC as described above and monitored with a refractive index detector. Terpinen-4-ol (purity: 86%, **1** in Chart 2) and elemol (purity: 97%, **2** in Chart 2) were separated from Fr. C as active compounds (Chart 1). After repeated purification by RP-HPLC, these materials were identified by comparing their NMR spectra with those of authentic compounds and analyzing their GC-MS spectra.

Chiral Separation of Terpinen-4-ol Chiral analysis of **1** was carried out by GC using a Hewlett Packard 5890II model equipped with a CP-cyclodextrin- β -236M column (oven temperature: 100 °C). This terpene was shown to be a mixture of optical isomers, thus, we performed chiral separation of **1** by HPLC. Chiral separation was carried out using a Waters 600 system under the following conditions: column, Chiralpak AD (Daicel Chemical Industries, Ltd., 10 mm i.d.×250 mm); mobile phase, *n*-hexane/2-propanol=95/5; flow rate, 1.2 ml/min; detection, refractive index detector. *d*-Terpinen-4-ol (**1a** in Chart 2, purity: 98%, optical purity: 95.2% ee, $[\alpha]_D^{25}$: +30.15°) and *l*-terpinen-4-ol (**1b** in Chart 2, purity: 98%, optical purity: 92.3% ee, $[\alpha]_D^{25}$: +30.63°) were separated (Chart 3). Optical purity was determined by measuring the specific rotation using a Jasco DIP-300 polarimeter.

Animals Wistar-ST or SD male rats (7 to 9 weeks old,

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Chart 1. Isolation of Antiulcer Principles from Leaf Essential Oil of *Cryptomeria japonica*

[], antiulcer activity (%); dosage, 100 mg/kg except for 228 mg/kg shown at a).

Chart 2. Terpinen-4-ol (1, 1a: *d*-Form and 1b: *l*-Form) and Elemol (2)

Chart 3. Chiral Separation of Terpinen-4-ol

[], antiulcer activity (%). a) 25 mg/kg, b) 50 mg/kg.

Japan SLC, Hamamatsu) were used. The animals were bred in quarters where the temperature and the relative humidity were kept at $24 \pm 1^\circ\text{C}$ and 50–60%, respectively. They were fed standard laboratory chow over a week. The animals were fasted for 24 h before experiments, but were allowed free access to drinking water.

Effects on HCl/Ethanol- or HCl/Aspirin-Induced Gastric Lesions The experiment was carried out according to the methods of Mizui and Doteuchi⁵⁾ and Guth *et al.*⁶⁾ Each sample suspended in 0.5% Tween 80 or vehicle was given orally to rats 1 h before oral administration of 0.15 N HCl/60% ethanol or 20 mg aspirin (Bayer)/ml in 0.15 N HCl (5 ml/kg). Two hours later, the animals were sacrificed. The stomach was removed and inflated by addition of 8 ml of 10% formalin to the gastric lumen. The stomach was incised along the greater curvature and examined for lesions. The length (mm) of each lesion was measured under a dissecting microscope with a grid and the sum per stomach was used as the lesion index.

Effects on Water-Immersion Stress-Induced Gastric Lesions The experiment was carried out according to the method of Takagi and Okabe.⁷⁾ Thirty minutes after administration of each sample, rats were placed in restraint cages and

Fig. 1. Antiulcer Activity of Cedar Essential Oil to Rat Ulcer Models

Each sample was orally given before administration of HCl/ethanol, HCl/aspirin, or water immersion, or intraduodenally after pylorus-ligation. Cedar essential oil (455 mg/kg in HCl/ethanol and water immersion, 228 mg/kg in HCl/aspirin and pylorus-ligation) or ecabect sodium (100 mg/kg) were administered to rats. Open column, control; closed column, cedar essential oil; hatched column, ecabect sodium. Each column shows the mean $\pm$ S.E. of 4–5 rats. *, **, Significantly different from control at $p < 0.05$ and 0.01, respectively (Student's *t*-test).

were then immersed in water ($23 \pm 1^\circ\text{C}$) for 5 h. At the end of this restraint, rats were sacrificed and the length (mm) of each lesion was measured as described above.

Effects on Pylorus-Ligation-Induced Gastric Lesions

The experiment was carried out according to the method of Shay *et al.*⁸⁾ Under ether anesthesia, the abdomen of each rat was incised and the pylorus was ligated. After intraduodenal administration of each sample, the abdomen was closed. Eight hours later, rats were sacrificed and the length (mm) of each lesion was measured as described above.

Effects on Secretion of Gastric Juice The operation was carried out as described for the method of pylorus-ligation-induced gastric lesions. Four hours later, celiotomy was conducted again to ligate the cardia and remove the stomach. Gastric juice was collected and filtered by mesh. The filtrate was used to measure volume, pH, acidity and pepsin activity. The acidity of gastric juice was determined by titration against 0.05 N NaOH using phenol red as pH indicator. Pepsin activity was measured according to the method of Anson.⁹⁾

RESULTS

Effects of Essential Oil on Gastric Lesions in Rats

Cedar essential oil at an oral dose of 455 mg/kg completely inhibited the formation of gastric lesions induced by HCl/ethanol and gastric lesions induced by water immersion by 73.3% (Fig. 1). Furthermore, this essential oil at an oral dose of 228 mg/kg prevented the gastric injury induced by HCl/aspirin and pylorus-ligation by 96.6% and 100%, respectively.

Oral treatment of ecabect sodium (Tanabe Pharmaceuticals) at 100 mg/kg potently inhibited the formation of gastric lesions induced by HCl/ethanol but only inhibited gastric lesions formed by water immersion by 38.6% (Fig. 1).

Separation of Antiulcer Compounds from Cedar Essential Oil Bioactivity-guided fractionation of cedar essential oil was carried out to isolate the antiulcer components. As shown in Chart 1, essential oil obtained from Tateyama-

sugi was distilled into three fractions (Fr. 1 to 3) under reduced pressure by Widmaier distillation. As Fr. 3 was the most active, further separation of this fraction was done by centrifugal partition chromatography. The fraction was separated into eight fractions, seven polar fractions (Fr. A to G), and one nonpolar fraction (Fr. H). Nonpolar Fr. H was further separated into four fractions (Fr. H1 to H4) by HPLC. Of these four fractions, Fr. H-1 showed the most potent antiulcer activity. This fraction was shown to include α -cadinene (62%) and longifolene (14%) by HPLC (data not shown). As Fr. C was the most active among the fractions examined, we carried out further separation of this fraction by HPLC. Compounds **1** and **2** were isolated from Fr. C as antiulcer compounds (Chart 1). When the antiulcer activity against the gastric ulcer induced by HCl/aspirin was compared, the activity of **1** was superior to that of **2** (Table 1).

Chiral analysis of **1** by GC showed that this compound was a mixture of optical isomers. The ratio of **1a** and **1b** isolated from cedar essential oil was 2 : 1. When the antiulcer activity of each isolated isomer was examined against gastric lesions induced by HCl/ethanol, **1a** and **1b** showed inhibition of 74.0% and 93.5% at 25 mg/kg and 94.8% and 98.8% at 50 mg/kg, respectively (Chart 3).

Effects of Terpinen-4-ol on the Secretion of Gastric Juice in Rats Basal secretion of gastric juice was 0.85 ml/h/100 g body weight in rats. When **1b** was administered to rats, the secretion clearly decreased to 0.16 ml/h/100 g body weight (Table 2). This compound also reduced the acid output and the secretion of pepsin in rats.

DISCUSSION

Leaves from *Cryptomeria japonica* contain various kind of compounds, such as essential oil, flavonoid, polyphenol and so on.²⁾ In the present study, we showed the antiulcer activity in essential oil and isolated the active principles.

Cedar essential oil exerted antiulcer activity in 4 kinds of animal model, with ulceration induced by HCl/ethanol, HCl/aspirin, pylorus-ligation or water immersion. The first

two models are effective for evaluation of antiulcer compounds, especially with regards to cytoprotective activity.^{5,6)} As essential oil was also effective in the formation of gastric ulcers induced by pylorus-ligation, this oil must include antiulcer principles with cytoprotective activity against gastric mucus and/or the antisecretory activity of gastric juice. Furthermore, cedar essential oil (455 mg/kg) showed potent antiulcer activity in water-immersion stress rats. These findings encouraged us to isolate the active components from cedar essential oil.

1, a monoterpene, and **2**, a sesquiterpene, were isolated by fractionation of cedar essential oil as potent antiulcer compounds. Of these terpenes, **1** showed the more potent antiulcer activity. It has been reported that some terpenes show antiulcer activity in animal models, for example, furanogermentone from *Zedoariae Rhizoma*,¹⁰⁾ costunolide from *Saussureae Radix*,¹¹⁾ zingiberene from *Zingiberis Rhizoma*,¹²⁾ and β -caryophyllene.¹³⁾ These terpenes are sesquiterpene, similar to **2**. Furthermore, the clinically used plaunotol is a diterpene.¹⁴⁾ It is interesting that **1**, a monoterpene, possesses potent antiulcer activity against gastric lesions induced by HCl/ethanol or HCl/aspirin. This terpene was a mixture of optical isomers, and each isomer (**1a** and **1b**) showed similar potency in the antiulcer assay. The activity was more potent than that of certain clinically used antiulcer agents (data not shown).

Next, we examined the effect of **1b** on gastric secretion to elucidate the mechanism of the antiulcer effect. This compound reduced the secretion and acidity of gastric juice and pepsin activity in rat stomach. β -Caryophyllene had no effect on gastric secretion at a dose of which formation of gastric ulcer was inhibited.¹³⁾ Plaunotol was reported to inhibit secretion only at the high dose of 300 mg/kg.¹⁴⁾ Compound **1** appears to be a more potent inhibitor of gastric secretion than the other two terpenes. Furthermore, the antiulcer activity of **1** in the the HCl/aspirin-induced model seems to be superior to that of β -caryophyllene¹³⁾ and plaunotol¹⁴⁾ in the aspirin- or indomethacin-induced model. As ulceration induced by HCl/ethanol or HCl/aspirin, used to estimate the effect of **1**, is hardly inhibited by drugs with antisecretory action, **1** may exert effect in the 4 animal models through cytoprotective activity on gastric mucus and/or antisecretory activity.

In conclusion, our results show that the antiulcer principles in cedar leaf oil are terpenes such as **1** and **2** and that these may be valuable as antiulcer agent with simple structure. Further studies will be presented on the antiulcer activity of terpenes in the near future.

Table 1. Antiulcer Activity of Terpinen-4-ol and Elemol to Gastric Lesions Induced by HCl/Ethanol or HCl/Aspirin

Dose (mg/kg)	Terpinen-4-ol		Elemol	
	100	200	100	200
Ulcer				
HCl/ethanol	100	99.7	94.5	100
HCl/aspirin	100	N.D.	64.2	100

Each sample was orally given 1 h before administration of HCl/ethanol or HCl/aspirin. Each value shows the mean of 3–4 rats. N.D., not determined.

Table 2. Effects of *l*-Terpinen-4-ol on the Secretion of Gastric Juice in Rats

	Gastric juice (ml/h/100 g BW)	pH	Acidity (μ eq/h/100 g BW)	Pepsin (PU/h/100 g BW)
Control	0.85 $\pm$ 0.07	1.10 $\pm$ 0.06	90.4 $\pm$ 9.8	4.61 $\pm$ 0.34
<i>l</i> -Terpinen-4-ol (100 mg/kg)	0.16 $\pm$ 0.04*** (81.2)	1.76 $\pm$ 0.14**	8.4 $\pm$ 2.2*** (90.7)	0.89 $\pm$ 0.23*** (80.7)

Each value shows the mean $\pm$ S.E. of 5 rats. The value in parentheses show the percent inhibition. **,***: Significantly different from control at $p < 0.01$, 0.001, respectively (Student's *t*-test).

REFERENCES AND NOTES

- 1) Present address: *Lead Chemical Co., Ltd., 77-3, Himata, Toyama 930-0912, Japan.*
- 2) Akamatsu K., "Wakanyaku," Ishiyaku Publishers Inc., Tokyo, 1970, pp. 662—663.
- 3) Misawa M., Kizawa M., *Pharmacometrics*, **39**, 81—87 (1990).
- 4) Boyd E. M., Sheppard E. P., *J. Pharmacol. Exp. Ther.*, **163**, 250—256 (1968).
- 5) Mizui T., Doteuchi M., *Jpn. J. Pharmacol.*, **33**, 939—945 (1983).
- 6) Guth P. H., Aures D., Paulsen G., *Gastroenterology*, **76**, 88—93 (1979).
- 7) Takagi K., Okabe S., *Jpn. J. Pharmacol.*, **18**, 9—18 (1968).
- 8) Shay H., Komarov S. A., Fels S. S., Meranze D., Gruenstein M., Siplet H., *Gastroenterology*, **5**, 43—61 (1945).
- 9) Anson M. L., *J. Gen. Physiol.*, **22**, 79—89 (1938).
- 10) Watanabe K., Shibata M., Yano S., Cai Y., Shibuya H., Kitagawa I., *Yakugaku Zasshi*, **106**, 1137—1142 (1986).
- 11) Yamahara J., Kobayashi M., Miki K., Kozuka M., Sawada T., Fujimura H., *Chem. Pharm. Bull.*, **33**, 1285—1288 (1985).
- 12) Yamahara J., Hatakeyama S., Taniguchi K., Kawamura M., Yoshikawa M., *Yakugaku Zasshi*, **112**, 645—655 (1992).
- 13) Tambe Y., Tsujiuchi H., Honda G., Ikeshiro Y., Tanaka S., *Planta Med.*, **62**, 469—470 (1996).
- 14) Ogiso A., "Pharmaceutical Research and Development," Vol. 2, ed. by Shibata S., Hirokawa Publishing Co., Tokyo, 1988, pp. 561—583.