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Relationship between antibacterial activity and constituents of *Cryptomeria japonica* essential oil

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Abstract
Cryptomeria japonica (Sugi) is a plant native to Japan that has attracted considerable attention because of its varied biological activities. We focused on the antibacterial activity of *C. japonica* and investigated whether the activity of Sugi oil can be estimated based on its components. Sugi oil exhibited antibacterial activity against the gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* and gram-negative bacteria *Escherichia coli*. A difference in activity was observed between individual plants with different Sugi oil constituent ratios. The common component was α–pinen, and its content differed between samples. No correlation between content of a-pinen and the activity was observed. These results suggest it is difficult to estimate antibacterial activity from the content of the common major component. Minor components might play a role as an enhancer of antibacterial activity.

Keywords: *Cryptomeria japonica,* antibacterial activity, terpenoid, enhanced activity

Introduction

Cryptomeria japonica is an evergreen tree widely distributed in Japan, and it has also been widely introduced in Taiwan and many provinces of mainland China as a forestry resource. Products made from *C. japonica* have a variety of applications, including as building materials and aromatherapy agents. Furthermore, research indicates that *C. japonica* exhibits antifungal, antipathogenic, and protective effects against insects. The leaves of *C. japonica* contain bioactive compounds associated with essential oil and flavones.

Studies of the terpenoid constituents of *C. japonica* essential oil have revealed that these constituents and their ratios can vary [1-3]. Whether the antibacterial activity associated with *C. japonica* essential oil depends on the terpenoid content remains unclear, however. Carson *et al*. [4] identified terpinen-4-ol as the component responsible for the antibacterial activity of tea tree oil. However, Inoue *et al*. [5] suggested that myrsene is critical for the activity of tea tree oil, even though its concentration is very low. In the present study, therefore, we examined the relationship between the antibacterial activity of essential oil obtained from *C. japonica* and the ratio of its constituents.

Materials and Methods

General

Sabinene, α-terpineol, terpinen-4-ol, and α-pinen were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Staphylococcus aureus* FDA209P, *Escherichia coli* NIHJ JC2, *Bacillus subtilis* ATCC6633, and *Pseudomonas aeruginosa* PAO1 were used as standard strains. All bacterial strains except for *S. aureus* were cultured in Mueller-Hinton broth (Becton Dickinson and Company, Franklin Lakes, NJ, USA); *S. aureus* was cultured in brain-heart infusion broth (Becton Dickinson and Company).

Plant material and extraction

Cryptomeria japonica specimens were collected at two different sites in northern Japan, Akita Prefecture and Iwate Prefecture. Essential oils were obtained from the specimens via steam distillation.

Constituents of *C. japonica* essential oil were analyzed by gas chromatography (GC)–mass spectrometry (MS). GC analyses were performed using a Shimadzu GC-17A system equipped with a DB-5MS column (30 m × 0.25 µm i.d., Agilent Technologies, Santa Clara, CA, USA) and flame ionization detector (FID**)**. MS analyses were performed using a 6890/5973 GCMS system (Agilent Technologies) equipped with a DB-5MS column. The oven temperature was programmed to increase from 40 to 280 °C at a rate of 4 °C/min and hold at 280 °C for 2 h.

The injector and FID detector temperature were maintained at 240 °C. Essential oil constituent ratios were determined based on peak areas of GC chromatograms.

Antibacterial activity assays

Antibacterial activity is expressed as the minimum inhibitory concentration (MIC) determined according to the micro–broth dilution method. The MIC was defined as the lowest concentration of the constituent at which visible growth in broth culture was not observed. Dimethyl sulfoxide was used to prepare stock solutions of *C. japonica* essential oil and its various constituents at a concentration of 8 mg/mL.

Results and Discussion

Table 1 shows the antibacterial activity (expressed as MIC) of essential oil of *C. japonica* at different constituent ratios. Essential oil of *C. japonica* specimens obtained from Iwate and Akita Prefectures in Japan inhibited the growth of *S. aureus* at a concentration of 0.2 mg/mL. This value was same as that of *C. japonica* essential oil of specimens obtained from Jeonju-si, Korea, and lower than that of essential oil of specimens obtained from Nonsam-si, Korea. Growth of another gram-positive species, *B. subtilis*, was inhibited by essential oil of *C. japonica* obtained from Iwate Prefecture, Japan, but not by essential oil of *C. japonica* obtained from Nonsam-si, Korea. These results indicate that *C. japonica* essential oil affects the growth of at least some gram-positive bacteria and that Japanese *C. japonica* essential oil has stronger activity against gram-positive bacteria than Korean *C. japonica* essential oil.

Table 1: Minimum inhibitory concentrations (MICs) of *C. japonica* essential oils against various microorganisms

Strain	Akita	Iwate	Nonsam-si1)	Jeonju-si2)
S. aureus	0.2	0.2	10.9	0.2
E. coli	0.2	>0.4	>21.8	12.8
B. subtilis	---	0.2	>21.8	----
P. aeruginosa	----	>0.4	>21.8	----

MIC values were determined in triplicate and expressed in mg/mL.

The growth of *E. coli* was suppressed by essential oil of *C. japonica* from Akita Prefecture but not by essential oil of *C. japonica* from Iwate Prefecture. A similar phenomenon was observed with Korean *C. japonica* essential oil. Essential oil of *C. japonica* inhibited the growth of *S. aureus*, but essential oil obtained from one *C. japonica* specimen from Akita Prefecture also inhibited the growth of *E. coli*. Thus, the antibacterial activity of *C. japonica* essential oil against gram-negative bacteria appears to be dependent on the ratio of various constituents.

Essential oil of *C. japonica* from Akita Prefecture inhibited the growth of *E. coli*, a gram-negative bacteria, but essential oil of *C. japonica* from Iwate Prefecture did not. By comparison, essential oil of *C. japonica* from Jeonju-si inhibited the growth of *E. coli*, but essential oil of *C. japonica* from Nonsam-si did not. The MIC values of essential oil of *C. japonica* from Akita Prefecture were lower than those of essential oil of *C. japonica* from Jeonju-si, Korea. The growth of *P. aeruginosa*, another gram-negative bacteria, was not inhibited by the *C. japonica* essential oils tested. The essential oil of *C. japonica* from different locations thus exhibited differences in antibacterial activity. We therefore analyzed the constituents of the *C. japonica* essential oils to elucidate the relationship between oil composition and antibacterial activity.

Table 2 shows the major constituents of *C. japonica* essential oils. Only α-pinen was common to all four *C. japonica* essential oils examined, but the concentration of α-pinen in each oil varied. Thus, *C. japonica* essential oil has a varying composition. We then focused on the major constituents present in the highest concentrations and investigated the correlation between oil composition and antibacterial activity.

Table 2: Major constituents of *C. japonica* essential oils (Area %)

Constituent	Akita	Iwate	Nonsam-si [1]	Jeonju-si [2]	Taiwan [3]
Kaur-16-ene	6.02	3.0	31.4	---	40.62
Sabinene	9.14	---	11.06	8.86	---
Elemol	7.13	---	8.6	11.2	---
β-Eudesmol	6.40	12.3	4.8	---	5.90
α-Pinen	23.25	9.8	4.2	6.07	3.08
Terpinen-4-ol	5.2	6.1	---	9.77	---
α-Terpineol	---	---	---	6.13	---
Limonene	7.41	4.3	---	---	---
Cadenene	1.08	---	---	7.16	---
α-Bulnesene	---	9.8	---	---	---
γ-Terpinene	1.40	5.9	---	---	19.91
Others	32.97	48.8	39.94	22.54	30.49

The MICs of α-pinen, sabinene, and terpinen-4-ol were determined against *S. aureus* and *E. coli* (Table 3). All four different *C. japonica* essential oils exhibited antibacterial activity against *S. aureus.* The common constituent was α-pinen, with an MIC against *S. aureus* of >0.4 mg/mL in our analysis, versus 6.4 mg/mL reported in a previous study. Neither sabinene nor terpinen-4-ol pure compounds exhibited the same antibacterial activity against *S. aureus* as *C. japonica* essential oil. This suggests that no single constituent is responsible for the antibacterial activity against *S. aureus*.

The greater antibacterial activity of the intact oil against *S. aureus* could be due to a multiplier effect involving a combination of some of the constituents. The growth of *B. subtilis* was inhibited by essential oil of *C. japonica* from Iwate Prefecture. This result was attributed to the similar cellular structures of *S. aureus* and *B. subtilis*, both of which are gram-positive bacteria.

Essential oil of *C. japonica* from Akita Prefecture exhibited antibacterial activity against *E. coli*, and essential oil of *C. japonica* from Jeonju-si, Korea, also reportedly inhibits *E. coli*. The common constituents in these oils were identified as α-pinen, elemol, sabinene, and terpinen-4-ol. Elemol might not play a significant role in the antibacterial activity against *E. coli*, as essential oil of *C. japonica* from Nonsam-si, Korea, contained a higher concentration of elemol than essential oil of *C. japonica* from Akita Prefecture but did not exhibit antibacterial activity against *E. coli*. The MICs of α-pinen, sabinene, and terpinen-4-ol against *E. coli* are listed in Table 3. None of the constituents exhibited activity similar to that of the *C. japonica* essential oils. A previous report indicated that the activity of α-pinen is similar to that of *C. japonica* essential oil and that sabinene, α-terpineol, and terpinen-4-ol exhibit superior activity compared with *C. japonica* essential oil [2]. The essential oil of *C. japonica* from Akita Prefecture exhibited the lowest MIC against *E. coli* but contained the highest concentration of α-pinen. As shown in Table 2, essential oils of *C. japonica* from Akita Prefecture, Japan, and Nonsam-si and Jeonju-si, Korea, contained similar levels of sabinene, but their MICs differed. A similar result was observed in the case of elemol. Terpinen-4-ol was present in essential oil of *C. japonica* from Jeonju-si, Korea, at comparatively higher levels and also present in essential oil of

C. japonica from Akita and Iwate Prefectures, Japan, at lower levels than essential oil of *C. japonica* from Jeonju-si, Korea. These data suggest that α-pinen plays an important role in the expression of antibacterial activity against *E. coli* via interactions with other constituents.

Table 3: MICs of major constituents of *C. japonica* essential oils

	vs *S. aureus*		vs *E. coli*	
	Our data	**Jeonju-si [2]**	**Our data**	**Jeonju-si [2]**
α-Pinen	>0.4	6.4	>0.4	12.8
Sabinene	0.4	1.6	>0.4	1.6
α-Terpineol	---	1.6	---	1.6
Terpinen-4-ol	0.4	1.6	>0.4	1.6

MIC was measured in triplicate and was expressed in mg/mL.

Pseudomonas aeruginosa possesses a unique system to suppress the passage of compounds across the cytosolic membrane and excrete antibacterial compounds via an active efflux pump [6, 7]. These characteristics of *P. aeruginosa* could be responsible for higher MICs of *C. japonica* essential oils and their constituents.

In conclusion, the results of the present study indicate that the antibacterial activity of *C. japonica* essential oils of specimens obtained from different locations cannot be estimated by summing the activities of the individual constituents. The antibacterial activity of *C. japonica* essential oil appears to be mediated in part by α-pinen, a major common component, in conjunction with various minor components that might play roles as enhancers.

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