Antifungal properties of Japanese cedar essential oil from waste wood chips made from used sake barrels

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Abstract. In this study, we prepared essential oil (EO) from waste wood chips made from used sake barrels (USBs) of Japanese cedar (i.e., EO-USB) by steam distillation. We found that EO-USB and three commercially purchased EOs derived from xylem tissue of Japanese woods, such as Japanese cedar (*Cryptomeria japonica*), Japanese cypress (*Chamaecyparis obtusa*) and false arborvitae (*Thujopsis dolabrata*), suppressed fungal growth activity against *Trichophyton rubrum*, which is the cause of tinea disease. The magnitude of the suppressive effects of the EOs ranked as follows: *T. dolabrata* > USB = *C. japonica* > *C. obtusa*. These EOs also inhibited the activity of DNA polymerase in an extract from *T. rubrum* mycelia with the following ranking: *T. dolabrata* > USB = *C. japonica* > *C. obtusa*. In addition, 50 μg/ml of EO-USB showed antifungal properties, killing *T. rubrum* mycelia at 27-42˚C in 20 min. By gas chromatography/mass spectrometry analysis, the main sesquiterpenes in EO-USB were δ-cadinene (25.94%) and epi-cubenol (11.55%), and the composition of EO-USB was approximately the same as that of EO-*C. japonica*. Three prepared sesquiterpenes, δ-cadinene, epi-cubenol and β-eudesmol, inhibited the fungal growth and DNA polymerase activities of *T. rubrum*, and epi-cubenol showed the strongest inhibition among the compounds tested. These sesquiterpenes had no inhibitory effects on the activities of other DNA metabolic enzymes, such as DNA topoisomerase II, IMP dehydrogenase, polynucleotide kinase and deoxyribonuclease from *T. rubrum*. Taken together, these results suggest that EO-USB containing epi-cubenol may be useful for its anti-tinea disease properties, which are based on DNA polymerase inhibition.

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Abbreviations: EO, essential oil; USB, used sake barrel; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; DMSO, dimethyl sulfoxide; IC_{50}, 50% inhibitory concentration

Key words: essential oil, used sake barrels, Japanese cedar, *Cryptomeria japonica*, antifungal, *Trichophyton rubrum*, tinea, DNA polymerase inhibition

Introduction

Dermatophytosis caused by *Trichophyton sp.*, *Candida sp.* or other microorganisms is a significant disease threat for people with weakened immune systems. Among various conditions of dermatophytosis, tinea pedis caused by *Trichophyton sp.* is widespread in humans and recurs frequently. Tinea pedis is usually treated by the administration of topical or oral medicines; however, essential oils (EOs) are also effective for treating dermatophytosis (1-3).

Over the years, it has been reported that EO from the Japanese cedar, *Cryptomeria japonica*, has various medicinal properties, including antifungal activity (4-7). *C. japonica* is one of the most commercially important conifers in Asia. 'Sake' is a rice-based alcoholic beverage of Japanese origin - the Japanese term for this specific beverage is Nihonshu, meaning 'Japanese alcohol'; in English, it is also referred to as rice wine. In Japan, a type of sake known as 'taru-sake' is traditionally distributed in Japanese cedar barrels, although nowadays it is generally sold in bottles. Moreover, bottled taru-sake is now more popular as it is a more convenient way to enjoy the woody aroma. Nevertheless, this type of sake makes up a part of the sake market.

In this study, we focused on used sake barrel (USB) wood chips as a way to recycle/reuse the USBs. We demonstrate that EO can be extracted by steam distillation from waste wood chips made from USBs of Japanese cedar (*C. japonica*). To determine whether the extracted EO-USB has the potential to treat tinea disease, we investigated the effectiveness of EO-USB in inhibiting *Trichophyton rubrum*, one of the major dermatophytes causing tinea pedis, as compared to other EOs derived from xylem tissue of Japanese woods.

We have established *in vitro* assay methods to identify direct inhibitors of DNA metabolic enzymes, such as DNA polymerase (8-10), DNA topoisomerase II (11), IMP dehydrogenase (12), polynucleotide kinase (8) and deoxyribo-

nuclease (8) and, over the past 15 years, we have discovered more than 100 inhibitors of these enzymes from natural materials (13-15). In this study, using these assays, we investigated whether the components of EO-USB can inhibit the activities of such enzymes from *T. rubrum*; in addition, we discuss the antifungal mechanism used by EO-USB against *T. rubrum*.

Materials and methods

Extraction of EO from the waste wood chips made from USBs. The waste wood chips were made from USBs that had been constructed from *C. japonica* grown in Nara Prefecture, Japan. The wood chips were subjected to steam distillation; EO was produced in a yield of 0.1% (v/w) when the distillate was twice the weight of the wood chips.

EOs and chemicals. EOs derived from xylem tissue from major and popular Japanese woods, including Japanese cypress (*Chamaecyparis obtusa*), false arborvitae (*Thujopsis dolabrata*) and Japanese cedar (*Cryptomeria japonica*), were purchased from the Tree of Life Co., Ltd. (Tokyo, Japan). δ-cadinene and β-eudesmol were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. Epi-cubenol was separated and purified from EO-USB by column chromatography with Wakogel 50C18 (Wako Pure Chemical Industries, Ltd.) eluted with methanol and then further purified on Wakogel C-300HG (Wako Pure Chemical Industries, Ltd.) eluted with *n*-hexane. The purity of each of these prepared sesquiterpenes was >90%.

Microbial strains. Two microorganisms, *T. rubrum* NBRC5467 and *Staphylococcus epidermidis* NBRC12993, were obtained from the National Institute of Technology and Evaluation (NITE) (Chiba, Japan). *T. rubrum* is the cause of tinea disease and *S. epidermidis* is a gram-positive bacterial strain that is commonly present on human skin and sometimes causes human illness.

Preparation of microconidia suspension from T. rubrum. *T. rubrum* was cultured at 27˚C for at least 1 week on one-tenth Sabouraud dextrose agar. Microconidia were harvested by adding PBS containing 0.1% Tween-80 (PBS + Tween, pH 7.0) to the slant culture using a pipette. After being washed with PBS + Tween, the suspension was filtered through sterile gauze to remove fragments of mycelia, yielding a dense suspension of microconidia.

Microbial growth suppression assays. Broth microdilution methods were performed in flat-bottomed 96-well microplates to determine the minimum inhibitory concentration (MIC) values for each EO and for the main components of EO-USB. Serial 2-fold dilutions of the test compounds were prepared to give a final concentration in each well ranging from 9.8 to 10,000 μg/ml. Mueller-Hinton broth containing 0.1% Tween-80 was inoculated with *T. rubrum* microconidia suspension to give a final concentration of $2.5x10^5$ microconidial cells/ml, and incubated at 27˚C for 7 days. The absorbance was measured at 630 nm using a plate reader (Multiskan Ascent; Thermo Fisher Scientific Inc., Waltham, MA, USA), and the MIC was defined as the lowest concentration of the compound that resulted in the not detected absorbance value, as compared to the control absorbance value.

After determination of the MIC, a 100-μl aliquot was removed from wells that showed no fungal growth and spread onto potato dextrose agar plates without the test compounds. The plates were incubated at 27˚C for 3 days. The minimum fungicidal concentration (MFC) end-point was defined as the lowest concentration of antifungal agent at which no visible growth was observed on the plates.

The suppression of bacterial growth activity against *S. epidermidis* was determined in the same way, except that plates were incubated at 30˚C for 24 h for the MIC test and for 3 days for the minimum bactericidal concentration (MBC) test.

Antifungal (sterilization of T. rubrum) assay. The antifungal activity of EO-USB against *T. rubrum* was estimated according to the method described by Inouye *et al* (1). In brief, the microconidial suspension (10^7 conidia) was spread uniformly on a Petri dish (8 cm in diameter) containing 15 ml of potato dextrose and 3% agar. The plates were incubated at 27˚C for 3 days. Agar plates with uniform mycelial growth were used to obtain agar blocks (7 mm in diameter) with a cork borer. These blocks, which had hyphae on the surface, were immersed in 10 ml of sterile water containing 1% dimethyl sulfoxide (DMSO) (control), or EO-USB (50 and 100 μg/ml) solution in 1% DMSO at 27, 37 or 42˚C for 20 min. After immersion, the agar blocks were washed with 10 ml of sterile saline solution, blotted onto filter paper to remove excess saline and then placed on 1.5% Sabouraud dextrose agar plates with the mycelial side on top. The agar plates were incubated at 27˚C for 8 days, and the diameter (long and short axis) of the colony formed was measured using a vernier caliper. The number of cells surviving on the agar blocks was calculated from a standard curve, which was constructed by plotting the number of conidia originally implanted on the agar blocks (10^7, 10^6, 10^5, 10^4 and 10^3 per plate) against the diameter of the colony after incubation. All results are the means of 4 assays.

Assay of DNA metabolic enzymes in the extract from T. rubrum mycelia. The mycelia of *T. rubrum* (5 mg) cultured on potato dextrose agar plates were collected, and then sonicated for 5 min in 2 ml of 50 mM Tris-HCl (pH 7.5) buffer containing 1 mM EDTA, 5 mM 2-mercaptoethanol, 15% glycerol, 1 μg/ml leupeptin and 100 μM phenylmethylsulfonyl fluoride (PMSF). The supernatant obtained by centrifugation (16,000 x g, 10 min, 4˚C) was considered as the extract (enzyme solution) from *T. rubrum* mycelia. This extract was diluted with PC-buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM 2-mercaptoethanol and 15% glycerol], and used for the DNA metabolic enzyme assays.

The *in vitro* activities of DNA polymerase (8-10), DNA topoisomerase II (11), IMP dehydrogenase (12), polynucleotide kinase (8) and deoxyribonuclease (8) of *T. rubrum* mycelia extract were assayed at 37˚C as described previously (8-12). The enzymes were used at 0.05 units each.

Gas chromatography/mass spectrometry (GC-MS) analysis. The composition of EOs from Japanese cedar were analyzed by a gas chromatograph mass spectrometer QP2010 plus (Shimadzu, Kyoto, Japan) operating in EI mode. The instru-

Table I. *T. rubrum* MIC and MFC values of EO-USB and the commercially purchased EOs from xylem tissue of Japanese woods.

Origin of EO	MIC (μg/ml)	MFC (μg/ml)
USB (Japanese cedar)	313	313
Cryptomeria japonica (Japanese cedar)	313	313
Chamaecyparis obtuse (Japanese cypress)	62.5-1,250	2,500
Thujopsis dolabrata (False arborvitae)	78.1-156	156

EO, essential oil; USB, used sake barrel; MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration.

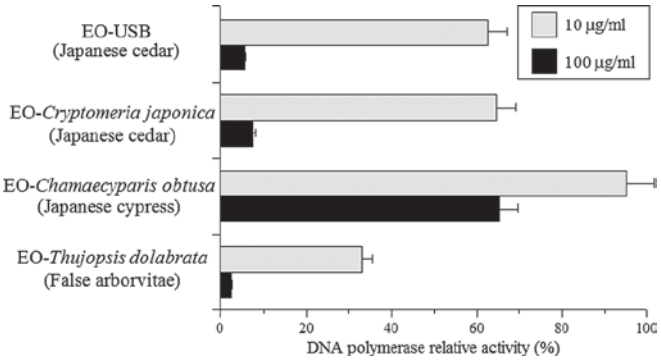

Figure 1. Inhibitory effects of EO-USB and the commercially purchased EOs from xylem tissue of Japanese woods on the activity of *T. rubrum* DNA polymerase. DNA polymerase activity (0.05 units) in the absence of EO was taken as 100%, and relative activity is shown. Data are presented as the means ± standard error (n=3). EO, essential oil; USB, used sake barrel.

ment was equipped with a fused silica column ULBON HR-SS-10 (50 m x 0.25 mm, film thickness 0.25 μm; Shinwa Chemical Industries Ltd., Kyoto, Japan) for sesquiterpenes. Helium was used as the carrier gas at a flow rate of 2 ml/min.

Prior to GC-MS analysis, EOs were diluted 1,000 times with *n*-hexane. *R*-(-)-carvone dissolved in ethanol was used as the internal standard. For analysis of sesquiterpenes, the oven temperature was maintained at 60˚C for 1 min, increased from 60 to 220˚C at a rate of 5˚C/min, and then held at 220˚C for 7 min.

Results and Discussion

Suppressive effects of EOs on T. rubrum growth. Initially, we investigated the inhibitory effects of EO-USB on the fungal growth of *T. rubrum*, which is related to tinea pedis, using broth microdilution methods. We found that the MIC (the lowest concentration of EO resulting in no growth of the fungus in broth) and the MFC (the minimal fungicidal concentration of EO resulting in no growth of *T. rubrum* on potato dextrose agar transferred from the broth after the MIC test) were both 313 μg/ml (Table I). The effects of EO-USB were the same strength as those of the commercial EO from xylem tissue of *C. japonica*, as these 2 EOs are derived from the same origin, Japanese cedar.

Among the 3 commercially purchased EOs, the strongest inhibitory effects against *T. rubrum* were shown by *T. dolabrata* (false arborvitae), followed by *C. japonica* (Japanese cedar including EO-USB) and then *C. obtusa* (Japanese cypress) (Table I). We also determined that the MFC/MIC ratio was nearly 1 for EO-*T. dolabrata* and EO-*C. japonica* (including EO-USB), indicating that these EOs primarily acted as fungicides. On the contrary, EO-*C. obtusa* appeared to be fungistatic in action as the MFC/MIC ratio was >2.

Effects of EOs on in vitro DNA polymerase activity from T. rubrum. We then investigated the *in vitro* biochemical action of EOs. As shown in Fig. 1, 100 μg/ml of EO-USB inhibited the activity of DNA polymerase in the extract from the mycelia of *T. rubrum*, and the 50% inhibitory concentration (IC_{50}) value was 21 μg/ml. The inhibitory activity of the commercial EO-*C. japonica* (Japanese cedar) was the same as that of EO-USB. Among the EOs tested, EO-*T. dolabrata* (false arborvitae) showed the strongest inhibition, and the inhibitory effects of the EOs were ranked as follows: *T. dolabrata* (false arborvitae) > USB = *C. japonica* (Japanese cedar) > *C. obtusa* (Japanese cypress). Therefore, the inhibitory effects of the EOs on the activity of *T. rubrum* DNA polymerase displayed the same order was as those on *T. rubrum* growth suppression (Table I). These results suggest that the suppression of *T. rubrum* growth by the EOs may be caused by the inhibition of DNA polymerase activity.

Inhibitory effects of EO-USB on T. rubrum mycelia activity. As a way to reuse the waste wood chips of USBs, this study focused on EO-USB, and thus its ability to inhibit *T. rubrum* mycelia activity was subsequently investigated. Agar blocks covered with *T. rubrum* mycelia were immersed in EO-USB solution, and the number of mycelia that survived was determined according to the method described by Inouye *et al* (1). As shown in Fig. 2, 50 and 100 μg/ml of EO-USB significantly decreased the number of viable cells after 20 min at 27, 37 and 42˚C. The percentage of mycelial cells surviving in 50 μg/ml of EO-USB was decreased to 5.2% at 27˚C, 16.0% at 37˚C and 10.4% at 42˚C; therefore, stronger antifungal effects were observed at low temperatures (27˚C) than at higher temperatures (37 and 42˚C). By contrast, with 8 μg/ml of itraconazole, which is a major antifungal agent prescribed to patients with fungal infections, such as tinea disease, the survival ratio of *T. rubrum* mycelia was ~2% at 27-42˚C. Therefore, immersion treatment in EO-USB seems to be as effective as itraconazole treatment. Inouye *et al* reported that mycelia of *T. mentagrophytes* were 10-100-fold more sensitive to EOs than conidia (1). Similarly, 100 μg/ml of EO-USB exhibited strong antifungal activity against *T. rubrum* mycelia with a killing ratio of 98%. Among EO-USB, the 50% sterilizing concentration against *T. rubrum* mycelia at 37˚C and the IC_{50} value for *T. rubrum* DNA polymerase inhibition at 37˚C were very similar (26 and 21 μg/ml, respectively); therefore, mycelial DNA polymerase inhibition may directly have antifungal effects.

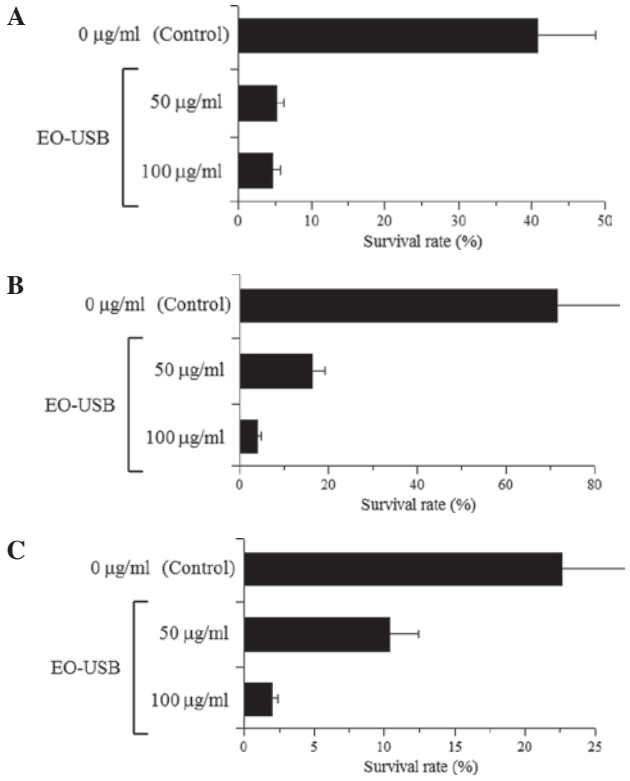

Figure 2. Effects of immersion in EO-USB solution on the viability of *T. rubrum* mycelia. Agar blocks implanted with *T. rubrum* were immersed in 1% DMSO solution containing 0, 50 or 100 μg/ml of EO-USB for 20 min at (A) 27˚C, (B) 37˚C and (C) 42˚C. Surviving cells (%) = viable cells/initial inoculum x 100 ± standard error (n=4). EO, essential oil; USB, used sake barrel; DMSO, dimethyl sulfoxide.

Suppressive effects of EOs on S. epidermidis growth. The microorganisms that normally inhabit the skin act as an anti-microbial shield and contribute to normal defense at the epidermal interface (16,17). We therefore investigated the MIC and MBC values of EOs against *S. epidermidis*, a normal inhabitant of human skin. EO-*T. dolabrata* (false arborvitae) showed strong anti-bacterial activity (MIC, 156-312 μg/ml; MBC, 5 mg/ml), whereas other EOs, including EO-USB, did not inhibit *S. epidermidis* growth (Table II). EO-*C. japonica* (Japanese cedar) monoterpenes, such as α-pinene and sabinene, have been reported to show strong activity against all bacteria tested, especially oral bacteria, with MIC values of 25-500 μg/ml (18). Monoterpenes are often present in EOs prepared from leaves and/or bark. Therefore, one possible reason for the observed lack of anti-bacterial activity of EO-USB is because it was produced from heartwood without monoterpenes (7). EO-*T. dolabrata* had significant inhibitory effects (IC_{50} value 60 μg/ml), whereas the other EOs had no inhibitory effects on the activity of DNA polymerase from *S. epidermidis* (IC_{50} values >1,000 μg/ml). These results suggest that EO-USB may be preferable for antifungal therapy as it shows anti-*Trichophyton* activity, while still preserving the normal microbial population on human skin.

Chemical composition of EOs from Japanese cedar. GC-MS analysis of the 2 Japanese cedar EOs, namely EO-USB and the commercial EO from xylem tissue of *C. japonica*, resulted in the identification of 14 different sesquiterpenes. Together, these sesquiterpenes accounted for 77% of the total compounds in these EOs. The identified compounds are listed in Table III according to their elution order from the capillary column. Although the amount of sesquiterpene in EO-USB was 0.95-fold lower than that in the commercial EO-*C. japonica*, the 2 EOs had a similar composition - in particular, the sesquiterpenes present in the highest and second highest amounts were δ-cadinene and epi-cubenol in both EOs. Thus, based on DNA polymerase inhibition, the antifungal activity of EO-USB was the same strength as that of the commercial EO.

Table II. *S. epidermidis* MIC and MBC values of EO-USB and the commercially purchased EOs from xylem tissue of Japanese woods.

Origin of EO	MIC (μg/ml)	MBC (μg/ml)
USB (Japanese cedar)	>10,000	>10,000
Cryptomeria japonica (Japanese cedar)	>10,000	>10,000
Chamaecyparis obtusa (Japanese cypress)	>10,000	>10,000
Thujopsis dolabrata (False arborvitae)	156-312	5,000

EO, essential oil; USB, used sake barrel; MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration.

Table III. Sesquiterpene composition of EOs extracted from Japanese cedar.

Compound	EO-USB		Commercial EO (*C. japonica*)	
	μg/ml	%	μg/ml	%
α-Cubebene	24.05	3.03	17.16	2.05
α-Muurolene	73.85	9.29	65.74	7.86
δ-Cadinene	206.13	25.94	190.98	22.84
Gleenol	17.15	2.16	11.34	1.36
Cubenol	50.07	6.30	38.68	4.63
Epi-cubenol	91.81	11.55	71.14	8.51
Elemol	22.99	2.89	56.35	6.74
T-Cadinol	3.58	0.45	14.87	1.78
γ-Eudesmol	12.98	1.63	18.00	2.15
Torreyol	30.23	3.80	29.95	3.58
α-Eudesmol	19.53	2.46	32.54	3.89
β-Eudesmol	36.77	4.63	48.36	5.78
Kongol	5.35	0.67	6.38	0.76
Cryptomerione	19.69	2.48	39.70	4.75
Others	180.50	22.72	195.11	23.32
Total	794.68	100.00	836.28	100.00

EO, essential oil; USB, used sake barrel.

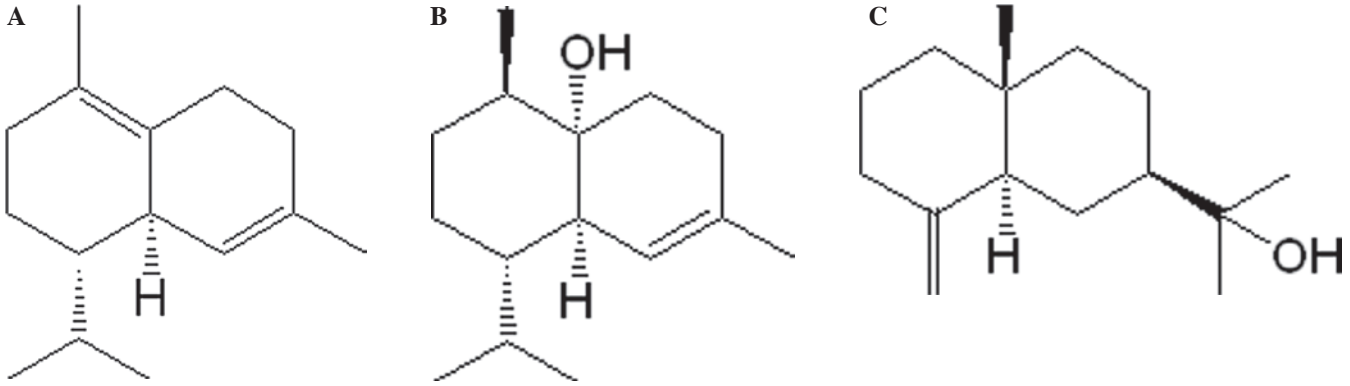

Figure 3. Structures of the sesquiterpenes present in EO-USB. (A) δ-cadinene, (B) epi-cubenol and (C) β-eudesmol. EO, essential oil; USB, used sake barrel.

Table IV. *T. rubrum* MIC values of sesquiterpenes from EO-USB (Japanese cedar, *C. japonica*).

Compound	MIC (μg/ml)
δ-Cadinene	500
Epi-cubenol	250-500
β-Eudesmol	1,000

EO, essential oil; USB, used sake barrel; MIC, minimum inhibitory concentration.

Suppressive effects of sesquiterpenes from EO-USB on T. rubrum growth. We prepared 3 major sesquiterpenes (>90% pure), δ-cadinene, epi-cubenol and β-eudesmol (Fig. 3), representing components with the 1st, 2nd and 5th highest amounts in EO-USB, respectively (Table III). The MIC values of these sesquiterpenes are listed in Table IV. Epi-cubenol, which accounted for 11.55% of the total sesquiterpenes in EO-USB, showed the strongest fungal growth suppression of *T. rubrum*. Other constituents, including the most abundant compound δ-cadinene (25.94%), showed smaller inhibitory effects than epi-cubenol. The combination of components in EO-USB must result in a synergistic antifungal effect against *T. rubrum*, because EO-USB was as effective as the strongest inhibitor, epi-cubenol, in terms of MIC.

Inhibitory effects of sesquiterpenes from EO-USB on the activities of T. rubrum DNA metabolic enzymes. The 3 major sesquiterpenes in EO-USB inhibited the *in vitro* activity of *T. rubrum* DNA polymerase, and the IC_{50} values of these compounds ranked as follows: epi-cubenol > δ-cadinene > β-eudesmol (Table V). These results suggest that the inhibition of DNA polymerase by these sesquiterpenes is positively correlated with their fungal growth suppression (Table IV). The IC_{50} values of epi-cubenol and EO-USB against *T. rubrum* DNA polymerase were 42 and 21 μg/ml, respectively (Fig. 1); therefore, this compound may be one of the DNA polymerase inhibitors in EO-USB. By contrast, these compounds had no effects on the activities of other DNA metabolic enzymes, such as DNA topoisomerase II, IMP dehydrogenase, polynucleotide kinase and deoxyribonuclease, in the extract from *T. rubrum* mycelia.

Table V. IC_{50} values of sesquiterpenes from EO-USB (Japanese cedar, *C. japonica*) against the activities of DNA metabolic enzymes from *T. rubrum*.

	Compound (μg/ml)		
Enzyme	δ-Cadinene	Epi-cubenol	β-Eudesmol
DNA polymerase	90	42	185
DNA topoisomerase II	>1,000	>1,000	>1,000
IMP dehydrogenase	>1,000	>1,000	>1,000
Polynucleotide kinase	>1,000	>1,000	>1,000
Deoxyribonuclease	>1,000	>1,000	>1,000

EO, essential oil; USB, used sake barrel.

In this study, we used an *in vitro* assay method to determine DNA polymerase activity in the fungal mycelia extract. This assay could be completed within 3 h and did not need culture of the target fungi; therefore, it must be a useful and convenient screening technique for antifungal agents.

In conclusion, EOs from *C. japonica* (Japanese cedar) heartwood contain sesquiterpenes, such as epi-cubenol and δ-cadinene, in greater quantities than present in sapwood, making them very effective for inhibiting the survival of skin pathogenic fungi without harming the microorganisms that normally live on the skin. Since USBs are made from *C. japonica* heartwood, the EO extracted from waste wood chips made from USBs may be useful as drugs and/or agents against tinea disease.

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