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Biological activity of extracts from *Cupressus lusitanica* cell culture

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Abstract Ethyl acetate extracts of *Cupressus lusitanica* suspension cell cultures were examined for their biological activities (viz., tyrosinase inhibitory, antioxidant, and antimicrobial activities). The extract from elicitor-treated cells showed all of the biological activities, whereas the extract from cultures without elicitor was not bioactive to a discernible extent. The biological activities shown by the cell culture extracts were almost solely accounted for by β-thujaplicin contained in the extracts. These results suggest that the ethyl acetate extract of *C. lusitanica* cells treated with the elicitor is a valuable bioactive source without isolation or purification of β-thujaplicin.

Key words Biological activity · *Cupressus lusitanica* · Cell culture · β-Thujaplicin · Antioxidant activity

Introduction

Some reports have shown production of compounds by plant cell cultures to be a useful method for obtaining valuable bioactive compounds such as shikonin,[1] anthocyanin,[2] tropane alkaloids,[3] and paclitaxel.[4,5] On the other hand, it is known that β-thujaplicin, a heartwood component of the Cupressaceae, shows some antimicrobial activity against a wide spectrum of microorganisms from bacteria to fungi[6] and is widely used as an ingredient for hair lotion, toothpaste, and cosmetics.[7] β-Thujaplicin also shows phytogrowth-regulating activity,[8] cytotoxic effects on mammalian cells,[9] scavenging activities against active oxygen species,[10] and inhibitory activity against some enzymes including tyrosinase, catechol-*O*-methyltransferase,[11] and mitochondrial ATPase.[12]

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We have maintained callus cultures of *Cupressus lusitanica* that can produce β-thujaplicin as a phytoalexin when stimulated with an yeast-derived elicitor.[13] It would be beneficial that crude extracts from the *C. lusitanica* cell cultures exhibit such biological activities without separation and purification of β-thujaplicin. In this paper, the ethyl acetate extract of *C. lusitanica* cell suspension culture which contained β-thujaplicin was examined for tyrosinase inhibitory, antioxidant, and antimicrobial activities. We also estimated the biological activities of the extract from *C. lusitanica* callus cells that did not contain β-thujaplicin owing to differences in culture conditions. The results were compared with the activities of authentic β-thujaplicin.

Materials and methods

Cell culture and extract preparation

The callus of *C. lusitanica* has been maintained and subcultured monthly for several years on a solid Gamborg B5 medium[14] containing 2% sucrose, 10 μM naphthylacetic acid, 0.01 μM 6-benzylaminopurine and 0.27% gellan gum in the dark at 25°C.

Cell suspension cultures were prepared by transferring about a 10-g (fresh weight) aliquot of the callus grown on the solid medium for 4 weeks, to 100 ml of a modified B5 liquid medium, IS-2,[15] in a 500-ml Erlenmeyer flask. To produce β-thujaplicin, the cultures were treated with aqueous solutions (10 ml) containing partially purified yeast extract as the elicitor[13] and incubated at 25°C with shaking at 70 rpm in the dark for 4 days. After the incubation, the cells were filtered and homogenized three times for 1 min with a Polytron (Kinematica Ag, Littau, Luzern Switzerland). Homogenized cells and the filtrate were combined and extracted with ethyl acetate to obtain extract 1 for biological activity assay. The callus culture of *C. lusitanica* without the elicitor was also extracted with ethyl acetate to obtain extract 2. The amount of β-thujaplicin in each extract was determined by high-performance liquid chromatography (HPLC).[16]

In preliminary experiments, use of less polar solvents such as hexane and diethyl ether resulted in incomplete extraction of β-thujaplicin. Methanol extracts contained large amounts of water-soluble contaminants.

Biological activity assay

Tyrosinase inhibitory activity

Tyrosinase inhibitory activity was measured as described by Morita et al.[17] with slight modifications. Mushroom tyrosinase solution (0.1 ml, 1250 U/ml) (Wako Pure Chemical Industries, Osaka, Japan), 2.0 ml 0.1% L-tyrosine, 0.7 ml McIlvain buffer (pH 6.8), and 0.2 ml methanol with or without any samples were mixed and then incubated at 37°C for 20 min. The percent tyrosinase activity was calculated as follows:

$$\text{Tyrosinase activity (\%)} = 100(B - A)/(D - C)$$

Here, A and B represent the absorbances at 475 nm in the presence of a sample before and after incubation; and C and D are the absorbances without a sample before and after incubation, respectively. The 50% inhibitory concentration (IC_{50}) of tyrosinase activity of each sample was calculated and compared with that of kojic acid, which was used as a positive control.

Antioxidant activity assay

Antioxidant activity was measured by the rabbit erythrocyte membrane ghost method[18] and the linoleic acid autoxidation method[19] with some modifications. The extracts from cell cultures of *C. lusitanica* (extracts 1 and 2) were used as antioxidant samples. Butylhydroxytoluene (BHT), butylhydroxyanisole (BHA), and α-tocopherol were used as positive controls.

For the ghost method, rabbit blood (40 ml) was diluted with 120 ml isotonic buffer (10 mM phosphate/152 mM NaCl, pH 7.4) and centrifuged (1500 g, 10 min). The precipitated blood was washed three times with 24 ml isotonic buffer, lysed by suspending it in 10 mM phosphate buffer (pH 7.4), and centrifuged (20000 *g*, 40 min) to precipitate erythrocyte membrane ghosts, which were then diluted to give a suspension (2.5 mg protein/ml). The protein concentration was determined by the Bradford method.[20] Peroxidation of the erythrocyte membrane ghosts was induced by the addition of *t*-butyl hydroperoxide. A mixture of 0.9 ml of the membrane ghosts suspension, 0.05 ml of 40 mM *t*-butyl hydroperoxide aqueous solution, and 0.05 ml of the test solution in dimethylsulfoxide was incubated for 30 min at 37°C. After the incubation, 1 ml of 2.0 M trichloroacetic acid/1.7 M HCl was added to stop the reaction. The mixture was colored by heating with 2 ml of 0.67% thiobarbituric acid (TBA)/1.0 N NaOH solution for 10 min in boiling water. The quantity of the TBA-reacting substance was estimated from the absorbance at 532 nm with a spectrophotometer. Lipid peroxidation was calculated as follows:

$$\text{Lipid peroxidation (\%)} = 100\ (A - C)/(B - C)$$

where, A, B, and C denote absorbances at 532 nm after incubation with a sample, after incubation without a sample, and before incubation without *t*-butyl hydroperoxide or a sample, respectively.

In the linoleic acid autoxidation method,[19] 5.0 ml 0.05 M phosphate buffer (pH 7.0), 5.0 ml linoleic acid solution (1.4% v/v in ethanol), 0.1 ml antioxidant sample solution in methanol, 0.1 ml 1.87 mM $FeCl_2$ aqueous solution, and 0.1 ml 12.5 mM ascorbate aqueous solution were added to 2.2 ml water in a test tube; the mixture was kept at 50°C. After every 48 h, 0.2 ml reaction mixture was taken and colored by vigorously mixing and incubating with a solution containing 9.4 ml of 75% aqueous ethanol, 0.2 ml 30% ammonium thiocyanate, and 0.2 ml 0.02 M $FeCl_2$/3.5% HCl for exactly 3 min at room temperature. The absorbance of the colored solution was measured at 500 nm with reference to a solution incubated without linoleic acid or an antioxidant sample. A blank test was performed without any antioxidant samples.

Antimicrobial activity assay

As a measure of antimicrobial activity, the minimal inhibitory concentrations (MIC) of extracts and β-thujaplicin were determined against bacteria – *Escherichia coli* (ATCC 25922) and *Bacillus subtilis* (IFO 4640) – on a glucose-peptone agar medium and against fungi – *Coriolus versicolor* (IFO 6482) and *Tyromyces palustris* (IFO 0507) – on a potato dextrose agar medium for 7 days at 30°C.

Results and discussion

Heartwood of the Cupressaceae usually contains exceedingly small amounts of β-thujaplicin (often less than 0.1%), and it sometimes contains closely related tropolones such as γ-thujaplicin and β-dolabrin, which are difficult to separate from β-thujaplicin by means of simple methods such as distillation and crystallization. However, a cell suspension culture of *C. lusitanica* accumulates β-thujaplicin in about 20 mg/g cell dry weight,[16] which is easily extracted with ethyl acetate from the cell culture. The content of β-thujaplicin was 9.2% in extract 1 (obtained from the elicitor-treated suspension culture of *C. lusitanica*), whereas extract 2 (from the callus without elicitation) contained only trace amounts of this compound.

Authentic β-thujaplicin exhibited IC_{50} on tyrosinase 0.57 ppm, which was much smaller than that of kojic acid a known strong tyrosinase inhibitor. This result implies that β-thujaplicin is a strong tyrosinase inhibitor (Table 1). The IC_{50} of extract 1, which contained 9.2% β-thujaplicin, was about 10 times that of β-thujaplicin; and extract 2 did not show any activity. This indicates that the tyrosinase inhibitory activity of extract 1 is almost solely dependent on β-thujaplicin in the extract. It is possible that tyrosinase inhibitory activity is expressed by the chelating activity

of β-thujaplicin with copper of the tyrosinase active center.

By the ghost method, antioxidant activity of β-thujaplicin was inferior to BHA and BHT but stronger than that of α-tocopherol (Fig. 1). On the other hand, β-thujaplicin's antioxidant activity based on the linoleic acid autoxidation was close to those of BHA and α-tocopherol but inferior to BHT (Fig. 2). According to Arima et al., scavenging activity of β-thjaplicin against active oxygen species was due to the supression of hydroxy radical formation, such as the Fenton reaction, by the formation of β-thjaplicin–Fe complex.[10] This assumption agreed with our results that superoxide anion scavenging and radical-capturing activities of β-thujaplicin were small (data not shown). However, it is interesting to note that even the β-thujaplicin–Fe complex had almost the same antioxidant activity as does β-thujaplicin itself in the ghost lipid peroxidation, as shown in Fig. 1, though the mechanism of the antioxidant activity exhibited by β-thujaplicin is not clarified at this stage of our study.

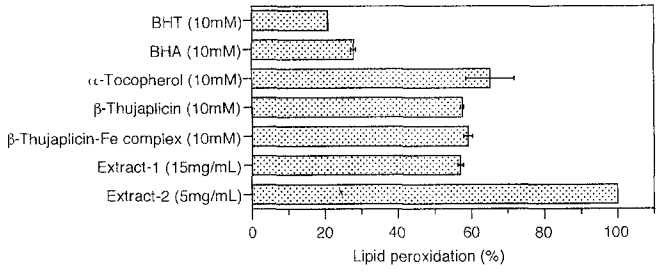

Fig. 1. Antioxidant activity of β-thujaplicin and extracts from *Cupressus lusitanica* cells. The smaller lipid peroxidation by the ghost method denotes stronger antioxidant activity. Error bars denote standard errors. *BHT*, butylhydroxytoluene; *BHA*, butylhydroxyanisole

Extract 1 at 15 mg/ml, which contained about 10 mM β-thujaplicin, had an antioxidant activity almost equal to 10 mM authentic β-thujaplicin by the ghost method. In the linoleic acid autoxidation method analyses, the oxidation rate for 5 mg/ml of extract 1 was similar to that for 0.5 mg/ml of β-thujaplicin. In the case with 15 mg/ml of the extract, the oxidation rate was similar to that with 10 mM β-thujaplicin. On the other hand, extract 2 did not show any discernible antioxidant activity by either assay method. These results suggest that the antioxidant activity of extract 1 is also dependent on the concentration of β-thujaplicin in extracts.

Table 1. Tyrosinase inhibitory and antimicrobial activities of extracts from *C. lusitanica* cell cultures and β-thujaplicin

Sample	Extract-1	Extract-2	β-Thujaplicin	Kojic acid
IC_{50} (ppm) for tyrosinase inhibition	5.01	>100	0.57	1.23
MIC (μg/ml)				
Escherichia coli	800	>3200	100	nd
Bacillus subtilis	800	>3200	50	nd
Coriolus versicolor	100	>3200	12.5	nd
Tyromyces palustris	100	>3200	25	nd

IC_{50}, concentration in which tyrosinase was inhibited by 50%; MIC, minimal inhibitory concentration.

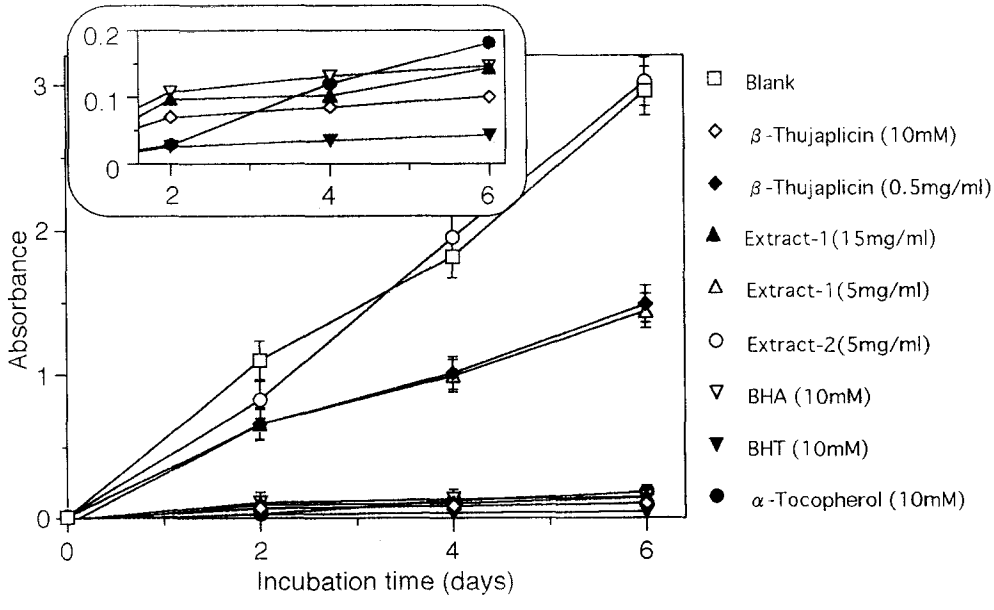

Fig. 2. Antioxidant activity of β-thujaplicin and extracts from *C. lusitanica* cells by the linoleic acid autoxidation method. The smaller absorbance denotes stronger antioxidant activity. Error bars denote standard errors

β-Thujaplicin exhibited a stronger antimicrobial activity against basidiomycetes than against bacteria (Table 1), as already pointed out by Okabe et al.[7] The MICs of extract 1 were four to six times that of authentic β-thujaplicin, whereas extract 2 had neither antifungal nor antibacterial activities. It is therefore expected that the extract 1 from *C. lusitanica* cells can be utilized as a source of antimicrobial material.

Conclusions

The ethyl acetate extract of *C. lusitanica* cell cultures that produce β-thujaplicin by treatment with the elicitor exhibited various biological activities. Therefore, the cell extract of cultured *C. lusitanica* might be a useful source of the bioactive component β-thujaplicin, without its isolation and purification.

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