ANTIFUNGAL SUBSTANCE IN BARK OF IJU (SHIMA WALLICHII SSP. LIUKIUENSIS BLOEMB.)

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ANTIFUNGAL SUBSTANCE IN BARK OF IJU (*SHIMA WALLICHII SSP. LIUKIUENSIS* BLOEMB.)

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Abstract

The methanol extract of bark of Iju (*Schima wallichii ssp. liukiuensis* Bloemb.) contains an antimicrobial substance. This substance inhibits the cell growth of yeast and fungi (eukaryote), but not bacteria (prokaryote). This substance is a triterpenoidal saponine whose components are A_1-barrigenol as sapogenine and four species of sugars, D-glucose, D-galactose, L-rhamnose and D-glucuronic acid in the mol ratio 1:1:1:1.

Introduction

There are many species of poisonous plants belonging to *Theaceae*. Some of them contain antimicrobial substances and antitermitic substances such as *Ternstroemia japonica* Thunb.[1]. Iju, a tall tree, commonly found on plantations at Amami and Lyukyu Islands of Japan, has been used for building materials of interior walls and posts. Fukui[2] reported a poisonous substance for fish in the MeOH extracts of this bark to be saponine-like material. Antitermitic substances were isolated from this bark by Shimamoto et al.[3] and from this wood by Takahashi et al.[4]. This report describes that the MeOH extract of this bark inhibits yeast and fungal cell growth but not bacterial cell growth. Moreover, the antifungal substance was determined as one of the triterpenoidal saponines.

Materials and Methods

Microorganisms Eight species of bacteria and eight species of yeast and fungi were used. The bacteria were: *E. coli* B, *B. mesentericus*, *B. mycoides*, *S. aureus* AHU 1537, *P. aeruginosa* IFO 3080, *P. fluorescens* IFO 3081, *V. parahaemolyticus*, *R. trifolii*. The yeasts were *S. rouxii* OUT 7133 and *S. cerevisiae* IFO 2193. The fungi were: *P. filamentosa*, *P. sasakii*, *Hormodendrun sp.*, *F. solani f. sp. pisi*, *F. oxysporum*. *A. niger* ATCC 6275.

Isolation of saponine. The bark of Iju was kindly supplied by Dr. Haga, of the Okinawa Local Foresty Office in Japan. This was chopped and extracted with hot MeOH,

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and then refluxed in a water bath for 4 days at 70°–80°. After drying, the extract was resuspended in distilled water and dialysed in visking tubes against tap water overnight at 18°. The inner solution was adsorbed to DEAE-cellulose column (200 mesh, column size: 25×400 mm) and the column eluted with 500 ml of 0.1 M NaCl at 20 ml/hr. The eluant was fractionated in a Sephadex LH–20 column (column size: 20×300 mm) at 10 ml/hr with MeOH. The antimicrobial activity of each fraction was checked using *Sacch. cerevisiae* as an indicator. After drying, the active fraction was a yellowish powder.

Determination of sapogenine. The saponine was refluxed with a 50% methanol-1N H_2SO_4 for 6 hrs at 100° and after the MeOH is removed, it was rehydrolysed with 2N H_2SO_4 in a sealed ampule for 20 hrs at 100°. The hydrolysate was extracted with EtOAc and then saponified with methanolic 1N KOH for 2 hrs at 100° and finally treated by steam distillation for 8 hrs at pH 3–4. The remaining part was fractionated in a column of SiO_2 (200 mesh, column size: 12×200 mm) with stepwise elution of the MeOH-$CHCl_3$ mixture (0, 2, 5, 10, 20% MeOH in $CHCl_3$). A colorless needle form crystal was obtained from eluted fractions of both the 2 and 5% mixture. The crystal has a mp of 294–296°.

Determination of the component sugars. The aqueous part of the hydrolysate was neutralized with saturated $Ba(OH)_2$. The ppt removed by centrifugation (10000×g, 20 min) and the upper solution desalted with H_2O in a Sephadex G–25 column (column size: 25×800 mm). Each sugar was determined by paperchromatography. The developing solvents were: A-soln: EtOAc-Py-HOAc-H_2O (5:5:1:3), B-soln: n-BuOH-Py-H_2O (6:4:3), C-soln: PHOH-H_2O (5:1). The papers were developed for 12 hrs (A-soln, B-soln) and for 30 hrs (C-soln) at 20°, using alkaline $AgNO_3$.

Component sugars ratio. Each sugar which had been separated by A-soln was extracted from the paperchromatogram and analysed quantitatively using the colorimetric PHOH-H_2SO_4 reaction. Since D-glucose and D-galactose are not separated this way, their ratio was determined by liquid-chromatography (JECOL JLC-6AS, column: LCR–3, anion-exchanger, 8×150 mm, detector: orcinol-H_2SO_4 method, 425 nm).

Measurement of growth inhibitory activity. The antimicrobial activity of purified saponine was measured using the inhibition ring cylinder plate method. The genera *Escherichia, Bacillus, Staphylococcus* and *Pseudomonous* were tested on a peptone agar medium (PA) for 18 hrs at 37° except for *P. fluorescens* which was incubated at 28°. *Vibrio sp.* was tested on the same medium to which 3% NaCl was added. Yeast was tested on Henneberg agar medium incubated at 28° for 18 hrs. Filamentous fungi precultured on potato-glucose agar medium (PGA) for 3 days at 30° were transferred, using a 5 mm diameter agar block containing the terminal zone of mycelia, to the center of another PGA plate on which stainless steel cylinders were previously positioned 25 mm from plate center. Each cup was then filled with 0.2 ml of saponine dissolved in H_2O (5.0, 0.5, 0.05, 0.005 mg/ml).

Inhibition test of liquid yeast culture. *Sacch. cerevisiae* was cultured for 30 hrs at 30° on rotary shaker in 5 ml of Henneberg liquid medium containing saponine at concentration of 10, 5, 1, 0.5, 0.1 μg/ml. The initial inoculum size was 10^3cells/ml. After the treatment surviving cells were determined by plating and counting colonies.

Results

Saponine. The yield of purified saponine was 7.5 g from 1 kg of fresh bark. The yellowish powder exhibited foaming property in aqueous solution and the Libermann-Burchard reaction was positive. The degradation temp was 224–226°.

Sapogenine. The sapogenine was converted to a colorless crystal of needle form; the molecular formula $C_{30}H_{50}O_5$, the MW of 490 was determined by GC-MS and the mp of 294–296° ascertained. This sapogenine was identified as A_1-barrigenol[5] by mp, IR, MS and NMR spectra of a mixture of our sample and an authentic sample.

Component sugars. The component sugars were determined to be D-glucose, D-galactose, L-rhamnose and D-glucuronic acid in the mol ratio 1:1:1:1 by paper-chromatography and liquid-chromatography.

Microbial assay. This saponine inhibited only yeast and fungi (eukaryote), but not bacteria (prokaryote). The strength of the inhibition was indicated qualitatively in 5 degrees (卅, 廾, +, ±, −) (Table 1). The inhibition of yeast cells in a liquid medium is shown in Fig. 1.

Microscorpic observation of saponine treated yeast cells. The yeast cells treated with saponine were observed to change to a square form and clump. The cytoplasm of these

Table 1. Antimicrobial Activity of Iju-Saponine

Saponine (mg/ml) / Microorganisms	5.0	0.5	0.05	0.005	cont.
E. coli B	−	−	−	−	−
S. aureus	−	−	−	−	−
B. mesentericus	−	−	−	−	−
B. mycoides	−	−	−	−	−
P. aeruginosa	−	−	−	−	−
P. fluorescens	−	−	−	−	−
V. parahaemolyticus	−	−	−	−	−
R. trifolii	−	−	−	−	−
S. rouxii	12.5	−	−	−	−
S. cerevisiae	19.0	12.9	−	−	−
P. filamentosa	廾	+	±	−	−
P. sasakii	廾	+	±	−	−
Hormodendrun sp.	卅	廾	−	−	−
F. oxysporum	±	−	−	−	−
F. solani f. sp. pisi	±	−	−	−	−
A. niger	廾	+	±	−	−

The strength degree expressed as follows;
卅 : very clear, 廾 : clear, + : slight clearing, ± : opaque, − : non inhibition. The number is diameter of inhibition ring (mm).

young cells was released through cell wall. When the saponine treated mature cells were transferred onto normal agar medium, they were able to form colonies.

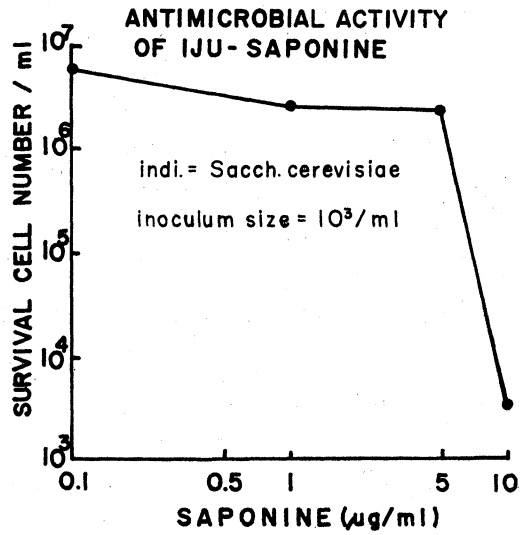

Fig. 1. Growth Inhibition of Yeast Cells in Liquid Medium Containing Iju Saponine.
Sacch. cerevisiae were cultured in 5 ml Henneberg liquid medium with saponine (inoculum size: 1×10^3/ml) by rotary shaker (30 rpm) for 30 hr at 30°.

Discussion

It has been known that higher plants usually contains many antimicrobial substances in the tissues and these substances are synthesized for the defense against bacteria and fungi in both air and soil. For example, some of these as phytoalexines[6] are synthesized in the plant tissues after infection by microorganisms. However, this Iju saponine is present before any infection. The fact that this saponine inhibits only the cell growth of eukaryotes suggests that it action is on the cell wall. However, the reason for these disparities was not determined. Furthermore, it is interesting that saponine-treated mature yeast cells although changed to a square morphology and clumped, were able to form colonies again on normal agar medium. The cytoplasm of yeast cells was released only from young weak cell walls as determined by microscopic viewing. It would appear that this saponine does not inhibit cell wall synthesis directly.

The traditional ways[7] of using Iju were reported in several papers to be either a fish poisons or an antitermitic material. Fukui[2] separated saponine-like substances from the bark of Iju and they reported the poisonous effect on fish. We exmained our purified saponine with cryprinodon as the indicator fish. The minimum fatal dose of this saponine was 5 ppm, for 30 min under room condition. Also, Takahashi et al.[4] separated saponines as antitermitic substances from the wood of Iju. They reported that A_1-barrigenol and R_1-barrigenol as sapongenine bound one mol of esterform to tiglic acid. The component sugars were rhamnose and galactose. However, our isolated saponine had only one kind of sapogenine, A_1-barrigenol, and no other organic matters was present. The component sugars of our compound were 4 species.

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