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Inducing Antibacterial Functions in Paper Fibers through the Chemical Addition of Western Red Cedar Extracts

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Abstract The 7-membered ring compounds, (thujic acid, β-thujaplicin (hinokitiol), nezukone, γ-thujaplicin and methyl thujate) distilled from Western Red Cedar (WRC) by high-pressure steam distillation method have antibacterial and antifungal activities. In our present study, we attempted to find a way to provide antbacterial and antifungal functions to the fabrics of paper fibers made of cellulose. Attention was focused on the bonding of thujic acid and β-thujaplicin with cellulose, and increase the yield of the compounds by adding auxiliary agents or metal ions. It was revealed that the hydroxyl group of β-thujaplicin reacted not only as carboxylic acid but also as phenol. That characteristic could be attributed to the molecular structure of the hydroxyl group resonating to the carbonyl group of β-thujaplicin. This reaction was verified by a model experiment. The quantity of those compounds which combined with the fabrics of paper fibers was determined by GC-MS. It was verified that the fabrics of the paper fibers possessed antibacterial and antifungal functions and that these functions remained even after machine-washing.
Key words : Antibacterial Functions, Thujic acid, β-Thujaplicin (Hinokitiol), Cellulose, Paper Fibers

1. Introduction

Western Red Cedar (*Thuja plicata:* WRC) belongs to Thuja and found on the coast of pacific side of north America. The wood is used for modern house siding, interior paneling, outdoor furniture, totem poles etc.[1], because the oil constituents of the wood have antibacterial and antifungal activity.[2] These constituents are unutilized resources since there is no technology to extract them.

The technology to distill the 7-membered ring compounds in WRC by high-pressure steam distillation method has been developed in our laboratory.[3] We could obtain these compounds by using this distillation technology. One of the effective means of utilizing the 7-membered ring compounds is to bind them with cellulose fibers. We have already found a method of inducing antibacterial and antifungal functions to the fabrics of cotton fibers by the esterification reaction between the carboxyl group of thujic acid and the hydroxyl group of cellulose of cotton.[4]

In our present study, we attempted to find a way the functions of antibacterial and antifungal activities to the fabrics of paper fibers. The experiments were conducted by the following two approaches. The first experiment was to find the optimum condition to increase the reaction between the carboxyl group of thujic acid and the hydroxyl group of cellulose. The second experiment was to determine the reactivity of the hydroxyl group of β-thujaplicin and its combination with the hydroxyl group of cellulose. We used paper fibers as the fabrics of the cellulose fibers because of the advantage of low weight and static-free ability. (The cellulose was utilized as the fabric of paper fibers in this report.)

The quantity of the chemical bonding between the carboxylic group of the thujic acid and the hydroxyl of cellulose was increased by adding the salts or metal ions. It was considered that the anion surface of cellulose[5,6] in the solution was changed to the cationic surface by adding the salts or the metals. It was investigated that the characterization of the hydroxyl group of β-thujaplicin by the reaction of both trimethyl-silyldiazomethane (TMDM) which reacts like diazomethane[7] and acetic acid anhydride. If β-thujaplicin is closer to the carboxylic group, it can react with the hydroxyl groups of cellulose. It was also conducted by model experiment between 1-butanol and β-thujaplicin. The relationship between the quantity of thujic acid which bonded with cellulose and the antibacterial activity was investigated.

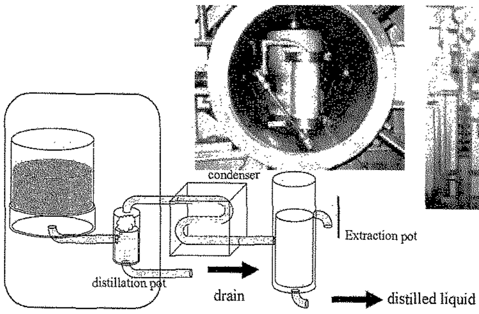

Fig.1 High-pressure steam distillation apparatus.

2. Experiment

2-1 The fixing of functional constituents of WRC distillation and testing for antibacterial activity

The WRC distillate was obtained by high-pressure steam distillation apparatus (Fig.1) .[3)]

The fabrics of paper fibers samples (5 cm×5 cm, 0.5 g) were soaked in 1.5 ml of WRC distillate for 1 hour at room temperature. The samples were heated at 120℃ for 5 min and the reaction was made to proceed after spraying the fabrics with low concentration of D-camphor-10-sulfonic acid in 1,4-dioxane, and machine-washed with water. After each process, the samples were dried.

Sodium chloride (NaOH) was used as auxiliary agents, Al^{3+} and Cu^{2+} was used as metal ions to increase the bonding of functional constituents of WRC distillation. 0.86 M, 1.0 M, 1.2 M, 1.4 M, 1.7 M of NaOH chloride were dissolved in WRC distillation and treated by the aforementioned method. 1 % of each of the ions Al^{3+} and Cu^{2+} were dissolved in water and soaked in the WRC distillation and treated aforementioned method.

All the samples prepared in the aforementioned method were machine-washed with alkali detergent to remove unbinding treatments, and the antibacterial activity was assayed in accordance with JIS L1902 criteria.

2-2 The quantity of the 7-membered ring compounds combined with paper fibers

The 7-membered ring compounds in WRC that were chemical bonded to the samples were eluted by hydrolysis. The sample was boiled in 3 % of sodium hydroxide for 1 hour after which 6 N hydrochloric acid was added until a pH 3. The ethyl acetate was added to the elution to extract the organic constituents, and 3,4-dimethoxy-benzyl alcohol was added as an internal standard. The acid constituents and water were removed from the organic constituents while the solvent was evaporated from the organic constituents. The organic constituents were then analyzed by the GC-MS (Shimazu QP5050) on a DB-5MS column (ϕ0.25 mm × 30 m, Shimazu) using helium carrier gas. For the first shot, the temperature of the pyrolyzer was maintained at 60 ℃ for 5 minutes, and then raised to 260 ℃ at a rate of 10 ℃/min and maintained for 5 minutes. The data was measured five times and the average was taken.

2-3 The reactivity of β-thujaplicin

Methylation: A quantity of 100 mg of β-thujaplicin was dissolved in a methanol and hexane (2:7) mixture. By a dropwise addition of the trimethylsilyldiazomethane (TMDM), the color of the mixture changed from transparent to yellow and later back to transparent. This process continued until the color was yellow and did not return to transparent. Hexane was added to the mixture to remove the TMDM by azeotropy after evaporation. The organic constituents were extracted from the mixture. And the organic constituents were analyzed by GC-MS and ^{1}H-NMR.

Acetylation: An amount of 10 mg of β-thujaplicin was dissolved in 2 ml acetic acid anhydride and 2 ml pyridine mixture. The acetylation was allowed to react at room temperature for 24 hours. The organic constituents were analyzed by the abovementioned method.

Etherification: An amount of 100 mg of β-thujaplicin was heated to reflux in 150 ml of anhydrous 1-butanol with 40 mg of D-camphor-10-sulfonic acid for 6 hours as condensed dehydration. The organic constituents were extracted from 1-butanol and the organic constituents were analyzed by GC-MS and ^{1}H-NMR. Thujic acid was used for comparison.

3. Results and Discussion

3.1 Fixing the functional constituents of WRC distillation and the testing for antibacterial activity

It found that the constituents in the distillation of WRC mainly consist of the five constituents. (thujic acid, γ-thujaplicin, β-thujaplicin, nezukone and methyl thujate) (Fig.2)

The quantity of the thujic acid which bonded with cellulose at adding of auxiliary agents and metal ions are shown in Table 1. It indicates that the quantity of the thujic acid increased until the concentration of 1.4 M of NaOH, and decreased over the concentration. The possible reason for the increase in the quantity of the thujic acid was that Na^+ ion positively increased the residual charge of the ξ-electron potential of cellulose surface,[5, 6)] allowing the thujic acid to approach the cellulose surface.

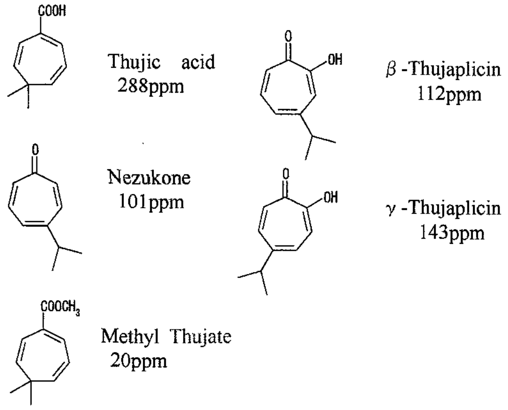

Fig.2 The 7-membered ring compounds and the concentration in the WRC distillation.

On the other hand, it was considered that the quantity of the thujic acid which bonded with cellulose in a treated of NaOH sample with a concentration over 1.4 M decreased by salting-out effect. The maximum quantity is brought about by the opposing effects of reducing negative ξ-electron potential of cellulose surface that increase the quantity of the thujic acid which bonded with cellulose and the salting-out effect that decrease the quantity of the thujic acid. The concentration of 1.4 M of NaOH was the maximum quantity of the thujic acid which bonded with cellulose.

Next, the testing for antibacterial activity with MRSA (*Methicillin resistant Staphylococus aureus)* was carried out. In the absence of NaCl as auxiliary agents, the value for the antibacterial activity and the biocidal activity were 0.99 and 3.14 respectively. Otherwise, it was significantly antibacterial with NaCl as auxiliary agents, and the value for the antibacterial and biocidal activities were over 2.38 and over 4.56 respectively. Based on the standard which stipulates a positive antibacterial activity and a bacteriostatic activity of over 2.2, these values without NaCl will be considered as minimum concentration of antibacterial activities for this bacterium. To observe the differences in antibacterial activity under various conditions, testing for antibacterial activities was carried out after the samples were cleaned by ultrasonic cleaner for 2 minutes. The results of testing for antibacterial activity are shown in Table 2.

Table 1 Content of thujic acid bonded to the fabrics of paper fibers with added chemicals

Treated Conditions	Thujic acid (μg/g of Paper Fibers)
None	1.63
0.86 M-NaCl	2.02
1.0 M-NaCl	2.75
1.2M-NaCl	3.04
1.4 M-NaCl	3.38
1.7 M-NaCl	2.75
1 % $Cu(OH)_2$	13.6
1% $AlK(SO_4)_2$	3.8

There was maximum antibacterial activity for *E.Coli.* in the 1.4 M-NaCl treated sample. The highest yield of thujic acid bonded with cellulose occurred in the 1.4 M-NaCl treated sample. It could be considered that the constituents which had antibacterial activity was the thujic acid.

3.2. The reactivity of β-thujaplicin

β-Thujaplicin rapidly reacted with the TMDM to produce methyl-β-Thujaplicin, indicating that the hydroxyl group of β-thujaplicin functioned as carboxylic group. β-thujaplicin also reacted with acetic acid anhydride and the product's quantity was about half that of phenol. The result showed that the hydroxyl group of β-thujaplicin also reacted as the hydroxyl group of phenol. The reactivity of the β-thujaplicin's hydroxyl group was not only close to carboxyl group[8] but also to that of phenol. This reaction could be attributed to the molecular structure of the hydroxyl group resonating to the carbonyl group.

The reaction between β-thujaplicin and the hydroxyl group of 1-butanol which was used as the hydroxyl group for cellulose model was investigated. The results are shown in Fig.3. The β-thujaplicin was observed at a retention time of 15.6 min and β-thujaplicin butylethers (isomers) were, 20.2 min and 20.7 min. These products represented 5.9 % of β-thujaplicin. Otherwise the produced thujic-butyl-ester was 31 % of thujic acid. The reaction speeds of β-thujaplicin-butyl-ethers were far fewer and slower than that of the thujic acid.

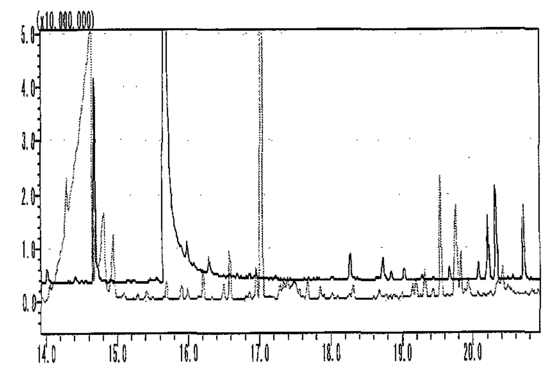

Fig.3 The retention time of GC of β-thujaplicin (15.6 min) and β-thujaplicin-butylethers (20.2 min and 20.7 min) (upper line); thujic acid (14.5 min) and thujic-butylester (17.1 min) (lower line)

Table 2 Antibacterial activity tests for *E.Coli* performed on paper yarn fabrics treated with WRC extracts.

	Viable Count(18h)	Biocidal Activity	Bacteriostatic Activity
Initial Number of fungi	1.09×10^5	–	–
Standard cotton	1.28×10^7	-2.07	–
None	9.10×10^4	0.08	2.15
0.86 M-NaCl	2.36×10^5	-0.34	4.16
1.0 M-NaCl	2.69×10^5	-0.39	2.73
1.2M-NaCl	2.133×10^5	-0.29	2.44
1.4 M-NaCl	below 20	above 2.74	above 4.81
1.7 M-NaCl	7.00×10^4	0.19	2.26

The standard for all experiment was untreated standard cotton sample.

4. Conclusion

Literature[2, 9] shows that tropilidine and tropone exhibited less bactecidal activity than β-thujaplicin. The unsubstituted 7-membered ring structure (tropilidine) has antibacterial activity, and that substitution with a single keto group (tropone) does not increase bacteristatic or bacteriocidal activity. However, the thujic acid is not studied in these literatures, because it could not be obtained before using the high-pressure steam distillation method.

From the results of testing for antibacterial activity, it was considered that the thujic acid had a strong antibacterial activity which was the main components bonded with the cellulose, even if the carboxylic groups had changed to ester groups.

The optimum quantity of thujic acid which bonded with cellulose is 1.4 M-NaCl sample, and the most antibacterial activity for *E. Coli.* was observed in the sample treated with 1.4 M-NaCl and the sample treated with Cu^{2+}.

The reactivity of the group of the β-thujaplicin was similar to that of the carboxyl group[8] as well as that of phenol. The β-thujaplicin could bond with the hydroxyl groups of cellulose and both the quantity of β-thujaplicin-ethers and the reaction speed of β-thujaplicin were far fewer and slower than that of the thujic acid.

It was verified that the fabrics of the paper fibers gained antibacterial and antifungal activities and that these functions remained even after machine-washing with alkali detergent.

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