Transactions of the Materials Research Society of Japan 28 [4] 1045-1048 (2003)

Products and Antibacterial Activity of Thermolysis of Apple Lees

Haruo Kitahara[1], Yukako Kitayama[1], Daigo Ishikawa[1], Taro Sakurai[1],
Jun Kawakami[2], Masahiko Nagaki[2], Tooru Fukui[3] and Toshihiro Okabe[4]

[1] Department of Chemistry, Faculty of Education, Hirosaki University

1 Bunkyo-cho, Hirosaki 036-8560, Japan

Fax: +81-72-39-3364, e-mail: hkyk1015@cc.hirosaki-u.ac.jp

[2] Department of Materials Science and Technology, Faculty of Science and Technology, Hirosaki University

3 Bunkyoucho, Hirosaki 036-8561, Japan

Fax: +81-172-39-3570, e-mail: jun@cc.hirosaki-u.ac.jp

[3] Byotai-seiri Laboratory

47-11 Kumanomachi, Itabashiku, Tokyo 173-0025, Japan

Fax: +81-3-3956-4101, e-mail: tooru.fukui@byotai.or.jp

[4] Industrial Research Institute of Aomori Prefecture

80 Fukuromachi Hirosaki 036-8560, Japan

Fax: +81-172-36-2105, e-mail: koug-okabe@net.pref.aomori.jp

Apple lees are used as a starting material in the manufacturing of woodceramics. The heating of apple lees with resin in a baking furnace produces woodceramics and, at the same time, produces a by-product known as wood vinegar. Wood vinegars consist of many chemical compounds and have a variety of bioactivities. In this paper, antibacterial activities of wood vinegar have been examined with separating extracts. From results, we confirmed that wood vinegar has antibacterial activity against staphylococcus aureus.

Key Words : Wood Vinegar, Antibacterial Activities, Apple lees, *Staphylococcus aureus*,

1. INTRODUCTION

In the manufacturing of woodceramics from apple lees, wood vinegars are produced as a by-product. It was well-known that wood vinegars consist of many chemical compounds and have a variety of bioactivities[1-3]. The chemical compounds consist of organic acids, phenolic compounds, carbonyl compounds and alcohols. The typical chemicals from wood vinegars are summerized in Table 1. However, there has been little scientific evidence of a correlation between chemical structures and bioactivities. Concerning this point, we tried to find the bioactive compound of wood vinegar.

Table 1. The typical chemicals from wood vinegars

Organic acid	acetic acid, propionic acid, crotonic acid
Phenol compound	guaiacol, p-cresol, vanillin, pyrogallol
Carbonyl compound	acetone, acetoaldehyde, cyclopentanone
Alcohol	methylalcohol, propylalcohol, allylalcohol

2. MATERIAL AND METHOD

Material

Wood vinegar made from apple lees in the manufacturing of woodceramics was provided by Hiroka corporation (Hirosaki, Japan).

Extraction

Wood vinegar (50ml) saturated with salt (20g) was stirred with ether (300ml) for 30min within a nitrogen-rich atmosphere. This procedure was performed four times and the combined ether extracts were evaporated and dried *in vacuo*. The weight of ether extracts and the aqueous layer were 2.70g and 86.08g, respectively.

Distillation

A short-pass distillation apparatus was used for distillation.

Separation

Two methods were performed for the separation of extracts from wood vinegar. One (Figure 2) was low pressure liquid chromatography (LPLC) on glass column (300 × 10 i.d.mm) with silica gel 60N (Merck Cat.No.37561-79) and the another (Table 3) was a flash chromatography apparatus (EYELA CERAMIC POMP VSP-2050, EYELA RI-UV MONITIR RI20UV). In the case of LPLC, extracts (7.12g) from wood vinegar (100ml) were separated with five solvents (200ml); n-hexane-AcOEt (5:1) , n-hexane-AcOEt (1:1) ,n-hexane-AcOEt(1:5), AcOEt and MeOH; Fr. Ⅰ (1.06g), Fr. Ⅱ (1.67g), Fr. Ⅲ (0.89g), Fr. Ⅳ (0.43g), and Fr. Ⅴ (0.31g). Separation of Fr. Ⅱ (94.6mg) with Flash chromatography (solvent rate 3ml/min) were separated to four more fractions, Fr. Ⅱ a (retention time 6-12min.,29.2 mg), Fr. Ⅱ b (retention time 12-33 min.,19.4 mg), Fr. Ⅱ c (retention time 33-48 min.,0.6 mg), Fr. Ⅱ d (retention time 48-56 min., 6.9 mg) with n-hexane-AcOEt (10:1, Fr. Ⅰ a-c.) and MeOH (Fr. Ⅱ d).

Biological Activity Test

Each sample was dissolved with dimethyl sulfoxide (DMSO) to 10 mg/ml, diluted with sterile and then distilled water to 1.0 mg/ml for biological activity tests. Minimum inhibitory concentrations (MICs) were determined by using an agar dilution method with 24-well tissue culture plates (Becton Dickinson, USA). The test was performed by Mueller Hinton Ⅱ Agar (BBL, Becton Dickinson, USA). Agar dilution susceptibility testing was performed as follows : a series of eleven two-fold dilutions in sterile distilled water were prepared for each test sample and 300 μl was added to the wells of each plate. The equivalent volume of two-fold concentrated medium, kept at 50℃ after autoclaving, was mixed and stirred rapidly. The sterile, distilled water was used as reference. The agar plates were cooled down to room temperature and S. aureus was cultured in Mueller Hinton Broth (BBL, Becton Dickinson, USA) at 35 ℃ for 18 hrs and the turbidity was adjusted to that of a No.0.5 McFarland standard (108 CFU/ml) with sterile physiological saline. The suspension of 1 μl (105 CFU) was spotted onto the samples containing Mueller Hinton Agar plate in each well. These tests were all duplicated. All plates were incubated at 35 ℃ for 18 hrs. The lowest concentrations with optically negative growth were MICs. When the MIC levels in duplicate tests were different, the higher concentration was adopted

3. RESULTS AND DISCUSSION

The two separation methods, ether extraction and distillation, comparing the antibacterial activities of each fraction were performed (Figure 1, Table 2 and Table 3).

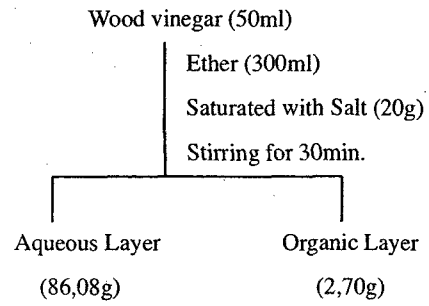

Figure 1. Ether extraction from wood vinegar

Table 2. Distillation of wood Vinegar.

Fraction	Temperature* (℃)	yield (%)
1	r, t. ~ 100	0,64g (1,3)
2	100 ~ 150	46,14g (92,3)
3	150 ~ 200	0,33g (0.6)
4	Residue	< 2.09g (4,2)

* Temperature is oil bath.

The ether extraction method has the advantage of good yield and good bioactivity. Extraction was the same as before, and ether extract was separated into five

Table 3. Antibacterial activity (MIC:mg/ml) of Distillation fraction and ether extract fraction

	Fusarium sp.	Botritis cinerea	Staphylococcus.aureus	E. coli	P. aeruginosa
Ether extract					
Organic layer	0,20	3,13	0,78	0,78	0,78
Aq. Layer	12,5	50	12,5	12,5	12,5
Distillation					
Fr, 1	5	1,25	5	5	5
Fr, 2	3.13	12.5	1.56	1.56	0.78
Fr, 3	5<	5<	5<	5<	5
Fr, 4	5	5	0.31	1,25	1,25
Reference					
Hinokithiol	0,05	0,1	0,1	0,1	0,2
Hiba oil	1,6	3,2	0,8	3,2	3,2

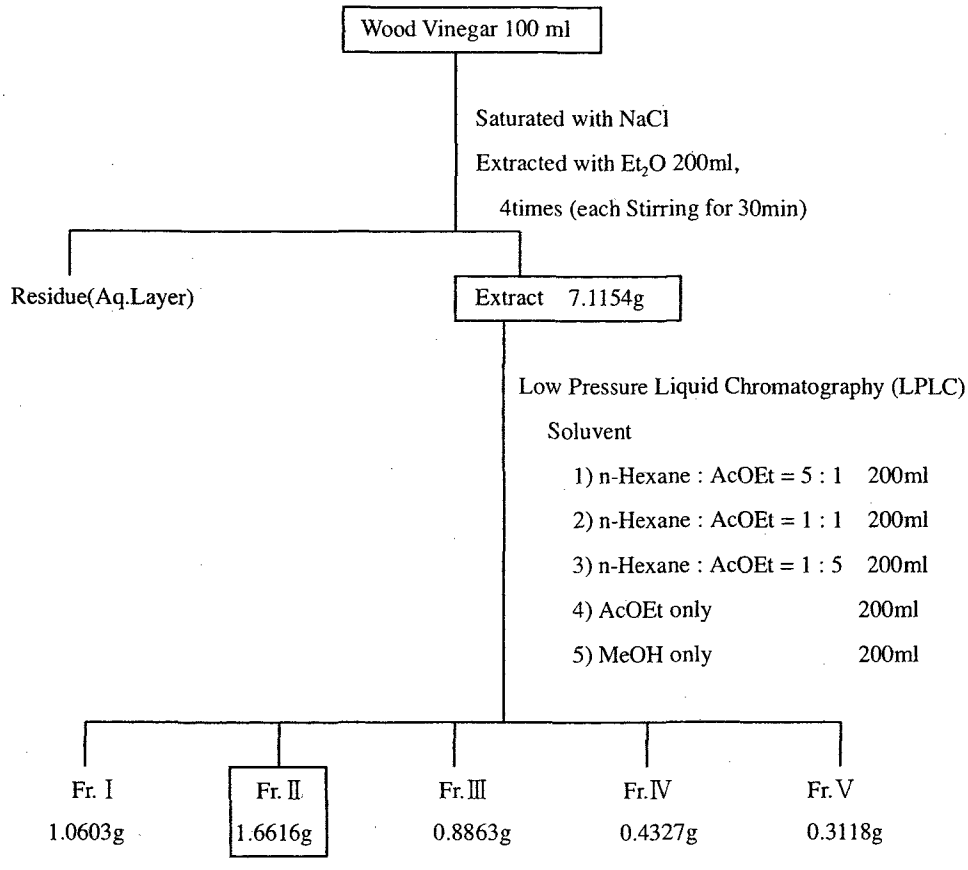

Figure 2. Extraction of wood vinegar and Separation with Low Pressure Liquid Chromatography

fractions (Fr. Ⅰ, Fr. Ⅱ, Fr. Ⅲ, Fr. Ⅳ and Fr. Ⅴ) by low pressure liquid chromatography (Figure 2). The five fractions were tested for antibacterial activities against the four species (Table 4). Fr. Ⅱ produced a good yield and good activity against Staphylococcus aureus (MIC 0.78). S. aureus causes a variety of suppurative infections and toxinoses in humans. It causes superficial skin lesions such as boils, styes and furuncles, and it also causes food poisoning by releasing enterotoxins into food. Nargenicin A and magunol have been known as the

Table 4. Antibacterial Activity of Fraction of Low Pressure Liquid Chromatography

	Staphylococcus aureus	Escherichia coli	Pseudomonas aeruginosa	Botrytis cinerea
Fr. I	3200	800	800	12800
Fr. II	6400	800	800	1600
Fr. III	3200	800	3200	1600
Fr. IV	1600	800	800	1600
Fr. V	6400	800	800	2000

Table 5. Separation of Fr. II (94. 6 mg) with Flash Chromatography apparatus

Fraction	Fr. II a	Fr. II b	Fr. II c	Fr. II d
Retention Time (min)	6 – 12 min	12 – 33 min	33 - 48 min	48 - 56 min
Yield (mg)	29.2	19.4	0.6	6.9

Table 6. Antibacterial Activity of fractionated samples against Staphylococcus aureus

Fraction	MIC (μ g/ml)
II a	500
II b	125
II c	15. 6
II d	250

typical antibacterial compounds against S.aureus[4]. However studies of antibacterial activity of wood vinegar against S.aureus have not been reported. Interested in these activities, the antibacterial activities against S. aureus was tested using fraction II. With the flash chromatography apparatus, four fractions (Fr. II a, Fr. II b, Fr. II c and Fr. II d) were created and tested for antibacterial activity (Table 5 and 6). Although the amount of fraction II c was the smallest (0.6 mg) of the four fractions, it has highest antibacterial activity (MIC 15.6 μ g/ml). No detection of this fraction in UV absorption suggestes that bioactive compound had not aromatic function. High antibacterial activity fraction II c consisted of three closely related compounds on a thin layer chromatography.

4. CONCLUSIONS

Antibacterial activities of wood vinegar has been examined. From the results we confirmed that wood vinegars had high antibacterial activity against staphylococcus aureus and we succeeded in obtaining strong antibacterial compounds from wood vinegars.

Continuing this research, we will work to isolate the antibacterial compound from fraction II c.

5.REFERENCES

1. M.Yatagai and G Unrinin, Mokuzai Gakkaishi, **35**, 1021-1028 (1989).

2. T.Uehara, Y. Horio, T. furuno and S.Jodai Mokuzai Gakkaishi, **39**, 1415-1420 (1993).

3. Y. Takahara,, K. Katoh, R .Inaba, and H. Iwata, Nippon Koshu Eisei Zasshi. **41**, 147-156 (1994). and there cite in.

4. U.Sankawa, " Comprehensive Natural Products Chemistry " , ed. by D.H.Barton and K.Nakanishi, Pergamon (1999), Vol.**1**.

(Received December 20, 2002; Accepted Octorber 11, 2003)