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Antimicrobial Effectiveness of Pine Needle Extract on Foodborne Illness Bacteria

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Abstract Fresh pine needles were collected and extracted with 95% methanol and the extract was concentrated to determine its antimicrobial activity. The methanol extract had a considerable inhibitory effect on the tested bacteria, such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Staphylococcus aureus*. The methanol extract of pine needles was further fractionated to chloroform, ethylacetate, butanol, and water fractions. Among these four fractions, the butanol and water fractions, which showed a relatively strong inhibitory effect on all of the tested bacteria, were purified and the minimum inhibitory concentration (MIC) was determined for each microorganism. The MIC ranged between 25 mg/ml and 45 mg/ml depending on the microorganism. The purified active fractions were applied to sterilized milk as a model food system to define the antimicrobial effectiveness and it was found that the antimicrobial activities in the water fractions were stronger than those in the butanol fractions.

Key words: Antimicrobial effectiveness, pine needle extract, foodborne illness bacteria

Although artificial food preservatives are generally used to inhibit the growth of food spoilage microorganisms, it has been recognized that antimicrobial substances that inhibit microbial growth exist in various plants [2, 14, 18]. Accordingly, a variety of research investigating the antimicrobial activities in plants and their application to food preservation has been conducted [3, 5, 7]. According to Beuchat [3], antimicrobials in plants, such as enzymes, specific proteins, organic acids, fatty acids, essential oils, the substances related to pigments, humulons and lupulons, oleuropein, caffeine, theophylline and theobromine, and phytoalexins, exist either naturally or are produced by physico-chemical stress. Among the many plants used as spices or flavoring materials, thymol [15], the essential oils of thyme and oregano; cinamic aldehyde [7], the extract of cinamon; eugenol [10] of cloves; and vanillin [5] of vanilla beans are all known as principal antimicrobial components. The flavonols, proanthocyanins, and anthocyanin present in the flowers of higher plants [9] are classified as nontoxic antimicrobial substances and are widely used as food colorants. Hartman [12] reported that pelargonidin-3-monoglucoside and its degraded product exhibit inhibitory activities toward *Escherichia coli* and *Staphylococcus aureus*, and Marwan *et al.* [16] reported that the antimicrobial activity of cranberries can be attributed to the flavonol and proanthocyanins on the growth of *Saccharomyces boyancis*.

The pine tree, which is an evergreen needle-leaf tree, has always been considered as a natural resource contributing to human health in east Asia [4]. Various parts of the pine tree, such as the pine needles, cones, cortices, and pollen, have been widely used for promoting health as folk medicine or as food. It has been documented that pine needles and pine cortices were used as food to relieve famine during severe drought [1, 21]. According to Buddhist scripture, pine needle extracts were commonly used as a tonic [22].

Recently, in Korea, there is growing public interest in incorporating pine needle-based products into diet because of their health-promoting properties. Despite this attitude, very little information exists currently concerning the nutritional value and systematic evaluation of the manufacturing methods of pine teas, and their antimicrobial substances. The objectives of this study were to (1) prepare a methanol extract of pine needles and then fractionate the extract with organic solvents and (2) determine the antimicrobial effectiveness of the solvent fractions toward four foodborne illness bacteria.

Materials and Methods

Preparation of Methanol Extract

Ten kilograms of fresh pine needles were selected and harvested from Korean red pine tree (*Pinus densiflora*

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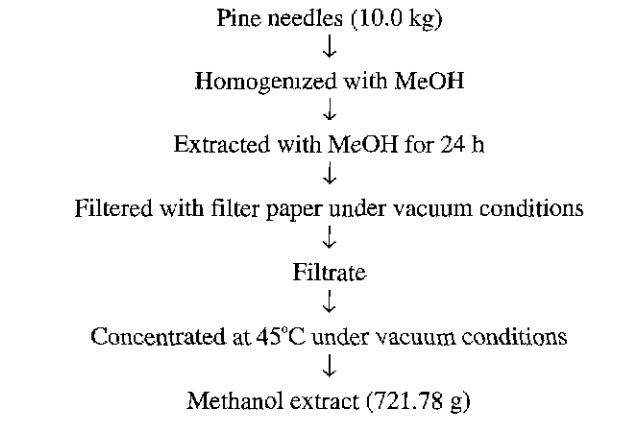

Fig. 1. Preparation procedure for methanol extract of pine needles.

Sieb. et Zucc.) in late April in Changseung, Korea. The samples were chopped with a knife into approximately 2-cm pieces, grounded for 1 min in an Osterizer (John Oster Manufacturing Company, Milwaukee, U.S.A.), and extracted by soaking in a ten-fold volume of methanol for 12 h. The same extraction procedure was repeated three times and all the extracts collected were combined and referred as the methanol extract of pine needles (Fig. 1). The methanol extract was filtered through Whatman No. 2 filter paper and concentrated in a vacuum evaporator (Type N-N, EYELA, Japan) at 45°C.

Solvent Fractionation of Methanol Extract

The methanol extract was initially fractionated with hexane, which is a solvent with a low polarity, followed by fractionation with chloroform, ethylacetate, butanol, and water, in an increasing order of their polarity (Fig. 2).

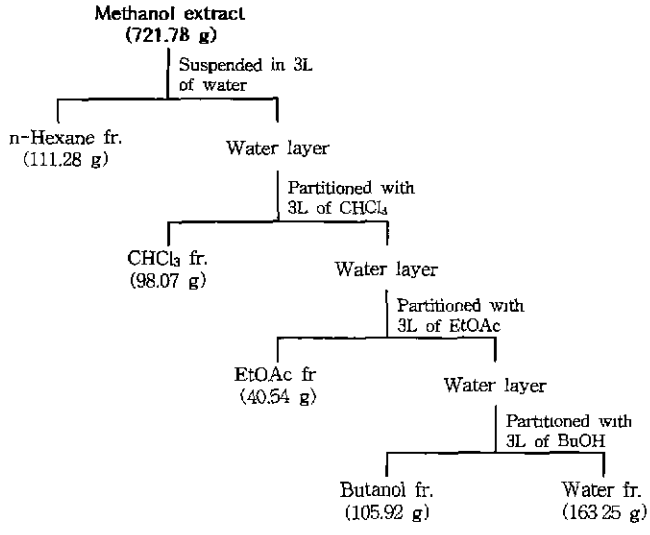

Fig. 2. Solvent fractionation of methanol extract of pine needles.

Test Microorganisms

The bacteria used in this study were obtained from the Department of Food Science and Technology, Washington State University, U.S.A., except for three strains of *S. aureus* which were purchased from the American Type Culture Collection (ATCC) for the antimicrobial activity test.

Media

The microorganisms were maintained on selective agar slants at 4°C and transferred monthly to maintain viability. A working culture was prepared by inoculating a loopful of culture into 5 ml of broth and incubated at 37°C for 18 h. All the *S. typhimurium* and *E. coli* O157:H7 strains were grown in a trypticase soy broth (TSB, Difco, Detroit, U.S.A.) whereas the *Listeria monocytogenes* and *S. aureus* strains were grown in a tryptose phosphate broth (TPB, Difco) and brain heart infusion broth (BHIB, Difco), respectively.

Determination of Antimicrobial Activity

The antimicrobial activity of the pine needle extract on the growth of the test microorganisms was determined using the paper disc method [19]. Culture solutions of the test bacteria were prepared by growth in broth media at 37°C for 18 h. During the stationary phase, the cells were serially diluted in a 0.1% (w/v) peptone solution to obtain initial microbial concentrations of approximately 10^4 cfu/ml prior to use as an inoculum. At first, 1 ml of the inoculum was transferred onto a petri dish and mixed with 18 ml of agar media by gentle shaking, and then the plate was maintained at 50°C in a water bath. The desired concentration for each fraction was loaded on a sterile filter paper disc (Ø 6 mm, Difco, Detroit, MI, U.S.A.), air dried, and then placed on the surface of an inoculated plate prepared in advance. The loaded extract on the paper disc was diffused into the agar plate by adding a 0.85% NaCl solution. The plate was incubated at 37°C for 24 h and the diameter of the resulting clear zone was then measured.

Purification of Butanol and Water Fractions

The water and butanol fractions containing significant amount of antimicrobial activities were purified through a column (4.1×50 cm) packed with silica gel by eluting with a methanol-chloroform mixture (3,500 ml), which consisted of a gradient from pure methanol to 100% (v/v) chloroform, at a rate of 1.0 ml/min. During the purification, the antimicrobial activity for each elution volume was examined prior to proceeding to the next purification step.

The eluate (500 ml) between 2,901 and 3,400 ml of the water fraction was confirmed to contain antimicrobials, and was further purified on a Sephadex-LH 20 column. The active antimicrobial eluate (85 ml) between 171 and

255 ml was collected and concentrated to a final weight of 58.0338 g.

The butanol fraction was purified following the same procedure as for the water fraction, and the antimicrobial activity was observed in the eluate (400 ml) collected between 2,801 and 3,199 ml. The active antimicrobial eluate (225 ml) on Sephadex-LH 20 column was collected from an elution band between 241 and 465 ml and concentrated to a final weight of 67.54 g.

Determination of Minimum Inhibitory Concentration (MIC)

After confirming their purities by thin layer chromatography (TLC), both the water and butanol fractions were subjected to determine the minimum inhibitory concentration (MIC) using the broth dilution assay method [22].

Two tenth ml of both fractions was added to 1.7 ml of a broth medium and mixed with 0.1 ml of the prepared inoculum with 10^3–10^4 cfu/ml as the initial number for the test microorganisms. The MIC value was defined as the lowest concentration by which the number of the colony was reduced by 10% after incubation at 37°C for 24 h.

Application of Active Fractions to Model Food System

To determine the antimicrobial effect of both fractions in sterilized milk (Haitai Beverage Inc., Korea) as a model food system, the water and butanol fractions with a five-fold concentration of the MIC were applied to sterilized milk and the same procedure was followed as for the MIC determination. The strength of the growth inhibition was defined as the viable cell counts on the plates after incubation at 7°C or 25°C for 7 days.

RESULTS AND DISCUSSION

Antimicrobial Activities in Solvent Fractions of Methanol Extract of Pine Needles

One half mg of each fraction, which is equivalent to a 1 g sample of pine needles, was loaded on a disc and compared with the activity of butylated hydroxyanisole (BHA), known to be an active antimicrobial additive, as a reference.

The hexane and chloroform fractions did not exhibit any activity, however, it was found that the ethylacetate, butanol, and water fractions inhibited the growth of the tested microorganisms (Table 1). The ethylacetate fraction had a comparatively weak activity producing clear zones with 9 to 12 mm diameters, whereas the butanol and water fractions had strong antimicrobial activities with inhibitory zones of 11 to 15 mm diameters, which were stronger than BHA. This indicates that the pine needle extract was highly active against both Gram-negative and Gram-positive bacteria, which is not common to other antimicrobial substances characterized in previous studies [14, 15].

Minimum Inhibitory Concentration (MIC) of Butanol and Water Fractions

The MIC value of the purified butanol on *S. typhimurium* and *S. aureus* was 25 mg/ml which was higher than

Table 1. Antimicrobial activities in solvent factions of methanol extract of pine needle against foodborne illness bacteria.

Microorganisms	Inhibitory zone (mm)					
	Hexane fr.	$CHCl_3$ fr.	EtOAc fr.	BuOH fr.	H_2O fr.	BHA[a]
Salmonella typhimurium DT104 WSU 2380[b]	-[e]	-	10[f]	12	13	10
Salmonella typhimurium DT104 WSU 2576	-	-	10	13	13	10
Salmonella typhimurium DT104 WSU 2582	-	-	10	12	14	9
Escherichia coli O157:H7 WSDH WSU 54[c]	-	-	10	11	13	11
Escherichia coli O157:H7 WSDH WSU 55	-	-	10	13	14	10
Escherichia coli O157:H7 WSDH WSU 251	-	-	10	11	13	11
Listeria monocytogenes ATCC 19114[d]	-	-	9	14	13	10
Listeria monocytogenes ATCC 19115	-	-	9	12	11	11
Listeria monocytogenes ATCC 19116	-	-	10	15	14	11
Staphylococcus aureus ATCC 12600	-	-	11	13	13	11
Staphylococcus aureus ATCC 13566	-	-	10	13	13	10
Staphylococcus aureus ATCC 25923	-	-	12	12	13	11

[a]Butylated hydroxyanisole.
[b]Dairy Technology.
[c]Washington State Department of Health, Washington State University.
[d]American Type Culture Collection
[e]Not inhibited.
[f]Inhibitory zone of paper disc (6.0 mm in diameter).

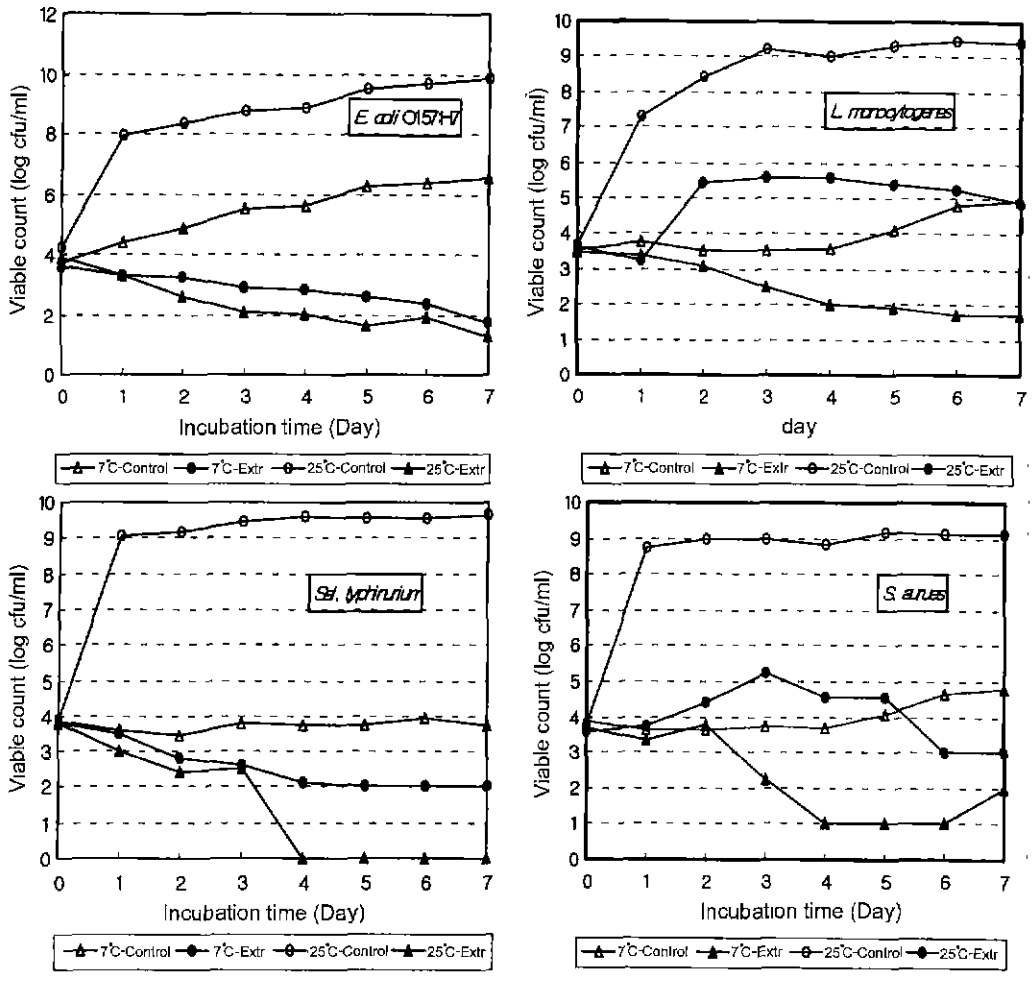

Fig. 3. Growth inhibition of H_2O fraction from methanol extract of pine needles against food-borne illness bacteria in sterilized milk as a model food system at 7°C and 25°C.

30 mg/ml on *E. coli* O157:H7 and 35 mg/ml on *L. monocytogenes*. This result indicates that the MIC values were not notably different according to the tested microorganisms. However, the MIC values in the water fraction were different depending on the testing bacteria, with the lowest value of 25 mg/ml with *S. typhimurium* and the highest value of 45 mg/ml with *L. monocytogenes*. Farag *et al.* [11] reported that the MIC values (mg/ml) of thyme oil were 1.25 and 0.25 for *St. aureus* and *S. typhimurium*, respectively. In addition, the present study indicated that there was no linear relationship between the antimicrobial activities and the MIC values, indicating that the susceptibility towards the natural antimicrobials differed depending on the microorganism tested [19].

Inhibitory Effectiveness in Model Food System

In testing milk as a model food system, the water fraction of the pine needles inhibited the growth of the four tested bacteria at 7°C and 25°C (Fig. 3). The growth of *S. typhimurium* and *E. coli* O157:H7 was inhibited from the initial period of incubation, however, the growths of *St. aureus* and *L. monocytogenes* were not much affected. Payne *et al.* [18] found that the phenolic compounds in a model milk system prevent the growth of *L. monocytogenes* for a 24 h incubation period.

The butanol fraction of the pine needles showed a different antimicrobial inhibition pattern on the tested bacteria compared to the water fraction (Fig. 4). The butanol fraction showed strong antimicrobial activities on the growth of *L. monocytogenes* and *S. aureus* at 7°C and 25°C, yet it produced the opposite effect, that is, no inhibition of the growth of *E. coli* O157:H7 and *S. typhimurium*. These results suggest that the antimicrobial activities of these fractions were affected by the strain of the tested microorganism and the incubation temperature, however, the lower temperature did not decrease significantly on the effectiveness of the antimicrobial substances contained [6]. The milk was selected as a model system for this study, since the pine needle extract changed the color and buffering capacity of the model milk system.

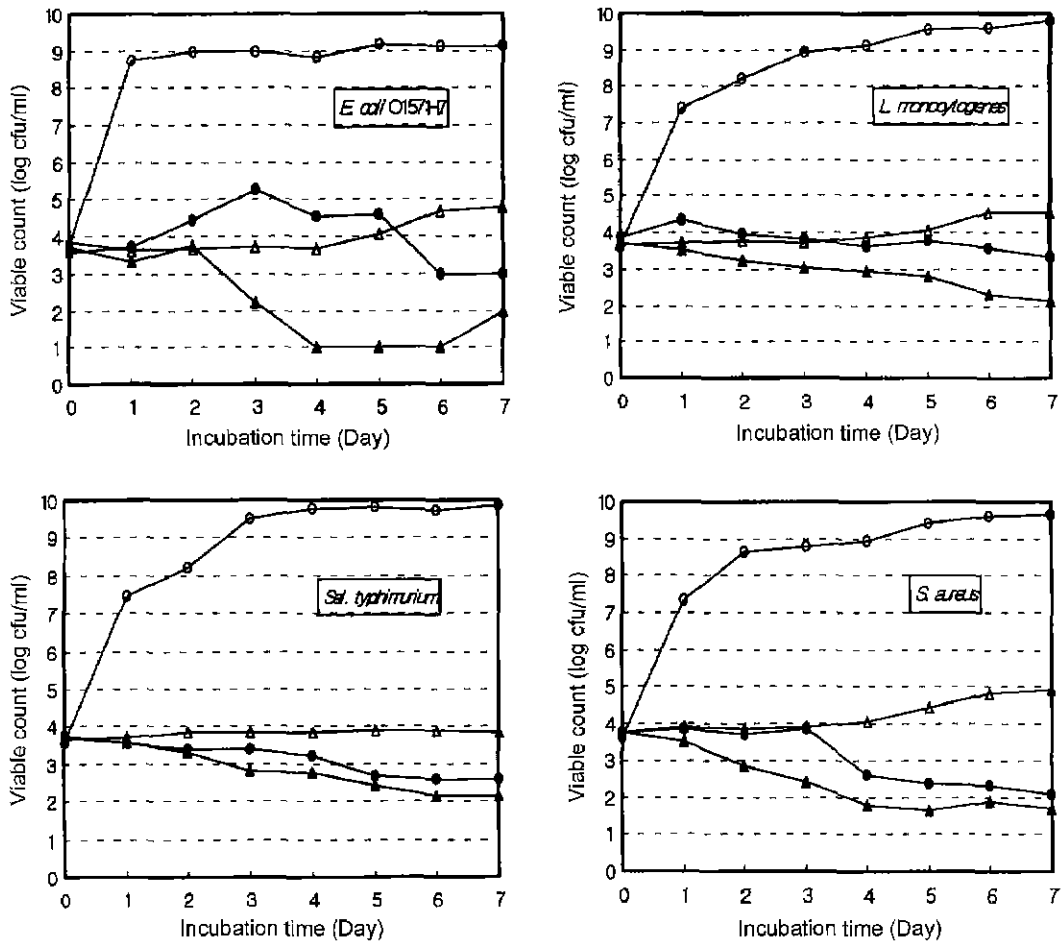

Fig. 4. Growth inhibition of BuOH fraction from methanol extract of pine needles against food-borne illness bacteria in sterilized milk as a model food system at 7°C and 25°C.
Symbols indicate -△- 7°C Control, -▲- 7°C Extr -○- 25°C Control, -●- 25°C Extr.

Acknowledgments

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