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Research article

Morphological characteristics and Antibacterial effects of various resinous concentrations of *Aquilaria crassna* on some pathogenic bacteria

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Abstract

We have examined *Agquilaria crasnna* (agarwood) essential oil, leaf teas and various woody parts for their antibacterial effects. The pathogenic bacteria tested were 3 Gram-negative (*E. coli*, *Enterbacter aerogenes*, *Pseudomonas aeruginosa*) and 1 Gram positive (*Staphylococcus aureus*) one. We found the very antagonistic results from Agarwood essential oil in our study compared with the general knowledge that pure essential oils have significant antibacterial effects. However, in our study, we have found that optimum content of essential oil (40% essential oil diluted with DMSO) had better antibacterial effects than pure Agarwood essential oil. Other diluted percentages of Agarwood essential oil (pure, 20%, 60% and 80%) had no significant bacterial inhibition effects. Other parts of Agar wood (woody chips and leaves in form of teas) also had no significant antibacterial effects. Agarwood essential oil seemed to be more effective against Gram positive microbes but showed very weak or no anitibacterial effects against Gram negative microbial strains.

Keywords: Antibacterial effect, Agar wood essential oil

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Introduction

The antibacterial effects of Agarwood have been well documented (Hendra et al., 2016). Agarwood belongs to the most precious fragrance wood from Aquilaria trees and is known with different names in different countries; Agarwood in EU countries, eaglewood in USA, Aloeswood in Singapore, and Krisanta in Thailand (Leelamanit et al., 2014). In Korea, it is called “Chimhyang” for which “chim” means “sinking” and “hyang” means “fragrance”, meaning “sinking fragrance wood”. Agarwood is the heartwood of the very special tropical hardwood tree. Agarwood is formed through the process, called the synthesis of resin, by Xylem cells of transport tissue in vascular plants. Agarwood is defined as a resinous wood formed within living Aquilaria trees with rich aromatic properties. They are used for incense and also, traditional medicine. *Aquilaria malaccensis* tree grows as high as 15 to 30 m tall and 1.5 to 2 m in diameter with white flowers (Ahmad et al., 2017). Similar characteristics were found in most Aquilaria genus. Agarwood belongs to the family of Thymelaeaceae. Agarwood essential oil is made by different methods, traditionally and commercially by hydro-distillation. However, unlike other trees, the yield of essential oil from Agarwood is very minimal, less than 2% compared to other trees (more than 10%) Ahmad et al., 2017). Under genus of Aquilaria, there are many

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species but more commonly at market; *Aquilaria sinensis* (mostly in China), *Aquilaria crassna* (mostly in Vietnam) *Aquilaria malaccensis* (mostly in Malaysia and Indonesia). Agarwood is endangered species registered by CITES in 2004 and IUCN in 2013. They are under very strict control by the governments but still some illegal hunters are seized in certain countries. People believe there are no longer wild agarwood trees existing and already almost extinct a decade ago. Export and import are banned for those wild agarwood by some countries. In countries like Cambodia, Laos, Indonesia and Papua New Guinea, there are some wild agarwood trees believed to be growing in natural forests but are found extremely rare. Agarwood has been used more for medicinal purpose in Asian countries, which has been found effective in mental relaxation, diabetes, hypertension and etc. For anti-bacterial effect, various studies have been reported. Hammer et al. (1999) reported that most essential oils from plants showed antimicrobial effects. Dash and Patra (2008) found that water extracts of bark and leaves of Aquilaria had antimicrobial effects but not the methanol extracts. Chen et al. (2011) studied that *Aquilaria sinensis* had antimicrobial effects against *S. aureus* and *B. subtilis* but not much significant to *E. coli*. One of the Agarwood components, B-Caryophyllene, has a significant microbial effect against *S. aureus* (Dahham et al., 2015). However, the antimicrobial effect against *E. coli* was not significant (Wetwitayaklung et al., 2009). The objective of our study is to investigate the antimicrobial effects of various Agarwood forms of essential oil, leaves (in form of teas) and woody chips against pathogenic bacteria of *E. coli*, *Enterbacter aerogenes*, *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

Materials and Methods

Morphological characteristics

Scanned Electron Microscopy (SEM) was used to detect some patterns of resinous parts for the partial and non-resinous parts of Agarwood. The SEM used was from the microscopic lab facility at Hankyong national university, Ansung city, Korea.

Samples of agarwood

A total of 10 samples of agarwood products were tested for their antibacterial effects, DMSO for negative control and Menaperom (Sigma) and Vancomycin (Sigma) for positive controls; 5 samples of Agarwood (essential oil contents diluted with DMSO; Oil 100% (no dilution; Sample 1), Oil 80% (Sample 2), Oil 60% (Sample 3), Oil 40% (Sample 4), Oil 20% (Sample 5), 2 boiled Agarwood (partially resinous chip (Sample 6), resinous chip (Sample 7), 3 samples of commercial agarwood leaf teas (Sample 8, Sample 9, Sample 10), which were all listed in Table 5. Each sample test was repeated 3 times.

Preparation of samples

Three different commercial products of *Aquilaria crassna* leaf teas (Sample 8,9,10 in Table 1.) were soaked in hot water for 24 h. Two types of agarwood chips were boiled for 30 min. and the hydrosol after boiling were used (Sample 6 and 7 in Table 1). Different parts of agarwood (1) essential oil (5 levels), (2) leaf teas (3 commercial products, (3) 3 types of wood chips with different degree of resinous residue (1=a whole resinous part, 2=partially resinous part (common raw material used for agarwood essential oil) and 3=woody part without resinous residue. An essential oil was diluted with DMSO for 5 different levels (1=no dilution, 2=80% oil; 3=60% oil; 4=40% oil;5=20% oil). The samples used in this study were summarized in Table 1 and positive controls used are shown in Table 2.

Microorganisms used in this study

Four Microorganisms were provided by the Microbiology Lab, Dept. of Biotechnology, Hankyong National University (Lab of Dr. W.T. Lim). The microorganisms tested were *Enterobacter aerogenes* ATCC13048 (=KCTC2190), E*scherichia coli* ATCC25922 (=KCTC 1682), *Pseudomonas aeruginosa*

Table 1: Samples used in this study for their antimicrobial effects

Samples	Property
Agarwood essential oil	(100%)
Agarwood essential oil	(80%, 20% DMSO)
Agarwood essential oil	(60%, 40% DMSO)
Agarwood essential oil	(40%, 60% DMSO)
Agarwood essential oil	(20%, 80% DMSO)
Partially resinous Chip	raw material used for hydro-distillation to produce essential oil
Resinous chip (whole dark brown)	mostly for smoke burning, incense stick or carved artistic stature
Tea A	Commercial product from A company
Tea B	Commercial product from B company
Tea B	Commercial product from C company
DMSO	Negative control
Meropenem® or Vancomycin® (Sigma)	Positive control (Sigma)

Table 2: Positive controls used for different bacteria in this study

	E. Coli	Enterbacter aerogenes	Pseudomonas aeruginosa	Staphylococcus aureus
Type	Meropenem	Meropenem	Meropenem	Vancomycin
w/v	0.001mg/ml	0.001mg/ml	0.1mg/ml	0.05mg/ml

Meropenem (Sigma); Vancomycin (Sigma).

Table 3: Microorganisms tested in this study

Microorganism	Type
E. coli ATCC 25922(=KCTC 1682)	Gram negative
Enterbacter aerogenes ATCC 13048 (=KCTC 2190)	Gram negative
Pseudomonas aeruginosa; Pseudomonas aeruginosa ATCC 27853 (= KCTC 2004)	Gram negative
Staphylococcus aureus ATCC 25923(= KCTC 1621)	Gram positive

Table 4: Degree of Antimicrobacterial effect assumed in this study

No effect	Weak	Intermediate	good	strong
0 (No clear zone)	+	++	+++	++++

+ = clear zone with diameter of filtered disk(7 to 9mm); ++ = clear zone with diameter of filtered disk(9 to 12mm); +++ = clear zone with diameter of filtered disk(12 to 15mm); ++++ =clear zone with diameter of filtered disk(>15mm).

ATCC 27853 (= KCTC 2004), *Staphylococcus aureus* ATCC 25923 (= KCTC 1621). These microorganisms were tested for their susceptibility against agarwood essential oil (*Aquilaria crassna*) and different forms of Agarwood products (Table 1). The microorganisms tested in this study were 3 Gram-negative (*E. coli*, *Enterbacter aerogenes*, *Pseudomonas aeruginosa*) and 1 Gram-positive (*Staphylococcus aureus*) are listed in Table 3.

Experimental media

LB media were used for cultivation of the 4 bacteria as shown in Table 2. The formulation of the media was same for all the 4 bacteria. For 1,000 ml of LB, 10.0g of tryptone, 5.0g of yeast extract, 10.0g of NaCl, 15.0g of sugar were mixed with distilled water for the rest to be a total of 1000ml LB agar media. The bacteria (1 ml of 108 cells/m) were spread onto LB agar media in Petri dishes. Filter paper discs with a diameter of 6 mm were soaked into each sample as shown in Table 1. Paper discs with sample 1 to 12 were placed onto the culture petri dishes. They were then incubated at 37°C for 36 hrs. The diameter of clear zone in each filtered disk paper was measured as an indicator of the microbial inhibition effect (antibacterial effect).

Results and Discussion

Morphological characteristics of Agarwood

Three different Agarwood samples used in this experiment were taken by Scanned Electron Microscopy (SEM) at Hankyong National University, Korea (Fig. 1). The Pictures were taken by magnification of 350 X. The reason for taking pictures by SEM was to see morphological characteristics of different parts of Agarwood commercially sold at market for their different usages. In fact, scammers use some fragrance chemicals and colors injected into the wood even the Agarwood has no resinous parts formed in the heartwood. The samples we used in this study showed the resinous parts of the Agarwood with different degradation levels of cellulose and lignin from the outer most to the inner part of Agarwood. The samples used were 3 different parts in Figure 1, A. resinous part, B. chips with some residue of resin (raw material for essential oil) and C. only woody part (no resin). As resinous part gradually accumulated toward the heartwood part, the woody characteristics of cellulose and lignin became well degraded. The structure of cellulose and lignin parts was distinctively different depending on the resinous accumulation in the wood as shown in Figure 1. The woody characteristics were almost not visible in the core resinous part of Agarwood (C) in Figure 1. The microscopic observation of the Agarwood could be one of the potential methods to identify the genuine Agarwood by checking the natural degradation degree of the woody characteristics of cellulose and lignin appearances.

Antibacterial effects

Several pathogenic bacteria were tested to find the antibacterial effects of Agarwood essential oil, leaf-tea and Agarwood chips. The main interest was to see antibacterial effects using different parts of Agarwood such as resinous parts, essential oil, leaves (in form of teas) and woody parts (wood chips). Antibacterial effects were shown in Fig. 2, where all pathogenic microbes were not significantly inhibited by Agarwood essential oil except by 40% of Agarwood essential oils which were weak or not effective at all against *E. Coli*, *E. aerogenes*, *P. aeruginosa* but effective only against mild against *S. aureus* (Fig. 2, 3), which was shown in Figure 1 and 2. All other percentages of Agarwood essential oil except 40% of it did not show any significant inhibition effect against the 4 pathogenic microbes. Therefore, disk paper figures of all other

Table 5: Inhibition effects of various parts of *Aquilaria crassna* on *E. coli*

Type of Bacteria	Sample	Content	Inhibition zone
E. coli	Sample 1	Oil 100%	-
	Sample 2	Oil 80%	-
	Sample 3	Oil 60%	-
	Sample 4	Oil 40%	+
	Sample 5	Oil 20%	-
	Sample 6	Boiled water of resinous chip	-
	Sample 7	Boiled water of woody chips with partially resinous	-
	Sample 8	Tea A	-
	Sample 9	Tea B	-
	Sample 10	Tea C	-
	Sample 11	DMSO	-
	Sample 12	Meropenem	++

(++++)=strong; (+++) = good; (++) = medium; (+) = weak; (-) = no effect.

Table 6: Inhibition effects of various parts of *Aquilaria crassna* on *Enterbacter aerogenes*

Type of Bacteria	Sample	Content	Inhibition zone
Enterbacter aerogenes	Sample 1	Oil 100%	-
	Sample 2	Oil 80%	-
	Sample 3	Oil 60%	-
	Sample 4	Oil 40%	++
	Sample 5	Oil 20%	-
	Sample 6	Boiled water of resinous chips	-
	Sample 7	Boiled water of wood chips with partially resinous	-
	Sample 8	Tea A	-
	Sample 9	Tea B	-
	Sample 10	Tea C	-
	Sample 11	DMSO	-
	Sample 12	Menaperem	++++

(++++)=strong; (+++) = good; (++) = medium; (+) = weak; (-) = no effect.

Table 7: Inhibition effects of of various parts of *Aquilaria crassna* on *Pseudomonas aeruginosa*

Type of Bacteria	Sample	Content	Inhibition zone
Pseudomonas aeruginosa	Sample 1	Oil 100%	-
	Sample 2	Oil 80%	-
	Sample 3	Oil 60%	-
	Sample 4	Oil 40%	+
	Sample 5	Oil 20%	-
	Sample 6	Boiled water of resinous chip	-
	Sample 7	Boiled water of woody chips with partially resinous	-
	Sample 8	Tea A	-
	Sample 9	Tea B	-
	Sample 10	Tea C	-
	Sample 11	DMSO	-
	Sample 12	Meropenem	++

(++++)=strong; (+++) = good; (++) = medium; (+) = weak; (-) = no effect

Table 8: Inhibition effects of various parts of *Aquilaria crassna* on *Staphylococcus aureus*

Type of Bacteria	Sample	Content	Inhibition zone
Staphylococcus aureus	Sample 1	Oil 100%	-
	Sample 2	Oil 80%	-
	Sample 3	Oil 60%	-
	Sample 4	Oil 40%	+
	Sample 5	Oil 20%	-
	Sample 6	Boiled water of resinous chip	-
	Sample 7	Boiled water of woody chips with partially resinous	-
	Sample 8	Tea A	-
	Sample 9	Tea B	-
	Sample 10	Tea C	-
	Sample 11	DMSO	-
	Sample 12	Vancomycin	++

(++++)=strong; (+++) = good; (++) = medium; (+) = weak; (-) = no effect.

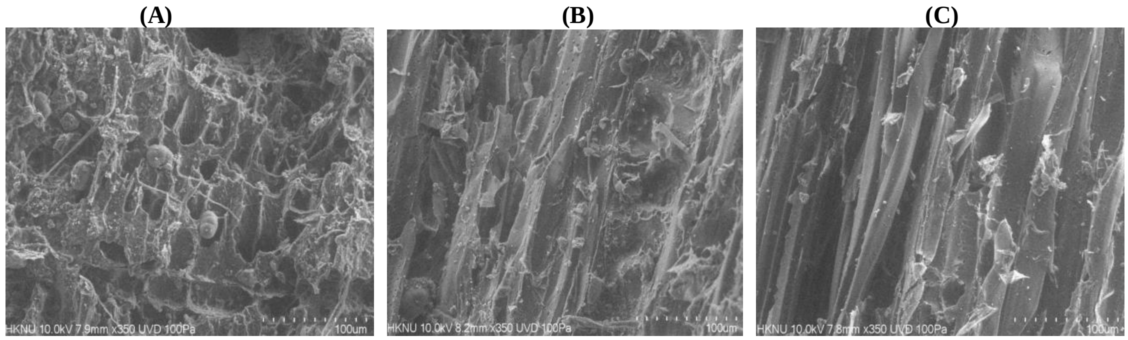

Fig. 1: Scanned Electron Microscopy (SEM: x350) of Agarwood chips. (A)=resinous part in the heartwood; (B)=chips with some residue of resin (raw material of essential oil); and, (C)=only woody part (no resin).

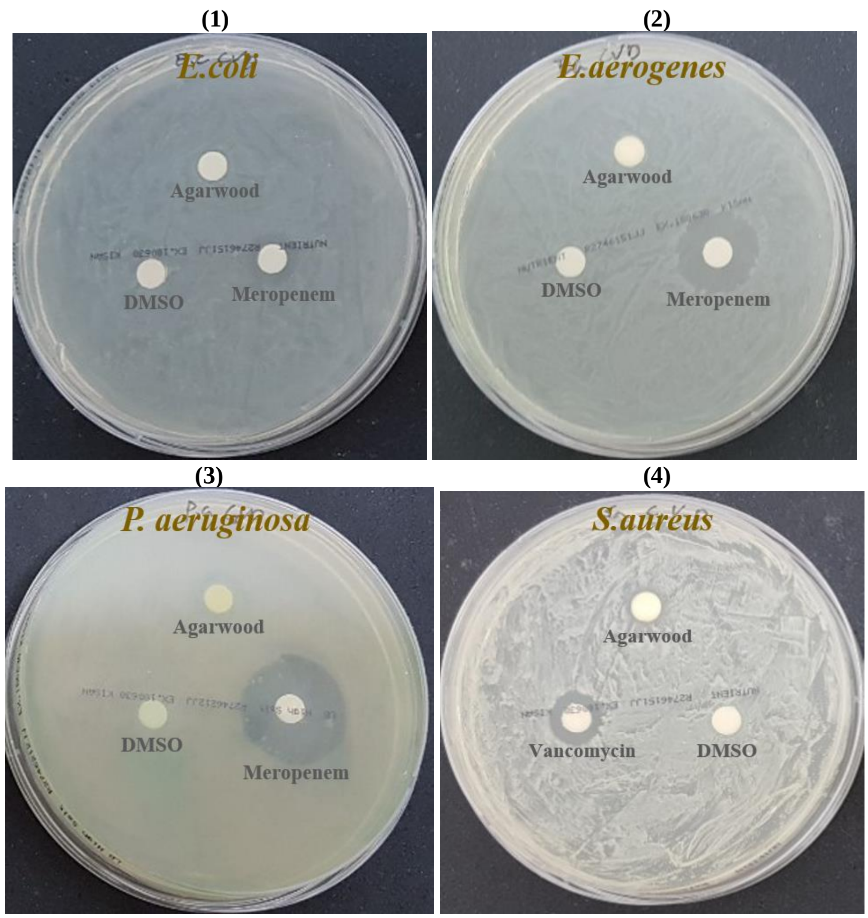

Fig. 2. Antibacterial effects of Agarwood for various pathogenic microorganisms by clear zones of disk papers (clockwise from top; *E. coli, Enterobactor aerogenes, Pseudomonas aeruginosa, Staphylococcus aureus*)

test results except 40% of Agarwood essential oil were not presented in our paper. For the antibacterial inhibition effects listed in Table 5 to 8, unlike the test result from Hendra et al. (2016), we had an antagonistic result such that no effects of Agarwood leaves (tea sample 8, 9 and 10) were found against *Staphylococcus aureus* but their result from alcohol extracts of Agarwood leaves had an inhibition effect against *Staphylococcus aureus*. However, a very similar result was found in our study that Agarwood essential oil had an antimicrobial effect against *S. aureus* but not against *E. coli* as was also studied by Wetwitayaklung et al. (2009). Dahham et al. (2015) found β- Caryophyllene, one of the compounds of Agarwood essential oil, has a very significant antimicrobial effect against *S. aureus*. Mei et al. (2008) also found *Aquilaria sinensis* has anti-MRSA effect. However, in general, it seems that Agarwood essential oil is not effective to inhibit *E. coli*, which was also supported by Chen et al. (2011) that most of the essential oils tend to be more active against Gram positive bacterial strains. In our study, Agarwood essential oil was also not significantly effective to inhibit the Gram negative microorganisms (*E. coli*, *E. Aerogenes* and *P. Aeruginosa*). No antibacterial effects were found for commercial leaf tea products (sample 8, 9 and 10) and also, similar results were found for the boiled Agarwood (sample 6 and 7). To conclude more concrete antibacterial effects of Agarwood of *Aquilaria Crassna*, further studies with more samples from different regions and different suppliers should be made.

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