Evaluation of three different artificial agarwood-inducing methods from *Aquilaria sinensis* using antimicrobial activity

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Abstract: The aim of this paper was to evaluate the effect of three different approaches for artificially inducing the formation of agarwood over time in young *Aquilaria sinensis* trees using antimicrobial activity. The antimicrobial activity was determined by a two-fold serial dilution method and minimum inhibitory concentration (MIC) against a panel of microorganisms (two bacterial strains, *Staphylococcus aureus and anti-methicillin-resistant Staphylococcus aureus,* and seven fungal strains: *Penicillium melinii, Penicillium adametzi, Penicillium urticae, Penicillium notatum, Paecilomyces varioti, Mucor saturninus Hagem* and *Aspergillus niger).* The results showed that artificial agarwood obtained by comprehensive stimulated method (formic acid plus fungal inoculation) and extended longer inducing time have better antimicrobial activity, which is similar to the result of chemical analysis. Therefore, it is a beneficial exploration to the first use of antimicrobial activity to evaluate artificial agarwood obtained by different producing methods and different culture time.

Keywords: Agarwood, antimicrobial activities, minimum inhibitory concentration, evaluation, artificial induction.

INTRODUCTION

Agarwood, namely "Chen-Xiang" in traditional Chinese medicine, is a resinous wood from the tree of *Aquilaria sinensis* (Lour.) Gilg (Thymelaeaceae), which is distributed in Fujian, Guangdong, Guangxi and Hainan provinces of China (Institute of Materia Medica of Chinese Academy of Medical Sciences, 1997). It has long history being used as sedative, analgesic, antiemetic and carminative (Hashim, 2016). In the natural process, agarwood was formed in the stem, branch or root of *Aquilaria* trees with a very slow speed through natural infection by a variety of fungi such as *Aspergillus* spp., *Botryodyplodia* spp., *Diplodia* spp., *Fusarium bulbiferum*, *F. laterium*, *F. oxysporum*, *F. solani*, *Penicillium* spp. and *Pythium* spp. (Soehartono, 1997) and naturally wounded by wind, lightning strikes, the gnawing of ants or insects.

In natural forests, only 1%~2% of *A. sinensis* contain agarwood and the resources were unreasonably developed (Pripdeevech, 2012). With the increase in demand and commercial value, trade development of agarwood has intensified in recent years which caused wild *Aquilaria* have been largely deforested and destroyed all over the world. These reasons lead to serious shortage of natural resource, thereby severely limiting drug supplies and causing prices to increase for up to US$ 0.1 million/kg for superior, pure material. To protect the wild plant resources and to meet the demand for sustainable agarwood production, *Aquilaria* trees are now being planted in large area by using a variety of artificial cultivation mode.

Deliberate wounding of trees with large knives and hammering nails into tree trunks were used as traditional agarwood-inducing methods in many countries, but the breeding cycle is long and the yield is very low. Over the years of development, the practice has applied to include the use of certain chemicals and microorganisms (Tamuli, 2000; Mohamed 2014; Cui, 2013; Mohamed, 2010). A formic acid or formic acid plus fungal infected combined method has also been developed recently (Tian, 2013), which yielded mixed results concerning the quantity and quality of agarwood (Chen, 2011). Thus, we need to find the evaluation of artificial agarwood-inducting approaches.

Phytochemistry investigation showed that the main active constituents in agarwood were sesquiterpenes and 2-(2-phenylethyl) chromone derivatives (Naef, 2011; Chen, 2012; Hashim, 2016; Yang, 2016; Li, 2016a). Therefore, chemical analysis methods were widely used in the evaluation of artificial agarwood-induction. Characteristics, extract and color reaction were employed to evaluate the quality in commercial agarwood and similar products (Yu, 2010). The concentration of sesquiterpenes in the artificial inoculation agarwood was detected by using GC-MS (Tamuli, 2005) and the quality of 2-(2-phenylethyl) chromones in agarwood products was directly analyzed in real time/time-of-flight mass spectrometry (Chen, 2007). The agarwood materials were distinguished between wild and the cultivated using direct analysis in UPLC-ESI-QTOF-MS (Li, 2016b). These studies also clarified the change rule of chemical products by different inoculations at the different test times.

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It has been reported that the use of microorganisms is the main method of inducing-agar. However, agarwood formation was also reported not to depend on the activity of specific fungi, but to represent a general reaction of the host to injury or invasion, in which the host adopts a "self-injury" mechanism and products some chemical constituents to protect other tissues during fungal invasion (Wong, 2013). Recently, antimicrobial activity of agarwood has been explored, such as *Staphylococcus aureus* (Wetwitayaklung, 2009). The antimicrobial test was simple and easy. Since the self-protective response can be stimulated in the process of microbial induction, it was considered whether to evaluate the superior and low quality of agarwood by the antibacterial activity or not. Consequently, in this paper, we detected the antimicrobial activities in agarwood extracts of different inducing-methods with different incubation times and evaluated three different artificial agarwood-inducing methods by analysis of the results of antimicrobial activities.

MATERIALS AND METHODS

Twelve batches of agarwood, with thirty-six samples (table 1) were collected from the farm of Xinyi suburban district of Guangdong province in China, which is the test area of artificial cultivation of agarwood. A method of chemical stimulation or chemical stimulation plus fungal inoculation was selected for the training formation of resinous in 5-year-old *A. sinensis* trees. Glacial acetic acid (CH_3COOH, HAc) was used as chemical irritant. *Fusarium* sp. (A2) and *Botrysphaeria rhodina* (A13) (Herron, 1996), which were obtained from *A. sinensis* (Xinyi, Guangdong Province, China) and preserved at the Guang-dong Provincial Key Laboratory of Microbiol Culture Collection and Application, Guangdong Institute of Microbiology (Sokmen, 2004), were selected to inoculate *A. sinensis* trees. Artificial cultivation methods of agarwood are as follows: The *A. sinensis* trees were selected with approximately 3-4m height, larger than 10 cm in diameter and distances nearly 50-70cm between every two trees. The holes were dug with a diameter of 0.5cm and a depth of 4-5cm in the trunks of trees at a height of 1m. 1%HAc or 1%HAc following by fungal liquid fermentation product was injected slowly into the xylem part of the tree, which can stimulate the tree to produce resins. Then the agarwood was harvested at different times for 6, 8, 10 and 12months after agarwood induction. The plant was identified by Prof. Yan (College of Traditional Medicine, Guangdong Pharmaceutical University, China).

Preparation of agarwood extract

Agarwood samples were dried at 50°C and then crushed into small pieces. Dried medicinal materials (0.5g) were extracted in boiling 95percent ethanol (50ml) for 2h. The filtrates were concentrated at 40°C using a rotary evaporator under low pressure and then freeze-dried in a lyophilizer (FDU1200, TOKYO RIKAKIKAI CO., LTD.). The extract with a total yield of 5.1~11.9% was stored at -20°C until used.

The experimental bacterial strains and the growth conditions

The following two bacterial strains were used: *Methicillin-resistant Staphylococcus aureus* (MRSA) ATCC 43300 and *Staphylococcus aureus* ATCC 33591 were selected for the antibacterial experimen. Standard strains were obtained from Institute of Microbiology, Chinese Academy of Sciences and stored in Luria Bertani (LB) broth supplemented with glycerol (20%) at−80°C. The tested bacteria were scrapped from LB agar after growing for 24h at 36°C. The cell material was suspended in sterile water to obtain a suspension of 10^6 CFU ml^{-1}.

The experimental fungal strains and the growth conditions

Seven fungi (*Penicillium melinii* AS 3.4474, *Penicillium adametzi* AS 3.4470, *Penicillium urticae* IFFI 04015, *Penicillium notatum* IFFI 04013, *Paecilomyces varioti* IFFI 04015, *Mucor saturninus* Hagem AS 3.210 and *Aspergillus niger* AS 3.1858 were used as test strains. Standard strains were obtained from Institute of Microbiology, Chinese Academy of Sciences. Strains were cultured on Yeast Extract Peptone Dextrose Medium (YPD) plates (Senbeijia, Nanjin province, China) using aseptic procedures to avoid contaimination. Conidia were obtained from 5-day-old YPD cultures of the fungus incubated at 28°C. Inoculum was prepared by scraping spore material from the culture surfaces with a loop and resuspending it in YPD liquid medium. The concentration of conidia was determined using a hemacytometer and adjusted to 10^6 CFU ml^{-1}.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the extract was evaluated by a modified resazurin microtitre plate assay as reported by Sokmen *et al* (2004). Briefly, a volume of 100μL of the nutrient medium solution was transferred into the first row of the 96 well plates. To all other wells, 96μL of the nutrient medium solutions added. 2μL of the agawood extracts which was dissolved in dimethyl sulfoxide (DMSO) in different concentrations or positive control were added. Finally, 2μL of bacterial/fungal suspension added. Each plate set three controls as follows: A column with broad spectrum antibiotics as positive control, a column with all solutions just without the test samples, a column with all solutions but using 2μL of the nutrient medium solutions instead of the bacterial/fungal solution as a negative control. The plates were incubated at 37°C for 24 h and 28°C for 48h for bacteria and fungi respectively. The absorbance was measured at OD 600 by micro quant for fungus and bacteria. All the samples were parallel tested at least three times. The MIC value was defined as the lowest concentration of the extract or antimicrobial standard.

STATISTICAL ANALYSIS

The results were calculated from triplicate data and were expressed as mean ± standard deviations (SD). The data were compared and analyzed using SPSS 20 software.

RESULTS

The antimicrobial activity of the ethanol extracts of twelve batches of agarwood (table 1) were assessed and the results are presented in tables 2 and 3. Followed by measurement of minimum inhibitory concentration (MIC), the results indicated that all the ethanol extracts of agarwood showed potent antibacterial activity against *MRSA* and *S. aureus.* With prolonging the induced culture time, the antibacterial activities of agarwood extracts obtained from three different artificial inoculation methods become stronger, and the extracts obtained after culturing 12months showed the strongest antibacterial activity in twelve batches of samples. In three different induction methods, the activity of sample induction by acetic acid and fungal agent displayed stronger activies than only by acetic acid.

In the antifungal screening experiments, seven strains were selected to be test. All the plant extracts showed poor activity against *Mucor saturninus Hagem, Penicillium adametzi* and *Paecilomyces varioti.* Unable to get MIC, the results about those three strains were not listed in table 3. Mild antifungal activities of all the samples were observed against *Penicillium notatum*, *Penicillium melinii* and *Aspergillus niger* (table3).

Accordingly, the inhibitory effect of these extracts on *Penicillium notatum* was the strongest, and the antifungal activity was the best after culturing for 12 months. At the same time, the extracts of artificial agarwood stimulated by formic acid plus fungal inoculation have better antifungal activity. Only stimulated by formic acid, the activity of artificial agarwood is poor.

DISCUSSION

We selected the twelve batches of artificial agarwood samples and tested their antimicrobial activity. These samples were obtained by using three different induction methods, and respectively harvested in the culturing of 6 months, 8 months, 10 months and 12 months. The antimicrobial activity of the ethanol extracts of twelve batches of agarwood (table 1) were assessed and the

Table 1: Artificial agarwood samples of *A. sinensis* in this study

Date of collection	Resin formation time (months)	Induction methods (Sample number)		
		HAc	HAc+A2	HAc+A13
2012.5.25	6	510	533	558
		511	536	560
2012.8.26	8	515	538	561
		516	539	562
2012.11.28	10	517	542	566
		519	543	568
2013.2.26	12	521	547	570
		524	548	571

HAc: CH_3COOH, A2: *Fusarium SP.,* A13: *Botrosphaeria rhodina.*

Table 2: Antimicrobial activity (MIC [a]) of ethanol extract of sample collected in differently time against selected strains [b]

Sample No.	*Staphylococcus aureus*	MRSA	*Penicillium melinii*	*Penicillium urticae*	*Penicillium notatum*	*Aspergillus niger*
510/511	245±9.50	170±3.5	NA [c]	141±6.36	NA	506±9.87
533/536	119±3.22	152±2.52	129±1.65	75±4.95	140±5.03	218±14.5
558/560	69±2.00	230±5.69	NA	121±1.53	115±2.83	231±7.51
515/516	88±2.01	154±6.43	555±5.51	63±2.83	NA	590±2.08
538/539	78±3.06	94±6.25	114±5.20	55±2.52	89±6.09	173±15.0
561/562	54±7.51	138±4.73	122±8.73	69±1.73	70±4.95	88±2.65
517/519	58±1.16	124±1.53	233±8.14	37±7.07	NA	284±10.44
542/543	56±0.58	96±4.36	50±3.46	25±1.41	52±2.08	84±8.51
566/568	41±3.79	97±4.04	200±4.58	30±0.58	51±4.24	122±7.21
521/524	50±0.58	112±1.16	113±4.04	39±8.00	NA	122±9.19
547/548	41±4.16	53±1.73	19±4.73	22±6.36	40±4.58	63±3.51
570/571	34±5.78	83±4.04	52±2.08	23±4.23	33±3.53	67±1.53

[a] Minimum inhibitory concentration, MIC (μg/mL). [b] Values are mean ±SD of three separate experiment. [c] NA: Not active

results are presented in table 2 and 3. The results indicated that all of the samples had some activities against the two tested bacteria and four fungi of seven tested strains.

Followed by the measurement of minimum inhibitory concentration (MIC), the results indicated that all the ethanol extracts of agarwood showed potent antibacterial activity against *MRSA* and *S. aureus*. The results of potent antibacterial activity were consistent with the previous studies: Mei et al (2008) showed that the essential oil from Chinese agarwood had anti-MRSA activity; Wetwitayaklung et al. (2009) found that the essential oil of agarwood (*A. crassna*) had antimicrobial activity against *S. aureus* and *C. albicans*. On the basis of determining the antibacterial activity, we firstly studied the antibacterial activity of artificial agarwood obtained with different

inducing methods and different harvest intervals. With the increasing culture time, the antibacterial activity was enhanced. The extracts obtained after culturing 12 months showed the strongest antibacterial activity in twelve batches of samples. This is probably because the contents of sesquiterpenes and chromone derivatives increased with prolonging the culture time. There are known as active components and usually possess antimicrobial activity (Chen, 2011). Comparing three different induction methods, it was concluded that the extracts of HAc+A2 and HAc+A13 had better antimicrobial activity than that of HAc! In order to confirm this conclusion, we carried out the antifungal activity test. In the antifungal experiments, seven strains were selected. All the plant extracts showed poor activity against *Mucor saturninus Hagem, Penicillium adametzi* and *Paecilomyces varioti*. Unable to get MIC, the results about those three strains were not listed in table 3.

The results listed in table 3 indicate that almost all the extracts showed good inhibitory activity against four fungal strains (*Penicillium melinii* AS 3.4474, *Penicillium urticae* IFFI 04015, *Penicillium notatum* IFFI 04013 and *Aspergillus niger* AS 3.1858), especially for *Penicillium urticae* IFFI 04015. In all the samples, the artificial agarwood samples harvested after culturing 12months showed the MIC can respectively reach 19μg/mL (*Penicillium melinii* AS 3.4474), 22μg/mL (*Penicillium urticae* IFFI 04015), 33μg/mL (*Penicillium notatum* IFFI 04013) and 22μg/mL (*Aspergillus niger* AS 3.1858), which seem to be the most potent bioactive samples. It also shows that the growth of the culture time could improve the antibacterial activity of the sample. There are few reports about the antifungal activities of artificial agarwood before, but this is the first report not only concerning he antifungal activities of artificial agarwood but also evaluating the effects of different induction methods on antimicrobial activity. Similarly, the agarwood samples of induced by HAc+A2 and HAc+A13 induced stronger antifungal activity than only by HAc. Although the potential samples are induced by acid and fungi, but for different fungi, the antifungal activity still had certain difference, such as *Penicillium urticae* IFFI 04015 and *Penicillium notatum* IFFI 04013, which indicated that the composition and the content of effective components generated by different inducing methods are different.

In a previous study of our team, we reported that induced time and stimulated method may lead to changes of endogenous metabolites in artificial agarwood (Gao, 2014). The results of this study also illustrated that the constituents of artificial agarwood obtained by comprehensively stimulating using formic acid plus fungal inoculation are much closer to the natural agarwood than those obtained by formic acid through analysis of 22 metabolism markers of agarwood by GC-MS. Chemical composition constitute the foundation of drug action, which reveals that changes in the composition inevitably cause difference in the effect. According to this study, the efficacy of agarwood was associated with it quality. The simple antimicrobial activity was selected and evaluated the effects of different induced methods and culture times on the quality of agarwood. The results showed that the comprehensive induction methods at longer culture time have better antimicrobial activity than the others, which is consistent with previous research results. Increased activity of the antimicrobial may be due to the comprehensively stimulated method (formic acid plus fungal inoculation) will lead to increasing sesquiterpene content slowly and fast augmenting the phenylethyl chromone derivatives. Therefore, this paper from a special perspective, evaluated the agarwood quality using antimicrobial efficacy directly. It also suggested that induced time and comprehensively stimulated method have a significant effect on metabolite levels of artificial agarwood, which are in agreement with our analysis through the MIC data of antimicrobial activity. So, we can use the determination of antimicrobial activity to evaluate the quality of artificial agarwood. This evaluation method was closer to the medicinal effect, simple and convenient, and could be applied to detect the effect of artificially inducing the formation of agarwood.

Therefore, agilawood quality, chemical composition and its efficacy should be organically combined in order to facilitate a comprehensive evaluation of agarwood in future. And how to use the antimicrobial effect and aitificial agarwood quality as the evaluating indicator of cohesion dose-effect needs further study.

CONCLUSION

Concluded that artificial agarwood obtained by comprehensively stimulated method (formic acid plus fungal inoculation) and longer induced time provide better antimicrobial activity. This study is a beneficial exploration as to the first use of antimicrobial activity to evaluate artificial agarwood obtained by different producing methods and different culture intervals.

ACKNOWLEDGMENTS

We thank the Foundation (81102418,31100496,81203006) by the National Natural Science Foundation of China. The work was also supported by the Scientific Research Innovation Team Foundation of the Logistics University of CPAPF (WHTD201304).

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