

## Antifungal Activity of Neutralized Wood Vinegar with Water Extracts of *Pinus densiflora* and *Quercus serrata* Saw Dusts

Velmurugan, N.<sup>1</sup>, Han, S. S.<sup>2</sup> and Lee, Y. S.<sup>1, 2\*</sup>

<sup>1</sup> Department of Forest Science & Technology, Institute of Agriculture and Life Sciences, Chonbuk National University. Jeonju, Chonbuk 561-756, Republic of Korea

<sup>2</sup> Institute of Agricultural Science & Technology, Chonbuk National University. Jeonju, Chonbuk 561-756, Republic of Korea

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**ABSTRACT:** The major objectives of this study were to find out the mechanism behind the sapstaining fungal hyphae penetration into wood elements using electron microscope and to evaluate the exact role of phenolic compounds existing in wood vinegar towards the sapstaining fungal growth inhibition. To formulate the new environmental friendly wood preservative, we neutralized (to pH 7) wood vinegar with NaOH and hot water extracts of *Pinus densiflora* and *Quercus serrata* saw dusts were used in the media instead of sterilized distilled water to enhance the fungal growth inhibition property of neutralized wood vinegar. The initial calibration of wood extract was done by calculating the specific gravity, tar calculation and total organic content. GC-MS analysis was performed to identify the phenolic compounds and other ingredients present in both neutralized wood vinegar and acidic wood vinegar by chloroform extraction method. Eleven major compounds were identified in acidic wood vinegar and 7 major compounds in neutralized wood vinegar. *Ophiostoma polonicum*, *O. ips*, *O. flexuosum*, *O. narcissi* and *O. tetropii* were the sapstaining fungi used in this study against neutralized wood vinegar, extracts of *Pinus densiflora* and *Quercus serrata*. More than 2.5% neutralized wood vinegar contained agar plates were showed strong antifungal activity against sapstaining fungi. The Scanning Electron Microscopy image of fungal growth on wood samples showed the phenomenon of fungal penetration into wood elements. *Pinus densiflora* was used as wood element for SEM analysis.

**Key words:** Antifungal activity, wood vinegar, Phenolic compounds, *Pinus densiflora* water extract, *Quercus serrata* water extract

### INTRODUCTION

A wide range of fungi can infect wood, including wood-decay fungi, staining fungi and insects (Zhou, *et al.*, 2006). Among these fungal groups, sapstaining fungi can cause staining in wood due to the production of melanin in ray parenchyma tissues and cell lumens of fungal hyphae (Geo and Breuil, 1998). This fungus does not affect the strength of the wood, but it can cause serious damage in natural color of wood. *Ophiostoma* sp. is one of the major genera of sapstaining fungi. Many authors studied about wood decay fungi and sapstain fungi on wood and several methods are used for controlling the fungal

growth on wood. Daniel *et al.* (2004) reported the new details for understanding the white-rot decay of lignocellulose using Cryo-FE-SEM & TEM. To study the phenomenon behind the fungal decay on wood is necessary to understanding the problem and define the basics of it, which will lead us to design the specific and eco-friendly preservative. This paper explains the direct effect of sapstaining fungal penetration on and in between wood elements and its potential to cause wood damage. Various kinds of preservatives were used in wood industries to protect the wood from fungal damage. The chemical protection of wood has been widely used to control fungal damage on

\*Corresponding author E-mail:ysoollee@chonbuk.ac.kr

wood. The major chemicals used in the wood industries as preservative materials are creosote, pentachlorophenol, chromated copper arsenate and ammoniac copper arsenate. Some environmental friendly preservatives are also used in the wood industries, like, ammoniac copper quaternary and copper azole. Natural resource materials, like wood vinegar and bamboo vinegar are not used in worldwide wood industries, but wood vinegar and bamboo vinegar used as folk medicine for skin diseases in Japan (Kimura, *et al.*, 2002). Wood vinegar has been used in agricultural fields, food industries and in house hold cleaning jobs also. Anti-dermatophyte activity of phenolic compounds in wood vinegar was already demonstrated (Ikerami, *et al.*, 1992). This paper deals with fungicidal activity of these natural resources against wood staining fungi at neutralized condition.

Wood vinegar (commonly called as “Mokusaku-eki” in Japan and S. Korea) is an acidic by-product of broad leaved trees charcoal burner and Bamboo vinegar is by-product of Bamboo charcoal burner. Both by-products contain more than 200 ingredients including phenolic, polyphenolic, organic acids and carcinogenic agents such as woodcreosote, benzo[a]pyrene, benzo[a]anthracene, and 3-methylcholanthrene (3-MCA) (Kimura *et al.*, 2002). Wood vinegar is commonly extracted from *Quercus serrata*, *Castanea crenata* and *Prunus jamasakura* (Kimura, *et al.*, 2002). Guaiacol, cresol, 4-ethyl-2-methoxyphenol and 2,6-dimethoxyphenol are major polyphenolic compounds present in wood vinegar with greater antifungal activity (Ikerami, *et al.*, 1992). But, still there is a controversy that whether the acidic nature of wood vinegar is responsible for antifungal activity or polyphenolic compounds. To overcome this question, in our research work, we neutralized the wood vinegar and tested against wood staining fungi. Saw dust extract of *Pinus densiflora* also has antifungal activity. Instead of using sterile distilled water in the preparation of growth medium, sterilized water extracts of *Pinus densiflora* and *Quercus serrata* saw dusts were added into MEA medium to enhance the antifungal activity of wood vinegar. To our knowledge, this is the first attempt to evaluate the antifungal activity of neutralized wood vinegar, hot water extracts of *Pinus densiflora*

and *Quercus serrata* saw dusts towards sapstaining fungal growth. GC-MS analysis was carried out for both neutralized wood vinegar and acidic vinegar to exhibit the presence of ingredients in the extracts. Hot water extracts of *Pinus densiflora* and *Quercus serrata* sawdust's were also used along with wood vinegar to study the prevention of the sapstaining fungal growth. *Ophiostoma flexuosum* was used to study the penetration phenomenon using scanning electron microscope.

## MATERIALS & METHODS

A total number of 5 fungal cultures *Ophiostoma flexuosum* (363175), *O. polonicum* (363181), *O. tetropii* (363182), *O. narcissi* (349579) and *O. ips* (363176) were obtained from CABI, Bioscience, UK Centre, formerly known as the International Mycological Institute (IMI). The fungal cultures were grown in 4 % MEA (Becton, Dickinson and Company, USA) as pre-inoculum. The fungal cultures were incubated at 25 °C for 4-7 days. Saw dusts of softwood *Pinus densiflora* and hardwood *Quercus serrata* were collected from wood industries near Chonbuk National University, Jeonju, South Korea. Samples were placed in a plastic bag, properly labeled, and then taken to the laboratory. 5 gms of both dusts were mixed with sterilized distilled water and boiled for 15 min. at 90 °C, separately. Extracts were filtered by Whatman No. 1 filter paper.

Commercially available wood vinegar was obtained from Jin-Jeop town wood industry, Gyeonggi-do province, South Korea. Dark brown color wood vinegar with pH 3.5 was neutralized (pH 7 ± 0.2) using NaOH (HI 98160, HANNA Instruments). The initial physico-chemical properties of extracts were calibrated based on the methods of Mun, *et al.* (2007). The tar content, the specific gravity and total organic content of the acidic and neutralized wood vinegar were calculated to find out the initial standardization of the extract. The data's of acidic wood vinegar calibration are not shown in this paper.

*Pinus densiflora* wood samples (uninfected) were collected from Gunsan City Wood Industry, Republic of Korea, in autumn of 2007. The samples (drum like shape) were collected from inside of wood blocks by using sterilized driller, the driller was sterilized by 70 % alcohol before collection

of every wood samples. The wood blocks were selected randomly. Samples (approximately 10 cm in length and 5 mm in depth) were collected from inside of wood blocks and those samples were placed in a plastic bag, properly labeled, and then taken to the laboratory. Samples were sterilized in autoclave at 121 °C for 20 min. Sterilized wood samples were sectioned into two (approximately 5 cm in length and 5 mm in depth) by sterile surgical knife.

Petri-plates were individually prepared with 20 ml of sterilized 2 % MEA medium, 6 mm mycelial plug of *Ophiostoma flexuosum* (363175) was placing in the centre of agar plates and plates were incubated at 20 °C until the fungal hyphae touches the end of plates. After complete growth of *Ophiostoma flexuosum*, sterilized wood sample was placed on each plate and incubated at 20 °C for 4 weeks. After 4 weeks, the wood samples were taken from agar plates for SEM analysis. Liquid nitrogen was poured on wood samples for 1-2 minutes and sectioned by sterilized knife to make small pieces then the samples were dehydrated through an ethyl alcohol series (20 %, 30 %, 40 %, 50 %, 60 % and 70 %, each 10 min.), after dehydration samples were allowed for air-drying then the samples were gold coated with gold by ion sputtering (JEOL JFC-1200 fine coater) and observed under SEM (JSM-5200). *Pinus densiflora*, *Quercus serrata* extracts and wood vinegar were used to prepare 3 different types of 2 % MEA media. First medium contains sterilized distilled water, second medium contains hot water extract of sawdust of *Pinus densiflora* (softwood) instead of distilled water, and third medium contains hot water extract of sawdust of hardwood *Quercus serrata* instead of distilled water. All the plates were sterilized at 121 °C for 20 min. After sterilization, media were allowed to pre-cool. Different concentrations (0.333, 0.667, 1.333, 2.5 and 3.0 %) of neutralized wood vinegar were mixed with all types of media. Filter sterilization of wood vinegar has been done before added into media. Finally media were poured into petri-plates and allowed for solidification in inside of laminar air flow chamber. *O. polonicum*, *O. flexuosum*, *O. tetropii*, *O. narcissi*, *O. ips* were inoculated into different concentrations of 3 MEA media, separately. Control was maintained without

wood extracts. Plates were incubated at 20 °C to evaluate the inhibition range of wood extracts.

Four replicates were maintained for 5 fungal samples at all concentrations including control. The difference between the mycelial growths of replicates were not significant, thus the average was used for antifungal index analysis. Antifungal index was calculated based on the method of Zhong, et al. (2007):

$$\text{Antifungal index (\%)} = (1 - Dt/Dc) \times 100$$

Where Dt = diameter of mycelial growth zone in test plate; Dc = diameter of mycelial growth zone in control plate. Results with significant difference  $P < 0.05$  were considered statistically (Ramos, et al., 1997).

The qualitative analysis of acidic wood vinegar and neutralized wood vinegar were performed using GC-MS (HP 6980 series, Hewlett Packard, USA). Five hundred µl of both extracts were mixed with 1 ml of chloroform, separately. The solution was mixed by vortex followed by centrifugation 13000 rpm for 10 min. One µl chloroform phase sample was injected in to gas chromatograph. DB-5 column, 30 m length, 0.25 mm ID, 0.25 µm film thickness was used for the separation. The oven temperature was elevated at the rate of 10 °C/10 min, from 90 °C to 300 °C. The final temperature at 300 °C was maintained for 5 minutes. The resulted peaks were compared with standard in MS spectroscopy. The MS was operated in electron ionization (EI) mode at 70 eV. Interface temperature was kept at 230 °C.

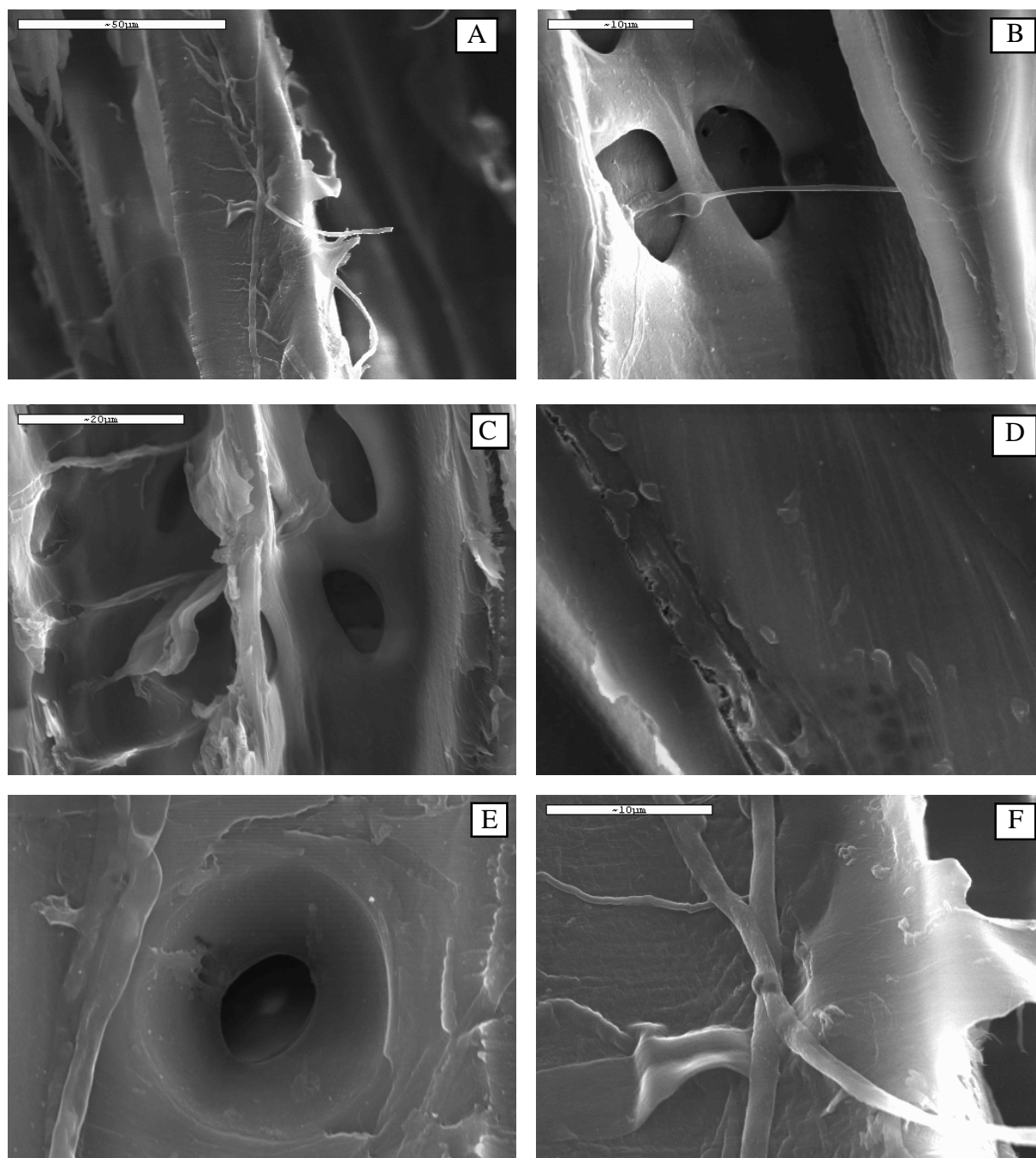
## RESULTS & DISCUSSION

The black color stain was observed in wood samples after 4 weeks of inoculation of wood samples on agar plates. The wood samples were sectioned into small pieces and observed under scanning electron microscope. The SEM studies provided information on fungal invention into *Pinus densiflora* pit membranes and degradation of pit membrane. Bordered pit degradation shown in (Fig. 1). Investigation also deals the surface cell wall degradation of wood elements. Some sapstaining fungus does not destroy the pit membrane, it grows in between the membranes and mechanical force externally plays the main role during the direct invention of fungal hyphae into wood. Wood decay by fungi involves

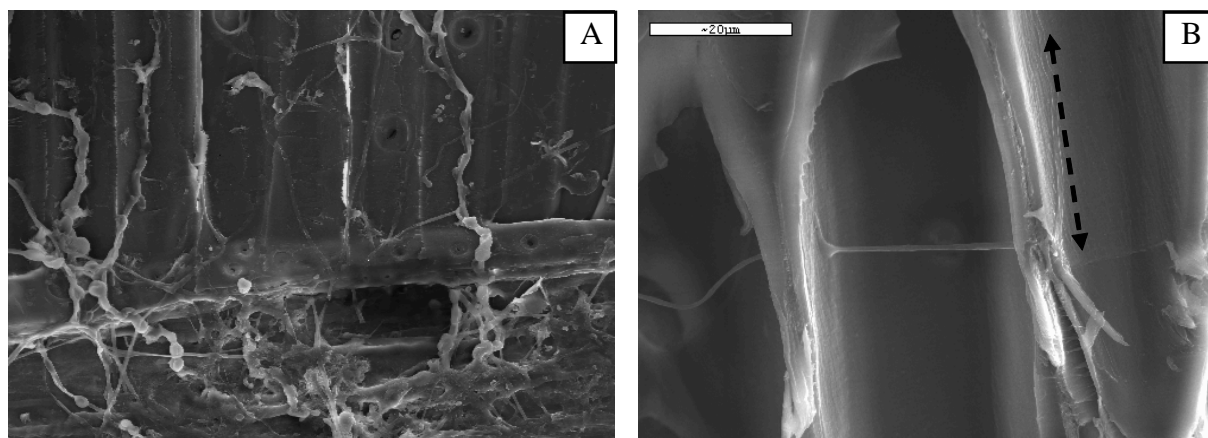
complicated chemical and enzymatic reactions. Initially, fungi colonize the surface of wood element. The hyphae penetrates the inner surface, the cell to cell passage was obtained by degrading the pit membranes. Some fungi directly penetrate the wood by physical forces. The SEM results

were shown in (Figs. 1 & 2). Radial longitudinal section of sample was shown in (Figs. 1 & 2) shows the direct penetration of hyphae to adjacent cell via cell wall.

Initial calibration of wood vinegar was calculated based on the values of specific gravity,



**Fig. 1. Development of fungal hyphae on wood cell wall (A); The intact pit membrane between fungal hypha and wood fibres. Traces of fungal hyphae (arrow) (B); Fungal hypha penetration through the cell wall and bordered pit degradation (arrow) (C); Disruption of wood cell wall by fungal hyphae (~ Bar in 20 µm) (D); Degradation of bordered pit by hyphae (Bar in photo ~10µm) (E); Development of fungal hyphae network on wood cell wall (F)**



**Fig. 2. A** Photograph of longitudinal section of *Pinus densiflora* colonized by sapstaining fungi; hyphae penetration without damaging Pit membrane (Arrow) (Bar in photo ~100µm) (A); Fungal hyphae penetration from one wood element to next wood element (White arrows). Black arrow shows the growth of fungal hyphae together with wood cell wall (B)

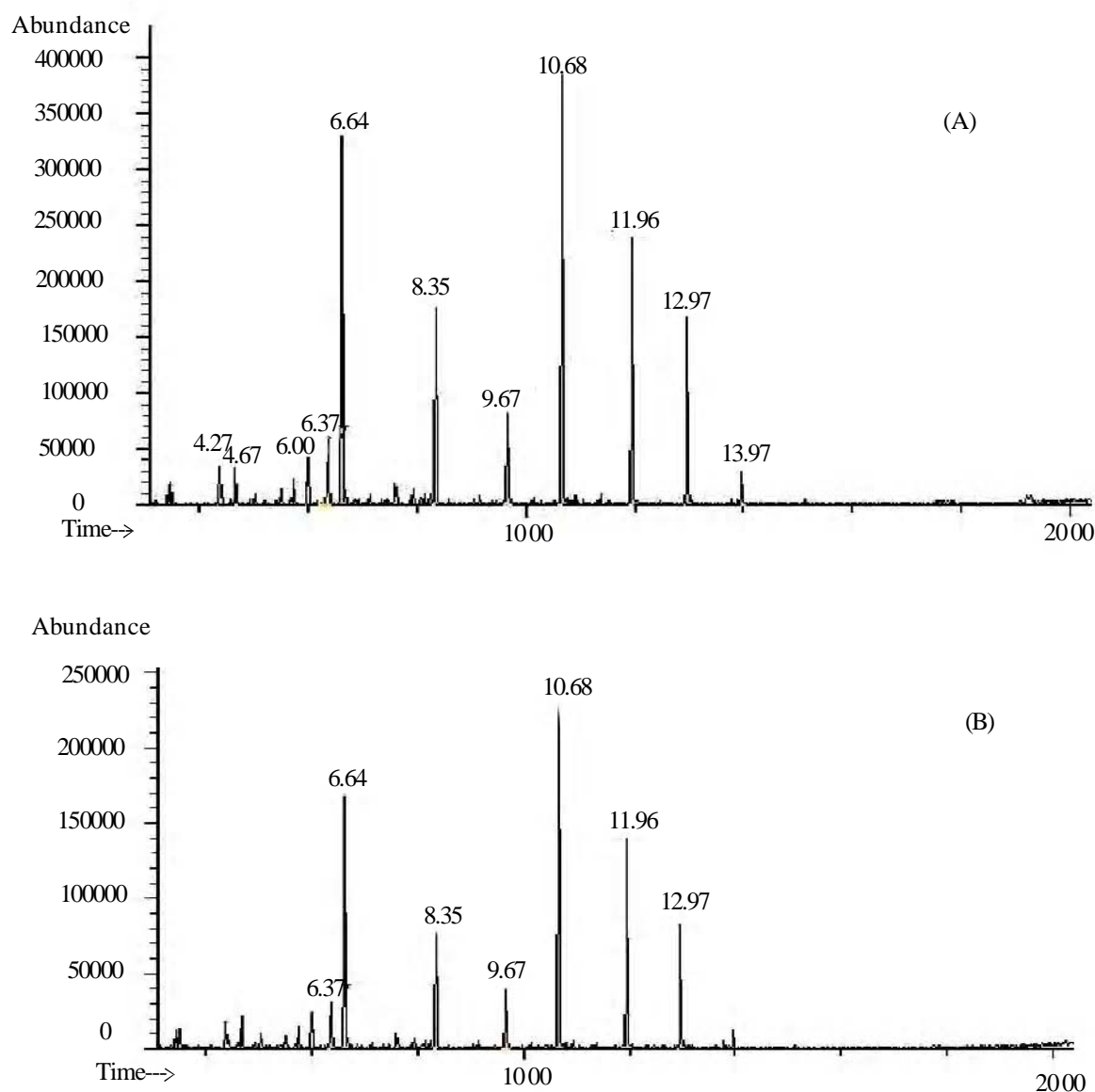
tar content and total organic content. The results of initial calibration for acidic wood vinegar are not shown. There are no much differences between acidic and neutralized wood vinegar. The tar content of neutralized wood vinegar was remains constant as acidic wood vinegar (0.30 %). The neutralized wood vinegar specific gravity was 1.000 and total organic content value was 10.28 %. The difference between the initial calibration values of acidic wood vinegar and neutralized wood vinegar are negligible.

The qualitative analysis of both acidic and neutralized wood vinegar was carried out by GC-MS spectrum. In acidic wood vinegar, peaks were

identified at RT (retention time) 4.27, 4.67, 6.00, 6.37, 6.64, 8.35, 9.67, 10.68, 11.96, 12.97 and 13.97 minutes (Fig.3). Seven phenolic compounds were identified using Mass spectrum and the major phenolic compound was detected as 2,6 dimethoxy phenol at RT 10.68. Other phenolic compounds were detected as Phenol (Izal), 2-methyl phenol (o-cresol), 4-methyl phenol (p-cresol), 2-methoxy phenol (guaiacol), 2-methoxy-4 methyl phenol and 4-ethyl-2-methoxy phenol. (Table 1). shows the complete list of compounds identified in acidic wood vinegar and area percentage of compounds. 2-furancarboxaldehyde and 2,3,5-trimethoxy toluene was also identified at RT 4.38 and 12.97, respectively.

**Table 1. Peak allocation and peak area percentage of chloroform fractions of acidic wood vinegar and neutralized wood vinegar**

RT (min)	Compounds	Area %	
		Acidic wood vinegar	Neutralized wood vinegar
4.38	4-Furancarboxaldehyde	1.64	-
4.67	Phenol (Izal)	1.88	-
6.00	2-Methyl phenol (o-cresol)	2.11	-
6.37	4-Methyl phenol (p-cresol)	4.59	5.46
6.64	2-Methoxy phenol (guaiacol)	19.53	20.40
8.35	2-Methoxy-4-methyl phenol	12.39	11.77
9.67	4-Ethyl-2-methoxy phenol	6.77	6.16
10.68	2,6-Dimethoxy phenol	25.32	32.06
12.97	2,3,5-Trimethoxy toluene	9.68	9.13
11.96	Dehydroacetic acid	14.14	15.02
13.97	Unknown	1.95	-



**Fig. 3.** GC chromatograph of acidic wood vinegar (A) and neutralized wood vinegar (B) (Column DB-5, 0.25 mm ID, 0.25  $\mu$ m film thicknesses)

4-methyl phenol (6.37), 2-methoxy phenol (6.64), 2,6-dimethoxy phenol (10.68) were identified as phenolic compounds in neutralized wood vinegar ( Fig. 3b). 2-methoxy-4-methyl phenol, 4-ethyl-2-methoxy phenol and 2,3,5-trimethoxy toluene were also identified at RT 8.35, 9.67 and 12.97. All the compounds identified were mentioned in Table 1. 2,6-dimethoxy phenol (10.38) was identified as major compound in neutralized wood vinegar.

Radial mycelial growth method was used to measure the percentage inhibition of sapstaining fungi. (Table 2). showed the fungal growth inhibition percentage on 2 % MEA medium

containing various concentrations of neutralized wood vinegar. The control growth of *Ophiostoma flexuosum*, *O. polonicum*, *O. tetropii*, *O. narcissi* and *O. ips* were 44, 82, 80, 20 and 72 mm in diameter, respectively. The results showed that neutralized wood vinegar alone not significantly affect the fungal growth, when minimum concentration of 0.33, 0.67 and 1.33 % used. The neutralized extract was active only at above 2.5 % used, almost 50 % fungal growth inhibition were identified at 3.0 % extract used. *Ophiostoma narcissi* was more resistant towards neutralized wood vinegar, complete growth was noticed at 0.33, 0.67 and 1.33 % used (Table 2).

Minor growth inhibition of *O. flexuosum*, *O. Polonicum*, *O. ips* and *O. tetropii* were observed at 0.67 and 1.33% plates (Table 2).

Significant fungal growth inhibition was observed in the plates containing neutralized wood extract and water extracts of *Pinus densiflora*. *O. polonicum* was more sensitive against *Pinus densiflora* extract (Table 2). Fourteen mm in diameter of fungal growth was observed in the plate containing 3.0 % neutralized wood vinegar and *Pinus densiflora* extract. The plate containing a mixture of 3 % neutralized wood vinegar and *Pinus densiflora* was more active against *O. flexuosum*, where only 8 mm of fungal hypha growth was observed. A considerable fungal growth inhibition was observed in all concentrations of mixture of neutralized wood vinegar and *Pinus densiflora* extract (Table 2). Minimum concentration (0.33 %) of neutralized wood vinegar is also active when it was used along with *Pinus densiflora* extract.

The antifungal effect of mixture of neutralized wood vinegar and *Quercus serrata* extract was not significant. *O. narcissi* was exhibited high resistance towards the mixture of Oak extract and neutralized wood vinegar (Table 2). *Ophiostoma flexuosum* was sensitive for this mixture at 2.5 and 3.0 % used.

The wood cell wall architecture is highly complex and in many instances, fungal degradation patterns are distinctive and are easily recognizable. However, to distinguish the degradation patterns caused by soft rot fungi and white rot fungi are not easy and every fungus has unique form of mode of action for decay. Ray parenchyma is main nutrient source for fungal growth in wood. Starch stored in ray parenchyma as food material. The abundant fungal growth was identified in longitudinal section of wood (Fig. 1). To obtain the mode of action of fungal penetration into the wood and study about the role of enzymes in the decay is essential to design the preservative for wood decay. Lignolytic enzymes were produced in hyphal tips to penetrate cell wall (Schwarze, 2007). Naturally wood elements consisting of polyphenolic compounds such as guaiacol, cresol, 4-ethyl-2-methoxyphenol, and these compounds also interrupt the growth of fungi in wood. It has already proved that sapstaining fungi are more pH

tolerant and can thrive in range between pH 2 to 10 (Panasenko, 1967; Land, 1987). The compounds detected by GC-MS analysis of chloroform extracts of both acidic wood vinegar and neutralized wood vinegar have been considered responsible for antifungal activity. Antifungal activity of neutralized wood vinegar was observed, when 2.5 % or more percentage used in the agar plates. Minimum concentrations (0.5-1.0 %) of acidic wood vinegar have more antifungal activity against sapstaining fungal growth in laboratory experiments (Data not shown). The antifungal property of phenolic compounds depends on the chemical structure of phenols and the performance could be due to the effect of pH on the ionization of phenolic compounds (Bortolomeazzi, *et al.*, 2007). Watarai and Tana (2005) reported that growth of *Salmonella enteritidis* inhibited in a medium containing wood vinegar (pH 7). The antifungal activities of Phenolic compounds are increased when ionized at pH 3.0 (Bortolomeazzi, *et al.*, 2007). At pH 7.0, the hydroxyl group from NaOH reacts with alkyl group of phenolic compounds and antioxidant property of phenolic compounds is responsible for fungal growth inhibition activity. Antioxidant activity of phenolic compounds could change due to the change in *para* position by pH modification (Bortolomeazzi, *et al.*, 2007). The electron donating ability of alkyl groups in the *para* position stabilizes the antioxidant capacity of phenolic compounds and this electron donation ability of *para* or *ortho* group responsible for inhibition of lipid oxidation. The inhibition of lipid oxidation originates the fungal growth inhibition (Bortolomeazzi, *et al.*, 2007; Rauha, *et al.*, 2000). The antioxidant ability of phenolic compounds depends on the number and presence of hydroxyl group and methoxyl group (Lopez, *et al.*, 2003). Due to the addition of NaOH into the wood vinegar, alkyl groups in *para* position of phenolic compounds accepting the electron and which leads the decrease in phenolic compounds ionization. Thus, the inhibition of lipid oxidation also decreased. It concluded that the antifungal property of wood vinegar ingredients especially phenolic compounds depends on the pH of the vinegar. However, still 2.5 and 3.0 % of neutralized wood vinegar inhibited the fungal growth in agar plates. GC-MS analysis shows the presence of many phenolic compounds in neutralized wood vinegar,

Table 2. Inhibition Percentage of neutralized wood vinegar, wood vinegar-soft wood extract and wood vinegar-hard wood extract on growth of *O. flexuosus*, *O. tetropii*, *O. polonicum*, *O. ips* and *O. naricissi* in 2 % MEA agar plates (Acidic wood vinegar data not shown)

Fungal	Control	Wood Vinegar					Wood Vinegar + Soft Wood Extract					Wood Vinegar + Hard Wood Extract				
Samples	(without	0.33 %	0.67 %	1.33 %	2.50 %	3.00 %	0.33 %	0.67 %	1.33 %	2.50 %	3.00 %	0.33 %	0.67 %	1.33 %	2.50 %	3.00 %
	preservatives)	%														
<i>O. flexuosus</i>	0	5	9	9	23	55	32	55	55	73	82	9	14	14	50	73
<i>O. tetropii</i>	0	2	5	5	22	47	25	27	27	37	75	7	12	17	32	52
<i>O. polonicum</i>	0	0	7	10	44	59	29	44	49	77	83	7	15	24	46	59
<i>O. ips</i>	0	6	6	6	28	39	14	14	17	44	78	11	11	14	22	50
<i>O. naricissi</i>	0	0	0	0	20	50	0	0	16	50	60	0	0	10	30	50



but those compounds were exhibited in non-ionized form. Accordingly some other compounds present in neutralized wood vinegar might also be responsible for fungal growth inhibition, but those compounds were weakly active to inactivate the fungal growth. This can be validated that 20-30 % of wood vinegar contains more than 200 ingredients other than phenolic compounds and 80-90 % occupied by water. GC-MS analysis of neutralized wood vinegar shows the presence of some benzene compounds. The antifungal activity of benzene compounds and its derivatives yet not been established.

Negative effects of water extracts from sawdust's of softwood and hardwood were demonstrated in various experiments. Suk and Kyung (2005) already demonstrated the effects of water extracts of *Pinus densiflora* sawdust on shiitake mushroom *Lentinula edodes* mycelial growth. Pine bark (*Pinus densiflora*) amended low molecular weight polyphenolic compounds (Mun and Ku, 2006). Pine phloem extract has been recognized for antioxidant and antimicrobial activity (Kwak, *et al.*, 2006; Rauha, *et al.*, 2000). S. K. Lee *et al.* (2005) reported the antifungal activity of pinosylvin, a constituent of *Pinus radiata*. Antifungal compound pinosylvin was extracted from leaf of *Pinus radiata*. Ethyl acetate fractions of *Pinus radiata* have potential antioxidant activity (Moure, *et al.*, 2005). In addition, the antioxidant activity of *Pinus radiata* fraction was well demonstrated, some phenolic compounds existing in *Pinus radiata* are water soluble and having greater antioxidant activity. Antimicrobial activity of ethyl acetate extracts of Oak hardwood was also demonstrated (Manter, *et al.*, 2007). Antimicrobial activity of Oak extracts also due to antioxidant activity of compounds existing in the fraction. Presence of free hydroxyl groups in the compounds determined the bactericidal and fungicidal activity. But our results shown that there was no significant growth reduction in the plates containing oak extract, this might be because of low solubility of compounds with antifungal activity in water. Oak hardwood also contains many nonvolatile compounds such as plicatic acid, plicatin and thujapicatin with weak antifungal activity (Barton and Macdonald, 1971). Some compounds existing in oak hardwood have greater antifungal activity against wood decay

fungi such as nootakatin (Rennerfelt and Nacht, 1955). But these compounds are soluble in ethyl acetate and acetone, thus it is recommended that the ethyl acetate and acetone extraction of these compounds are very useful for commercial purpose.

Based on our results, we concluded that the strong fungicidal activity of acidic broad leaved wood vinegar at lower concentration might be the combination of both acidic nature and presence of ingredients, such as phenolic, polyphenolic compounds and organic acids. Antifungal activity of phenolic compounds present in the wood vinegar arise from their chemical structure and pH of solution influence the changes on chemical structure. It confirms the statement that the fungicidal activity of extract is mainly based on phenolic compounds. The results of GC-MS analysis also support this statement.

## CONCLUSION

In conclusion, the acidic nature of broad leaved wood vinegar may create handling and environmental hazardous problem during commercial use. Thus neutralization of wood vinegar is essential in field and commercial uses. But higher concentration of neutralized vinegar (above 3 %) is only active as antifungal agent. To improve the efficacy of neutralized wood vinegar in the field at lower concentration, we could add hot water extract of *Pinus densiflora* and *Pinus radiata*. To minimize the quantity of extracts of *Pinus densiflora* and *Pinus radiata*, we should purify the antifungal compounds from hot water extracts either by ethyl acetate or acetone fractionation. This neutralized wood vinegar and hot water fraction mixture could be an eco-friendly material and would not create any hazardous problem in the field. This mixture could be a very good substitution for chemicals used to control sapstaining fungi in the field.

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