Biotechnology Research and Innovation (2019) 3, 47–53

Biotechnology Research & Innovation

http://www.journals.elsevier.com/biotechnology-research-and-innovation/

RESEARCH PAPER

In vitro evaluation of antimicrobial efficacy of pyroligneous acid from softwood mixture

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Received 6 December 2018; accepted 22 February 2019
Available online 15 March 2019

KEYWORDS
Pyroligneous acid;
Pyrolysis;
Antibacterial;
MIC;
Antifungal

Abstract A novel technology was developed to obtain a high amount of pyroligneous acid (PA) rich in organics. PA was obtained by atmospheric pyrolysis of a mixture of pine, spruce and fir wood particles, analyzed by gas chromatography-mass spectrometry (GC–MS) and evaluated for *in vitro* antibacterial and antifungal activity. Several microbial inhibitory compounds were observed in PA. Antimicrobial activity of PA was studied at both acidic pH (3.7) and neutral pH (7.0) of the liquid. Neutralized PA showed higher antibacterial activity than acidic PA against 5 pathogenic bacterial strains, and Minimum Inhibitory Concentration (MIC) obtained with neutralized PA were 0.3125% (v/v), 0.3125% (v/v), 0.625% (v/v), 0.3125% (v/v), 0.3125% (v/v) for *Escherichia coli*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Listeria monocytogenes* and *Enterococcus faecalis*, respectively. For fungal strains, acidic PA was found to be more effective than neutralized PA. The highest activity was against *Trametes versicolor*, followed by *Aspergillus niger* and *Aspergillus fumigatus*. The MIC of acidic PA with which fungal inhibition was seen was 0.125% (v/v) for *T. versicolor* and *A. fumigatus*, and 0.25% (v/v) *for A. niger*. Hence, the novel technology was found to be effective to produce a high yield of PA (40–45 wt% of dry biomass), rich in antimicrobial compounds, and the PA is proposed as a potential alternative to antibiotics, preservatives and/or chemical disinfectants that are currently in use.

Introduction

Pyroligneous acid (PA) commonly called ''Wood Vinegar'', is the aqueous phase obtained from the pyrolysis of

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https://doi.org/10.1016/j.biori.2019.02.004

lignocellulosic biomass. PA is highly acidic and includes several families of volatile organic compounds such as organic acids, phenols, aldehydes, phenols, alcohols, ketones, pyranfuran derivatives, and polyphenolic compounds. PAs can be obtained by slow or fast pyrolysis. Slow pyrolysis at 400–500 °C (at 10 °C/minute) with a longer vapor residence time (45–90 min) has been the conventional method used for the conversion of lignocellulosic biomass (Omulo et al., 2017). However, fast pyrolysis – in which biomass is rapidly heated to around 500 °C (at 1000 °C/s) for a short vapor residence time (less than 2 s), and then rapidly cooled – is gaining interest for the PA yield, which has been reported to be up to 75 wt% depending on the initial weight of the biomass as compared to ~25 wt% reported for slow pyrolysis (Bridgwater & Peacocke, 2000; Czernik & Bridgwater, 2004; Tiilikkala, Fagernäs, & Tiilikkala, 2010).

Previously, several studies have reported the antifungal properties of the oil phase obtained by pyrolysis (Mourant, Yang, Lu, Riedl, & Roy, 2009; Mourant, Yang, Lu, & Roy, 2007; Mourant, Yang, Riedl, & Roy, 2008). However, in recent times, PAs have been studied for their potential application as a termiticide, pesticide and synergistic insecticide in plant protection, due to their complex composition, many of which are known to have inhibitory properties (Pangnakorn, Watanasorn, Kuntha, & Chuenchooklin, 2009; Tiilikkala et al., 2010). Studies have reported the activity of PA against several plant pathogens (Chalermsan & Peerapan, 2009; Kim, Seo, Lee, & Lee, 2008).

The extensive use of antimicrobials in humans and animals has led to the emergence, resistance and spread of antibiotic resistance, and multidrug-resistant pathogens. Additionally, since the end of the ''Golden Age of Antibiotics'' in the 1970s, there has been a significant decline in the discovery of new antibiotics. This, coupled with the decreasing profitability of the development of novel antibiotics has led to the exploration of alternatives to antibiotics (Stanton, 2013). PAs are an attractive candidate for this, due to their perceived antimicrobial properties. With every component having a different mechanism of action, it would be difficult for microorganisms to develop wall-to-wall resistance. PA also potentially represents an environmentally friendly, and less toxic alternative to the chemical antifungal agents presently used (Velmurugan, Han, & Lee, 2009)

While several studies have been conducted on the advantages of using PAs in agriculture, few have looked into the activity of PAs against human pathogens. Also, there is a conflict regarding which aspect of the PA is responsible for the antimicrobial activity. Is it the pH or is it the chemical composition which varies with the feedstock and pyrolysis process conditions? Therefore, in this study the *in vitro* efficacy of acidic and neutralized PA obtained from pyrolysis of a softwood mix was evaluated against two Gram-positive and three Gram-negative bacteria that are known human pathogens. Additionally, the *in vitro* antifungal activity was also checked against three fungal strains known to cause wood rot and food contamination.

Materials and methods

Production and analysis of PA

The feedstock used for the production of PA was obtained from Belle-Ripe (www.belle-ripe.com), a biomass supplier company. The composition of the softwood collected was determined to be a mixture of wood particles of pine (80% v/v), spruce, and fir (20% v/v combined), with a particle size of 3/16 inches. The biomass pyrolysis process was carried out in Pyrovac Inc. facilities in Saint-Lambert-de-Lauzon, QC, Canada using continuous feed pilot equipment at a mass rate of 36 kg/h. Pyrovac makes use of a multiple hearth reactor indirectly heated with molten salts. One important feature is the vapor condensing system where the heavier oil was collected separately from the aqueous phase PA. The thermal decomposition reactions were performed at a temperature of 475 °C at close to atmospheric pressure conditions. The technique generates high yields of PA, rich in soluble organic compounds such as phenols and their derivatives, carboxylic acids, and aldehydes. The organic content reached 57% w/w and the rest was water. In this process, the first condensing tower contains the heavy bio-oils, while the second tower contains the water-rich, acid-soluble oxygenated compounds in water (Roy, Blanchette, & de Caumia, 2000).

The composition analysis of PA was done by GC–MS using an Agilent 5890 gas chromatograph. Separation was carried out on a 30 m × 0.25 mm I.D. DB-5 ms fused silica capillary column × 0.25 mm film thickness coated with 5% phenyl methylpolysiloxane. The GC oven was held at 50 °C for 2 min, and then programmed to 290 °C at 5 °C min^{-1} and maintained for 10 min. The injector temperature was 280 °C with split mode (1/30 split ration). The carrier gas was Helium with a flow of 1 ml per minute. The end of the column was directly introduced into the ion source of an Agilent model 5970 mass selective detector operated with electron impact ionization mode. Typical mass spectrometer operating conditions were as follows: transfer line 270 °C, ion source 250 °C, electron energy 70 eV. The mass range m/z: 30–500 Dalton was scanned every 0.8 s. The computerized match was manually evaluated to ensure the quality of identification. Identification of selected target compounds was confirmed by matching mass spectra and retention times with standard compounds. Quantification was carried out using a series of standard mixture solutions such as phenol, cresol, guaiacol, syringol, catechol, eugenol, and levoglucosan with different concentrations as calibration solutions.

Antimicrobial tests

Antimicrobial tests were carried out with native PA (pH 3.7) and neutralized PA samples. The initial pH (3.7) of PA was neutralized (to pH 7.0) using 12 M NaOH. The acidic and neutralized PA samples were filter-sterilized using a syringe filter, and the filtrate was used to test antibacterial and antifungal activities.

In vitro antibacterial activity

Bacterial strains used were *Escherichia coli* (ATCC 25922), *Enterobacter aerogenes* (ATCC 13048), *Pseudomonas aeruginosa* (ATCC 15442), *Listeria monocytogenes* (ATCC 191110) and *Enterococcus faecalis* (ATCC 29212). *E. coli* and *E. aerogenes* were cultured in a Luria-Bertani medium, *P. aeruginosa*, *L. monocytogenes* and *E. faecalis* in Tryptic Soy media. Strains were incubated overnight at 37 °C.

The qualitative test for *in vitro* antibacterial activity was done using a modified protocol for the well diffusion method as described by Tagg, Dajani, and Wannamaker (1976). To 25 ml of media with 0.75% (w/v) agar, 125 μl of 1:1000 dilution of the overnight bacterial culture was added, poured into Petri plates and allowed to solidify. Wells were punched into the agar plates using sterile 5 ml pipettes and 80 μl of filter-sterilized PA sample was added. The sterile broth was added as the negative control. Plates were incubated at 37 °C overnight, and then the inhibition zone diameters were measured.

The quantitative test to determine Minimum Inhibitory Concentration (MIC) value was done by the broth microdilution method as described by Fernandez, Le Lay, Jean, and Fliss (2013). To all wells of the 96-well plate, 125 μl of sterile broth was added. To the first well, 125 μl of PA sample was added and subsequent two-fold serial dilutions were done. Finally, 50 μl of a 1:1000 dilution of an overnight culture of the bacteria were added to each well to give a final concentration of $\sim 10^6$ CFU mL^{-1}. Controls with sterile broth only and no bacterial inoculum were maintained. Plates were incubated at 37 °C for 24 h and the optical densities were measured at 595 nm by a spectrophotometer (Infinite® F200 PRO, Tecan Inc., Durham, NC, USA).

In vitro antifungal activity

Fungal strains tested were *Trametes versicolor* (ATCC 2086), *Aspergillus niger* (NRRL 2001) and *Aspergillus fumigatus* (NRRL 163). Strains were cultured in potato dextrose broth at 30 °C till visible mycelia were observed. Filter sterilized PA sample was added at different concentrations (1%, 0.5%, 0.25%. 0.12% and 0.06% v/v) to potato dextrose agar (PDA) plates. Sterile filter paper disk was soaked into the liquid media containing fungi for absorption of mycelia, and this was placed in the center of the plate. Controls of PDA without PA were also maintained. *Trametes* plates were maintained at 30 °C for 6 days, and *Aspergillus* plates were incubated at 30 °C for 4 days, after which time the mycelial growth was measured (Jung, 2007). The inhibition rate was calculated using the following equation:

$$\text{Inhibition efficiency}\,(\%) = \left(1 \times \frac{D_t}{D_c}\right) \times 100, \qquad (1)$$

where D_t is the diameter of fungal growth in the test plate and D_c is the diameter of the fungal growth in the control plate

Statistical analyses

Qualitative and quantitative analyses were used to describe the antimicrobial activity of the PA samples. For testing antibiotic activity, tests were carried out in duplicates and results were expressed as the mean value with standard deviation. For antifungal activity, tests were carried out in triplicates and results were expressed as the mean value with standard deviation.

Table 1 Chemical composition of PA used in the current study.

Compound	Wt.%
Water	45.00
Catechol	8.72
4-Methylcatechol	7.41
Acetic acid	3.90
4-Ethylbenzenediol	3.54
3-Methylcatechol	3.24
Propanetriol	2.10
2-Hydroxy-3-methyl-2-cyclopenten-1-one	1.44
Cyclohexanone	1.05
Furfural	0.81
Dihydrofuranone	0.81
Vanillin	0.76
Cresol	0.74
Phenol	0.72
Unknown	0.60
4,5-Dimethyl-1,3-benzendiol	0.57
Guaiacol	0.38
Eugenol	0.36
4-Propylbenzenediol	0.36
2-Propanal	0.33
2,5-Dimethylphenol	0.33
Hydroxymethoxyethylfuran	0.28
Methoxypropene	0.25
Methylpropanol	0.25
Hexadienal	0.24
Furanmethanol	0.22
1-(4-Hydroxy-3-methoxyphenyl)ethanone	0.19
5-Methtyl-2-(propenyl)phenol	0.16
Acetic acid ethenylester	0.15
Thiazolidine	0.14
Ethylphenol	0.14
Isoeugenol	0.13
4-Propylguaiacol	0.13
2-Methyl-3-methoxy-1-propene	0.12
2-Methylpropanol	0.10
Unknown (insoluble in DCM and water)	7.8
Unknown (insoluble in DCM but soluble in water)	6.53
Total	100.00

Results

Characterization of PA

The pyrolysis of the softwood mixture used in the current study gave a high PA yield up to 40–45% on an anhydrous biomass basis. The chemical composition of the PA obtained in this study is given in Table 1. PA obtained in this study was found to have high concentrations of phenolics like catechol and methylcatechol. The thermal degradation of lignin leads to the formation of phenolic compounds, and the pyrolysis

Table 2 Diameters of the zone of inhibition for acidic and neutralized PA.

Bacterial strains	Diameter of inhibition zones (mm)		
	Acidic PA	Neutralized PA	Negative control
E. coli	20 ± 0.1	18.0 ± 0.1	–
E. aerogenes	25.5 ± 0.05	23.0 ± 0.1	–
P. aeruginosa	23.5 ± 0.15	18.3 ± 0.07	–
L. monocytogenes	31.0 ± 0.1	30.1 ± 0.11	–
E. faecalis	32.9 ± 0.1	25.0 ± 0.1	–

products formed depends on the structure of the lignin. Catechol and its derivatives are formed due to demethylation of 2-methoxyphenol, which is abundant in softwoods (Li et al., 2017). Other compounds that were seen in higher concentrations were acetic acid, aldehydes (such as furfural and vanillin), phenol and cresol, all of which are known inhibitors of microbial growth. Organic acids such as acetic acid and aldehydes like furans and ketones, which were also observed in higher amounts in PA, are produced by the thermal pyrolysis of xylans (Stefanidis et al., 2014)

Evaluation of antibacterial activity

Antibacterial activity of PA was tested against 3 Gram-negative (*E. coli*, *E. aerogenes*, and *P. aeruginosa*) and 2 Gram-positive (*L. monocytogenes and E. faecalis*) pathogenic bacteria. The diameters of the inhibition zones obtained with acidic and neutral PA are given in Table 2.

For the microbroth dilution test, the MIC values were obtained for both acidic and neutralized PA (Fig. 1). The MIC value for acidic PA was the least for *E. coli* and *E. aerogenes* (0.625 % v/v), and the highest for *P. aeruginosa*, *L. monocytogenes* and *E. faecalis* (1.25% v/v). Neutralized PA was found to be more effective for all strains as it gave a lesser MIC value than acidic PA. For *E. coli*, *E. aerogenes*, *P. aeruginosa* and *E faecalis*, the MIC value for neutralized PA was half the value of MIC for acidic PA. For *L. monocytogenes*, neutralized PA had a MIC value of 0.3125% (v/v), which was four times less than the MIC of acidic PA.

Evaluation of antifungal activity

Antifungal activity of PA was tested against *T. versicolor* (a wood rot fungus), *A. niger* (a common food contaminant) and *A. fumigatus* (a common cause of pulmonary infections in immunocompromised patients). As defined by Kartal et al., an inhibition efficiency greater than 20% is needed for the test fungus to be considered inhibited (Kartal, Imamura, Tsuchiya, & Ohsato, 2004). A representative image of the antifungal assay (for *T. versicolor*) is given in Fig. 2.

The inhibition efficiency of acidic and neutralized PA against the three fungal strains are given in Fig. 3. *T. versicolor* was found to be most sensitive to both acidic and neutralized PA. Acidic PA was found to inhibit *T. versicolor* completely even at a concentration of 0.12% (v/v), whereas neutralized PA gave 100% inhibition against *T. versicolor* only at 1% (v/v) concentration. For both species of *Aspergillus*, total inhibition was obtained only at a PA concentration of 1% (v/v). For *A. niger* and *A. fumigatus*, acidic PA was inhibitory at a minimum concentration of 0.25% (v/v). The MIC for neutralized PA for *A. niger* and *A. fumigatus* was 1% and 0.5% (v/v) respectively.

Discussion

Pyrolysis of biomass produces PA, pyrolysis oil, wood charcoal, and non-condensable gas, the ratio of which depends on several parameters such the feedstock used, vapor retention time and condensation system. The pyrolysis process that was used in the current study utilizes a novel technology for the condensation of organic vapors and steam, which enables high production of PA-rich in phenols, aldehydes, and organic acids. The PA yield obtained in this study at 40–45 wt% is rather high as compared to previous studies that reported PA yield of 7.26 wt% from mango tree wood (Mopoung & Udeye, 2015), 20 wt% from softwood bark (Garcìa-Pérez, Chaala, Pakdel, Kretschmer, & Roy, 2007) and 25 wt% from cotton stalk (Wu et al., 2015).

A high concentration of phenolic compounds such as catechol (8.72%) and methyl catechol (10.65%) was observed in the current Pyrovac PA sample, compared to those previously reported by Yang et al. at 5.2% and Fagernäs et al. at 0.16% (Fagernäs, Kuoppala, Tiilikkala, & Oasmaa, 2012; Yang et al., 2016). Catechols, phenols and cresols are formed by thermal decomposition of lignin. 2-Methoxyphenol is a major component of lignin. Catechol and its derivatives are formed due to demethylation of 2-methoxyphenol (Li et al., 2017). Additionally, it has been reported that the pyrolysis process at a temperature between 425 °C and 575 °C enables high yield of phenols (Butt, 2006). Other compounds that were seen in higher concentrations were acetic acid, aldehydes (such as furfural and vanillin), lactones (*e.g.* dihydrofuranone), phenol and cresol, all of which are known inhibitors of microbial growth. The yield of furfural obtained in this study (0.81%) was almost double of that reported by Mu et al. (0.44%) during the pyrolysis of bamboo at 480 °C (Mu, Uehara, & Furuno, 2004). Organic acids such as acetic acid, aldehydes and ketones are produced by the thermal pyrolysis of xylans (Stefanidis et al., 2014), whereas lactones are produced by hemicellulose degradation (Mathew & Zakaria, 2015).

For all the bacterial strains, inhibition zones obtained for acidic and neutralized PA were similar, indicating that both were equally effective against the strains. However, the MIC values obtained against bacterial strains were lower with the neutralized PA, as compared to acidic PA. The high activity of

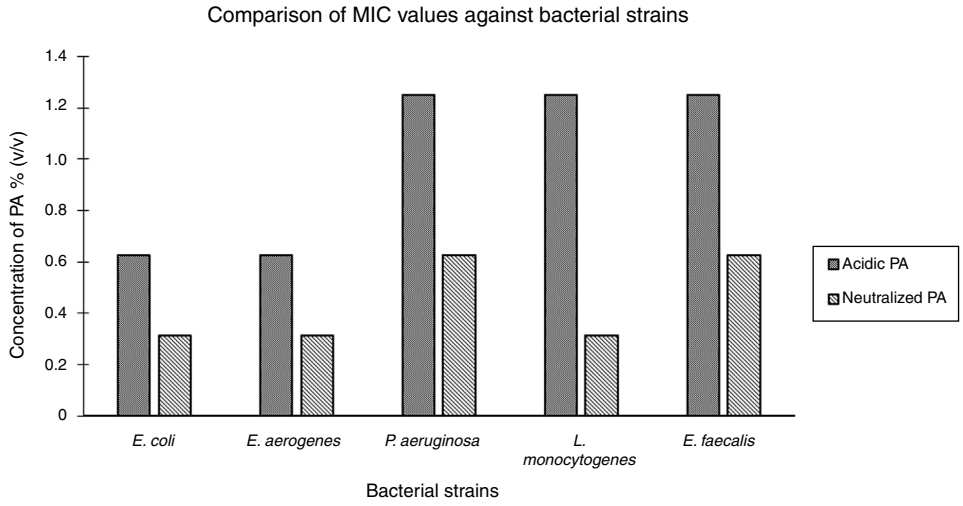

Figure 1 Comparison of MIC values for acidic and neutralized PA.

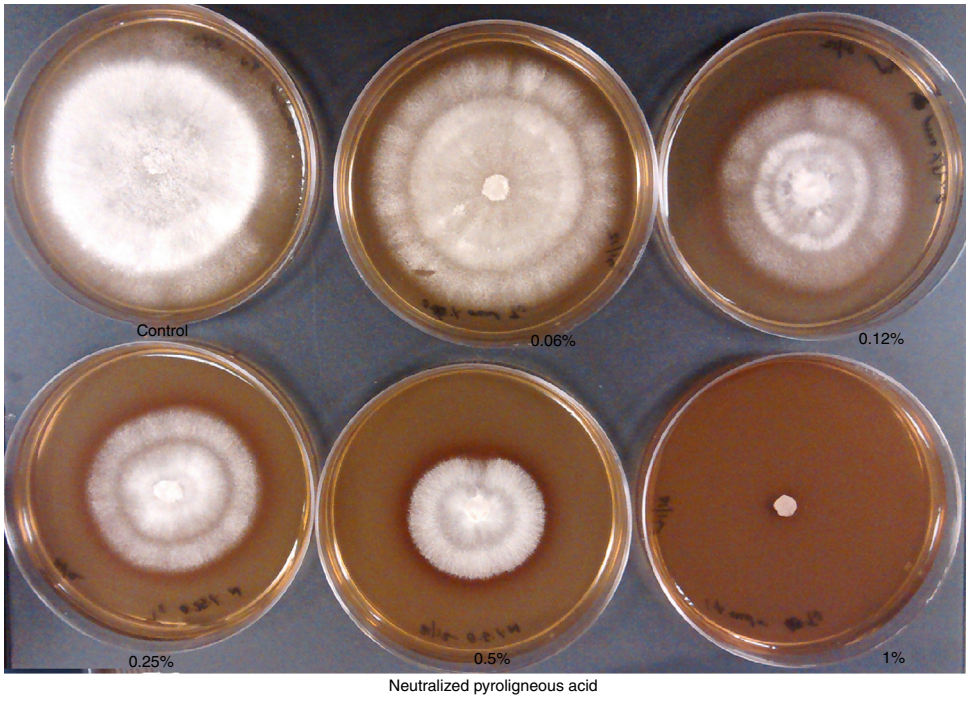

Figure 2 Effect of increasing concentrations of PA on the growth of *T. versicolor*.

neutralized PA indicated that the antibacterial property of the PA was due to its complex chemical composition, and not the massive presence of acetic acid. The antibacterial efficiency of the neutralized PA could also be attributed to the antimicrobial effect of ketones (furfural, vanillin.) which is enhanced at neutral pH. PA was found to inhibit both Gram-positive and Gram-negative bacteria. Previous studies have reported that Gram-positive bacteria are more susceptible to antibiotics as compared to Gram-negative bacteria due to the lipid layer present in the cell wall of Gram-negative cells (Ariffin et al., 2017; Yang et al., 2016). However, in this case, the MIC value of the acidic PA for the two Gram-positive bacteria was found to be higher than that of *E. coli* and *E. aerogenes*. A possible reason for this could be the protective effect exerted by the peptidoglycan layer in the Gram-positive cell wall (Mozin, Rosyidi, Sjofjan, & Widodo, 2015).

According to World Health Organization report (2017), *E. coli*, *E. aerogenes*, and *P. aeruginosa* are the leading causes of multidrug-resistant nosocomial infections and therefore belong to the high priority category, against which there is a need for novel antimicrobials (Tacconelli & Magrini, 2017). Drug-resistant strains of *E. faecalis* are known to cause nosocomial infections with a high mortality rate. *L. monocytogenes* (a psychotropic foodborne pathogen), is one of the most common contaminants in packaged meat products. Therefore a natural preservative agent would be of great interest to the meat industry. Therefore,

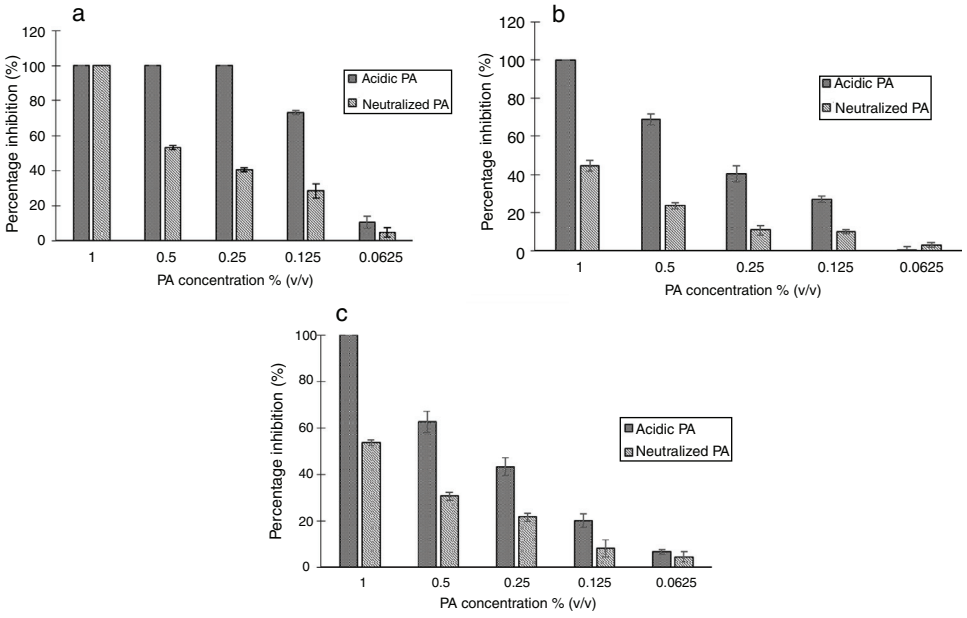

Figure 3 Inhibition efficiency of acidic and neutralized PA against (a) *T. versicolor*, (b) *A. niger* and (c) *A. fumigatus*.

neutralized PA can be proposed as not only an eco-friendly alternative to antibiotics but also as a natural preservative agent.

For all fungal strains, acidic PA was found to be more effective than neutralized PA. This could be due to a change in the ionization of the phenolic content of neutralized PAs (Baimark & Niamsa, 2009). Fungal inhibition is caused by the antioxidative property of phenolics, and previous studies have reported that the inhibition of lipid oxidation by phenolic compounds is enhanced at acidic pH. When PA is neutralized with NaOH, the alkyl groups in the para position of phenolic compounds to get reduced, causing a decrease in the ionization of the phenolic compounds. This causes a decrease in the lipid oxidation, and consequently a decrease in antifungal activity (Velmurugan et al., 2009). Studies have reported that the treatment of wood with phenolics and aldehydes can protect it from white and brown rot fungi (Singh & Singh, 2012; Voda, Boh, Vrtačnik, & Pohleven, 2003). Mourant et al. reported that the ethyl ether-soluble fraction of PA obtained by pyrolysis of softwood bark mixture showed 100% inhibition against *T. versicolor* at a concentration of 0.15 g/ml (Mourant et al., 2007). Therefore PA obtained in this study can be developed as an agent for surface application or treatment to protect wood from fungal rotting. The inhibitory effect of PAs on different (aflatoxin producing) strains of *Aspergillus*, has also been studied, however, these have not been very encouraging (Jothityangkoon, Koolachart, Wanapat, Wongkaew, & Jogloy, 2008; Uysal, Duman, Onal, Yasa, & Yanik, 2014). Hence, additional studies should be carried out with higher concentrations of neutralized PA to widen its sphere of application against *Aspergillus*.

Conclusion

The PA obtained in this study was found to have organic acids such as acetic acid, and aldehydes like vanillin and furfural, all of which are known as microbial inhibitors. Each of these inhibitory compounds has weak antimicrobial activities by themselves. It is therefore believed that with the PA samples used in this study, the synergistic activity of the various compounds could account for the high antimicrobial efficacy. Additionally, due to the multiple sites of action of each of these compounds, it is postulated that it would be more difficult for the pathogen to develop resistance against the PA. Therefore the development of PA obtained in this study into an effective antimicrobial agent is worth being pursued. Furthermore, since the chemical composition of PA depends on the feedstock used, it could be worthwhile to carry out future studies with different compositions of the biomass feedstock and evaluate the antimicrobial activity of the PA obtained.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The work was supported by the Natural Sciences and Engineering Research Council of Canada (Engage Grant 122842). The authors are sincerely thankful to Dr. Benoît Fernandez for assistance with the antimicrobial tests.

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