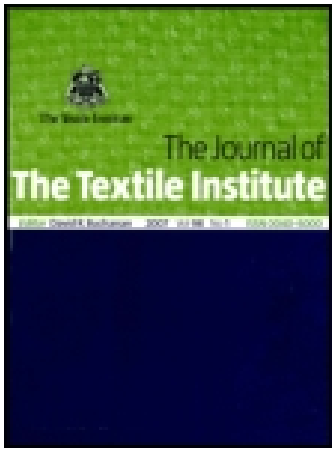

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The origin of the antibacterial property of bamboo

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The origin of the antibacterial property of bamboo

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Bamboo is an eco-friendly and multifunctional plant. Bamboo clothing has recently entered the textile market with a claim for its antimicrobial properties, but without scientific evidence. In this study, the antibacterial activity of plant extracts from Australian-grown bamboo (*Phyllostachys pubescens*) is investigated. Bamboo extracts were made using water, dimethyl sulphoxide (DMSO) and dioxane and their antibacterial properties were compared against Gram-negative bacteria, *Escherichia coli*. It was found that the extract made in 20% DMSO aqueous solution showed weak antibacterial activity, whereas the extract made using 90% dioxane aqueous solution exhibited strong antibacterial activity, even after 20 times dilution. The results indicate that antibacterial agents of *P. pubescens* are located in lignin, not in hemicellulose or other water-soluble chemical components.

Keywords: bamboo; antibacterial property; lignin; *E. coli*

Introduction

Bamboo is a bast fibre and considered as a "green" natural nanocomposites where cellulose nanofibrils are embedded in the matrix of lignin and hemicelluloses (Afrin, Tsuzuki, & Wang, 2010; Rao & Rao, 2005). Bamboo is well recognised for its multifunctionality and eco-friendly nature and has been serving the daily needs of over 1.5 billion of people for centuries (Austin, Levy, & Ueda, 1970; Liese, 2009). The use of bamboo in medicinal applications has a long history. It was shown that the leaves of some bamboo species have an antioxidative activity (Lu, Wu, Tie, Zhang, & Zhang, 2005). Bamboo's role in oral medicine is also portrayed where the crude extracts of *Polygonum cuspidatum* roots showed a wide range of antibacterial activities against both Gram-positive and Gram-negative bacteria due to the presence of phenolic compounds in its chemical constituents (Shan, Cai, Brooks, & Corke, 2008). Some researchers have also reported antibacterial activity of bamboo charcoal (Yang et al., 2009) and bamboo vinegar (Sulaiman, Murphy, Hashim, & Gritsch, 2005).

Recently, bamboo clothing have entered the textile industry and many commercial bamboo fabric products are claimed to be eco-friendly and antibacterial. However, most of the claims are made by the industry stakeholders where little scientific evidence was presented (Afrin, Tsuzuki, & Wang, 2009). In particular, the compound(s) responsible for antibacterial properties in bamboo has not been fully investigated. In some Asian countries, the antibacterial agent in bamboo plants is identified as "kun" that represents a hydroxyl functional group (–OH) in a direct translation, but it fails to describe the actual chemical compound and its location in bamboo.

Bamboo is a lignin–carbohydrate compound that is a glycoconjugate where hydrophobic lignin is chemically bound to hydrophilic polysaccharides, such as cellulose and hemicellulose (Koshijima & Watanabe, 2003). The extraction of lignin is commonly carried out using aqueous dioxane solutions (Björkman, 1954). The extraction of hemicelluloses is typically made in dimethyl sulphoxide (DMSO) (Al-Bakri & Afifi, 2007). Hence, in the present study, extraction of Australian-grown moso bamboo (*Phyllostachys pubescens*) was carried out in water, DMSO and aqueous dioxane, and the antibacterial activity of the extracts was investigated to elucidate the location of the chemical compounds(s) responsible for antibacterial properties in bamboo.

Experimental

Microorganisms and media

Gram-negative bacterium, *Escherichia coli* (*E. coli*)-ATCC 25922 was used as test organism. The bacterial inoculums were prepared to obtain a bacterial suspension in exponential growth of 8×10^8 colony

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forming units ml^{-1} in 5 ml of nutrient broth (modified Trypton soya broth from Oxoids). Trypton soya agar (from Oxoids) was used as the nutrient agar for the agar plates. The Atherton cyber series autoclave was used for sterilisation and media preparation at 121°C for 20 min.

Materials and methods

Bamboo (*P. pubescens*) plant samples were purchased from Earthcare Farm at Crystal Waters Permaculture Village in Queensland, Australia. They were matured culms and already dried. The bamboo was crushed into fine powder to give the possibly highest surface area while extracting with solvents. First, the raw bamboo specimen was crushed into a powder form in a vertical turret mill (Hafco, Super Power BM-52VF; Hare and Forbes Machinery house, Melbourne, Australia) and then shaker milling was further performed in a 8000M mixer/mill (Spex, Metuchen, NJ, USA) in a steel container with the steel balls of 0.9–1 cm in diameter with the weight ratio of sample:ball = 1:60.

Time-dependent water extraction was done with raw bamboo powder. Ten grams of bamboo is added to 300 ml of deionised sterile water. The extraction is carried out for 1, 3, 6, 12, 24 and 72 h. After extraction the solution was centrifuged (Eppendorf centrifuge, 5430R) and the supernatants were collected.

Ajax supplied the DMSO. It has been reported that the DMSO itself has an antibacterial activity (Ansel, Norred, & Roth, 1969). Therefore, the dependence of DMSO concentration in water on the antibacterial activity was studied within the concentration range from 0 to 100%. It was found that 20% is the best concentration to use for extraction, because the numbers of the colonies were in between 30 and 300, suitable for colony-counting. To make bamboo extracts, 10 g of milled bamboo powder was immersed into 300 ml of 100% DMSO and was kept at room temperature for 72 h with continuous stirring, followed by filtering to collect the supernatants, in which deionised sterile water were added to make 20% DMSO aqueous solution.

Reagent grade dioxane was purchased from Sigma-Aldrich (Sydney, Australia). The extraction was carried out at room temperature by keeping 10 g bamboo in 300 ml aqueous dioxane solution (water:dioxane = 1:9 v/v) for 72 h with continuous stirring. The powder–liquid mixtures were then filtered and the supernatant was collected. The dioxane was evaporated to make bamboo extracts in water so that there is no effect of the dioxane on the antibacterial activity. This was considered as 100% solution of milled wood lignin (MWL) and was further diluted with sterile deionised water to obtain 50, 25, 10 and 5% solutions.

The *E. coli* growth in nutrient broth was monitored by the optical density measurements using an Asys micro plate reader spectrophotometer at 550 nm (Expert plus UV; Type: G020151, ASYS Hitech GmbH, Eugendorf, Austria). Hundred microlitres of *E. coli* inoculum was added into 5 ml of bamboo extracts (water, DMSO extractions and MWL in water) and incubated for 18 h at 37°C in a shaker oven. Twenty percent DMSO was used as control for bamboo extracts in DMSO and sterile water was used as control for water extracts and MWL, respectively. After 18 h of incubation, 100 µl of the *E. coli* and or extract mixtures were plated (three of each) and incubated for further 18 h at 37°C. After incubation, the plates were observed on the light box and pictures were taken. A Ricoh 12 mega pixel camera was used for the photography of the agar plates with bacterial growth.

Physiochemical characterisation of bamboo powder

The morphologies of milled bamboo powder and lignin extracts were studied by scanning electron microscopy (SEM) using a Supra 55 VP. A Fourier transform infrared spectroscopy (FT-IR) was carried out to identify the chemical bonds with a Bruker Vertex 70 spectrometer (Etllingen, Germany) and associated software OPUS 5.5. A Malvern Mastersizer 2000 particle size analyser (Worcestershire, UK) was used to measure the particle size of the milled bamboo powder by static laser light scattering, with water as a dispersant. The amounts of cellulose, hemicellulose and lignin are measured according to Chinese standard method GB5889-8.

Results and discussion

Physical appearance of bamboo powders and MWL

The particle size of the bamboo powder after vertical turret milling was around ~500 µm in diameter. Further milling in a shaker mill reduced the particle size down to 5–20 µm as shown in Figure 1(a). The

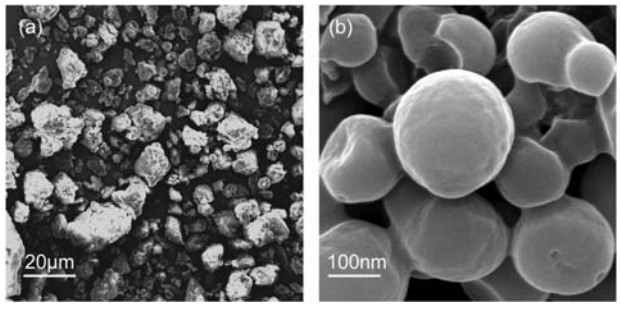

Figure 1. SEM images of (a) milled bamboo powder and (b) extracted bamboo lignin by using 90% aqueous dioxane.

particle sizing by laser-light scattering indicated that the average volume particle size was 30 µm. The larger particle size observed by laser light scattering may be caused by the fact that the particles swelled during the measurements in water, while the SEM image was taken for dried particles.

After extraction in 90% dioxane aqueous solution, the dioxane was evaporated and the bamboo extracts were collected in water that is termed as MWL. The SEM image of MWL is shown in Figure 1(b). These particles were found to be quite similar to the particles isolated by Liese (1998). This observation gives the indication of successful extraction of the gummy material, i.e. lignin.

Chemical constituents

Figure 2 shows the chemical constituents of the Australian-grown bamboo (*P. pubescens*) measured according to Chinese standard method GB5889-8. Cellulose, hemicellulose and lignin were identified as 53, 15 and 28%, respectively.

FT-IR spectroscopy was carried out on the untreated bamboo samples to reveal the chemical bonds, especially in the lignin region of the bamboo, as shown in Figure 3. It has been indicated earlier that the absorption peaks associated with lignin are located in the range from 1500 to 1750 cm^{-1} (Yueping et al., 2010). However, in this study, two major components of lignin are guaiacyl and syringyl and their stretching vibration rings are evident in a lower wavenumber range at 1230 and 1160 cm^{-1}, respectively. Also 1740 and 1675 cm^{-1} bands are identified as nonconjugated carbonyl stretching and conjugated carbonyl stretching, respectively. The 1600, 1505 and 1425 cm^{-1} bands are aromatic skeletal vibrations (Buta, Zadrazil, & Galletti, 1989; Sakakibara & Sano, 2001; Yueping et al., 2010). The spectrum also shows the typical cellulose finger print where 1050 cm^{-1} band is assigned to complex vibrations associated with the C–O, C–C stretching and C–OH bending in polysaccharides (Rodríguez-Lucena, Lucena, & Hernández-Apaolaza, 2009; Yueping et al., 2010). The 1375 cm^{-1} band corresponds to the C–H deformation in cellulose and hemicellulose. C–H deformation in cellulose is evident in 898 cm^{-1} band (Pandey & Pitman, 2003).

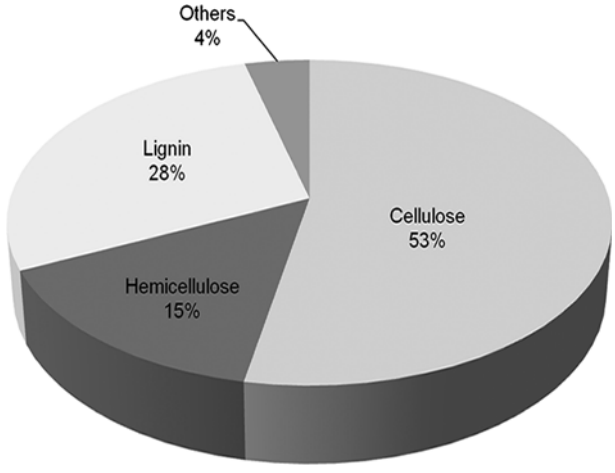

Figure 2. Chemical constituents of Australian-grown bamboo (*P. pubescens*) measured according to GB5889-8 standard.

Antimicrobial activity

Bamboo (time dependent) extracts in water

The antibacterial activity of bamboo extracts in water is presented in Figure 4. It is evident that the bamboo extracts in water could not inhibit or kill the growth of *E. coli* as the plates of control and the bamboo extracts in water have shown similar appearance in the bacterial lawn. Therefore, it is clear that the antibacterial compound(s) (if any) of bamboo (*P. pubescens*) is not water soluble.

Bamboo extracts in 20% aqueous DMSO

Figure 5 shows photographical images of *E. coli* colonies on Trypton soya agar that were plated with 20% DMSO solution (control) and the bamboo extracts in 20% DMSO after incubations for 18 h. We found that the colony size was significantly larger on the control (DMSO) plates than on the bamboo extracts plates. It gives the indication of inhibition of bacterial growth. However, the colony number was higher in the bamboo extracts plates than the control plates. DMSO has earlier been reported to be bacteriostatic for *E. coli* at 20% concentration (David, 1972). It is possible that the typical plant solvent DMSO could not properly extract the antibacterial compound out from bamboo. DMSO is also reported as the solvent for extracting hemicellulose (Haimer et al., 2010), and therefore, it is evident that the hemicellulose could not show prominent antibacterial activity.

Bamboo extracts in water made using dioxane

The MWL (the bamboo extract in an aqueous dioxane solution after removal of dioxane) was diluted with water into v/v 100% (undiluted), 50, 25, 10 and 5% and their antibacterial properties were tested against *E. coli*. Figure 6 shows photographical image of bacterial plaques that were plated with control solution, undiluted MWL and diluted MWL. The control plates had a full lawn of bacteria, whereas no bacterial col-

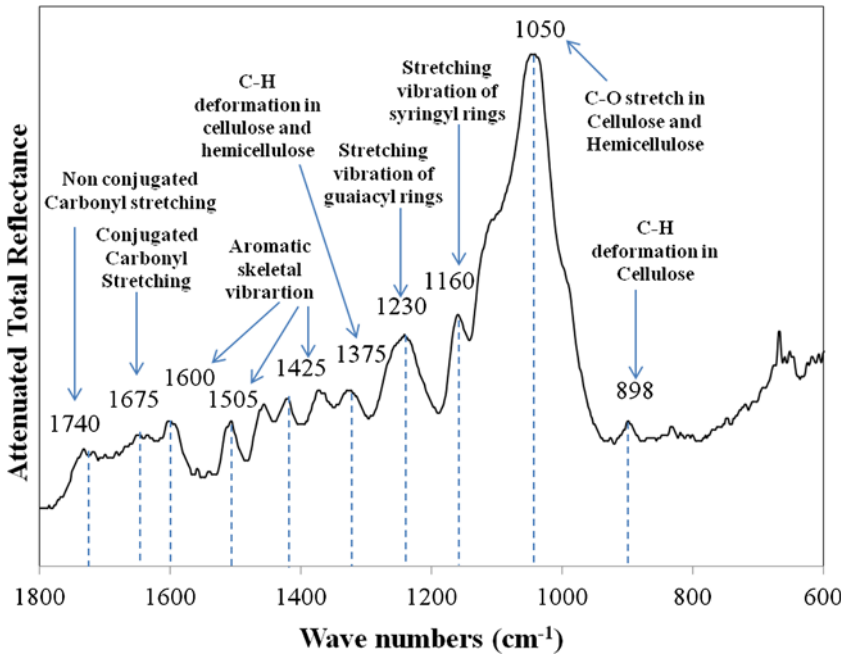

Figure 3. FT-IR spectra of untreated bamboo (*P. pubescens*).

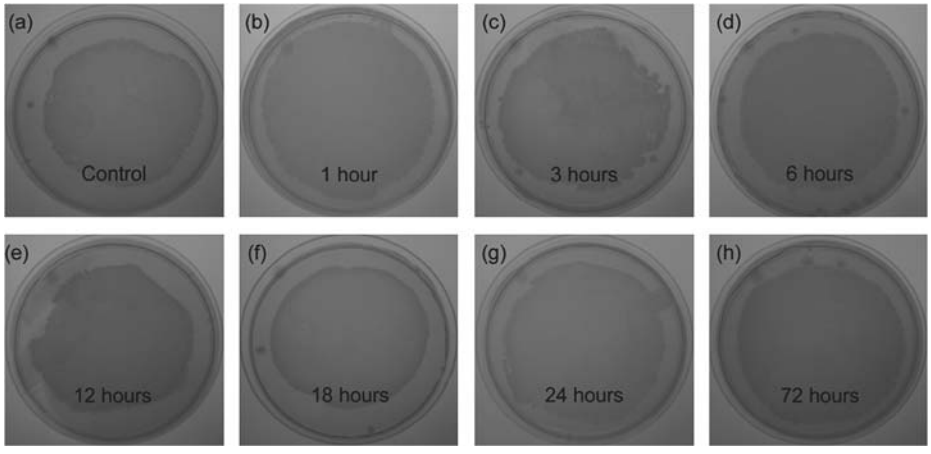

Figure 4. Photographical image of bacterial plaques that were plated with the bamboo extracts in water: (a) control, (b) 1 h, (c) 3 h, (d) 6 h, (e) 12 h, (f) 18 h, (g) 24 h, and (h) 72 h of incubation.

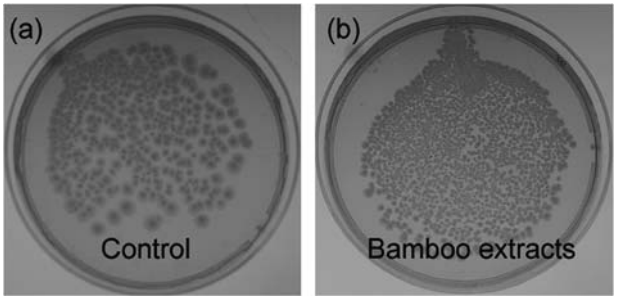

Figure 5. Photographical image of bacterial plaques that were plated with (a) 20% DMSO solution and (b) bamboo extracts in 20% DMSO.

ony was evident on the plates with bamboo extracts. It was demonstrated that at the lowest concentration, 5% of bamboo extract in water was sufficient to achieve 100% sterilisation rate against strong bacteria such as *E. coli*. Since dioxane was evaporated after extracting bamboo in aqueous dioxane (dioxane: water = 9:1), there was no effect of dioxane on the antibacterial activity. Moreover, dioxane is safely and commonly used to prove the antibacterial activity of the Schiff base metals (Johari, Kumar, Kumar, & Singh, 2009). Therefore, it is evident that the bamboo

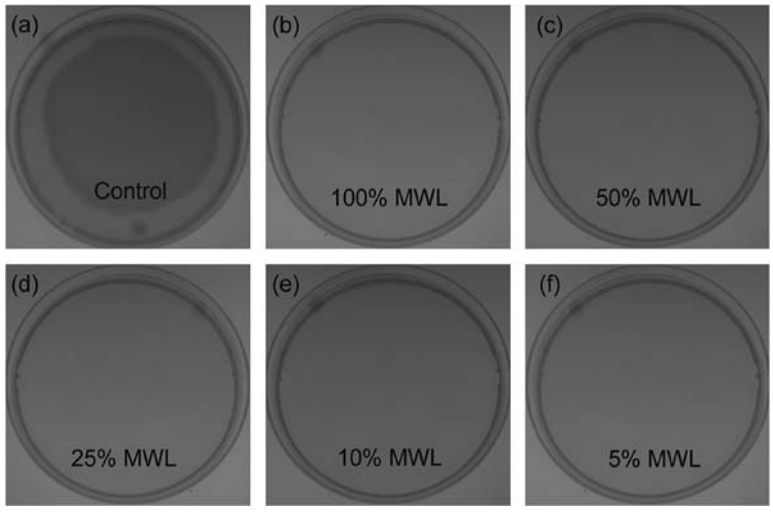

Figure 6. Photographical image of bacterial plaques that were plated with (a) sterilised deionised water and (b) 100%, (c) 50%, (d) 25%, (e) 10%, and (f) 5% MWL in sterile water.

(*P. pubescens*) lignin contains strong antibacterial compounds.

The location of antibacterial agents in P. pubescens

From the above antibacterial test results, it is evident that the antibacterial agents of *P. pubescens* are located in lignin, not in hemicelluloses. The water-insoluble nature of antibacterial compounds also suggests that the antibacterial agents reside in lignin which is almost insoluble in water (Walker, 2006).

The chemical constituent analysis proved the presence of a high amount of lignin (28%) in the bamboo powder used in this study. In general, lignin is an aromatic gummy material composed of guaiacyl, syringyl and *p*-hydroxyphenyl functional groups as well as *p*-coumaric acid that is esterified in the polymer systems (Higuchi, 1969). The lignin in softwood-like bamboo is composed of coniferyl alcohol as the principal monomer (Dimmel, 2010). Lignin is a complicated network of polymers made of oxidative coupling of three major C_6–C_3 (phenylpropanoid) units with many carbon-to-carbon and ether linkages and is formed by dehydrogenative polymerisation of three lignin precursors, *p*-hydroxycinnamyl, coniferyl and sinapyl alcohols (Xu, Sun, Sun, Fowler, & Baird, 2006; Zhanga, Liua, & Suna, 2010). FT-IR spectroscopy in this study revealed the presence of aromatic and carbonyl functional chemical groups in bamboo. Some edible plant extracts have shown antibacterial activity because of the presence of phenolic groups (Alzoreky & Nakahara, 2003). Other studies reported the separation of bioactive lignophenol antioxidants from bamboo lignin and described their neuroprotective activity (Akao et al., 2004; Ito et al., 2007). Zemek, Kosikova, Augustin, and Joniak (1979) also depicted antibiotic effects of synthetic compounds having guaiacyl and syringyl structures that are related to the structure of native lignin. As such, the existence of the aromatic and phenolic functional groups in lignin may be responsible for the antibacterial property of *P. pubescens*.

In order to produce antibacterial bamboo fabrics, lignin components need to be retained into the fibres while processing raw bamboo into fibre. However, current methods to process bamboo plants into fibres are based on the regeneration principle where bamboo plants are dissolved into solvents like alkali and carbon disulphide to reconstruct cellulose-rich fibres (Rydholm, 1965), through which the functional chemical compound like lignin is lost. Therefore, there is a strong need for the development of new fibre production methods that enables the retention of lignin in the final fibre products.

Conclusion

In this study, the origin of antibacterial property of Australian-grown bamboo was investigated. The bamboo extract in the typical plant solvent DMSO to extract hemicellulose showed the inhibition of bacterial growth but could not kill the bacteria. The MWL which was extracted in aqueous dioxane showed 100% antibacterial activity even after extensive dilution. Therefore, it is concluded that the antibacterial compound of bamboo is located in lignin. FT-IR results suggested that the antibacterial property may stem from

the aromatic and phenolic functional groups in lignin. Further antibacterial studies against a Gram-positive bacterium *Staphylococcus aureus* are under way.

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