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Treatment of rubberwood (*Hevea brasiliensis*) (Willd. ex A. Juss.) Müll. Arg. with maleic anhydride to prevent moulds

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ABSTRACT: The rubberwood samples were treated with 0.5–10% maleic anhydride (MA) solutions. The treatment of wood with 2.5% MA was adequate to prevent the growth of moulds on wood for 1 year at least. The viable count of *Aspergillus niger* van Tieghem PSU1 on MA treated wood indicated that fungal spores were not killed. The maleic anhydride treated wood slices had no antifungal activity. The concentration of MA released from treated wood in the leachate was 0.02 mg·m^{-3}. Agar well diffusion showed that the leachate from MA treated wood had no antifungal activity. However, after leaching MA treated wood still had a high resistance to mould growth. The moisture contents of MA treated and untreated wood samples were not significantly different. The MA treated wood showed almost a smooth surface while the untreated wood showed a rough surface. The cytotoxicity test showed that the leachates of both MA treated and untreated wood samples had a similar effect. So the treatment of rubberwood with MA is a safe method to prevent mould growth.

Keywords: wood modification; mold prevention; leaching; wood moisture content

Latex is the main source of income for farmers from rubber trees (*Hevea brasiliensis* (Willdenow ex. A. de Jussieu) Müller Argoviensis) for a period of 25 to 30 years before the trees are cut and the fields are replanted (Shigematsu et al. 2010; Ratnasingam, Grohmann 2014). Rubberwood is the final output from rubber trees which contains both heartwood and sapwood. The attractive properties of rubberwood are its creamy colour, low cost and good working properties (Muhammed et al. 2009; Teoh et al. 2011). In the South East Asia region, rubberwood has been widely used as a raw material in various industries such as furniture manufacturing, kitchenware and wooden toy industries (Lim et al. 2003). Rubberwood is highly susceptible to fungal attack due to the lack of heartwood formation (Priyadarshan 2011). Susceptibility of rubberwood to mould colonization on its surface is considered a major concern for wooden toy manufacturers and consumers. Today, the demand for

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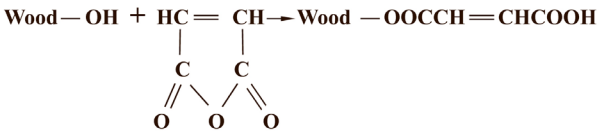

Fig. 1. Reaction of wood with maleic anhydride (Iwamoto, Itoh 2005)

low toxic wood protection to avoid the growth of moulds is increasing. In addition, there is a general concern about the environmental and health effects of chemical preservatives as well as the intention to reduce their use in wood products. Therefore, wood preservation methods with low toxicity to humans and a less negative impact on the environment are required.

An alternative method of improving the fungal growth resistance of wood without the use of chemical biocides is chemical modification of wood. The chemical modification of wood using di- and tricarboxylic acid anhydrides has been reported (Papadopoulos, Hill 2002; Iwamoto, Itoh 2005). Treatments of wood with various organic anhydrides have the potential to increase fungal resistance and to replace the use of biocides for wood preservation (Schiopu, Tiruta-Barna 2012). Wood chemical modification using anhydrides is accomplished by the reaction of hydroxyl groups of the cell wall polymers with the selected anhydride, to form a covalent bond without leaving toxic residues within the wood (Papadopoulos et al. 2008). If free hydroxyl groups are occupied and the access to water is prevented, the susceptibility to fungal attack is reduced (Hill et al. 2004).

Maleic anhydride (MA) is one of the chemicals that are usually used for chemical modification of wood (Clemons et al. 1992; Tjeerdsma et al. 2005; Hill 2006). This chemical bears non-polar endings capable of bonding with polar structures such as wood (Hill 2006). The reaction of wood with MA is displayed in Fig. 1. Maleic anhydrides do not yield a by-product when reacting with the hydroxyl groups of wood (Hill, Mallon 1998).

Previous studies showed that treatments of wood with MA enhanced the resistance of treated wood that was exposed to the attack of brown and white-rot fungi. Maleic anhydride treated samples of different softwoods have been tested against different standard fungal species, e.g. *Fomitopsis palustris* (Berkeley & M.A. Curtis) Gilbertson & Ryvarden and *Trametes versicolor* (Linnaeus) Lloyd and it has been observed that a better durability is achieved through this process (Fujimoto 1992, 1995; Iwamoto, Itoh 2005).

The information about the protective effect of MA in rubberwood, especially against moulds, is scarce. Therefore, the present study investigated the possibility of using MA as wood treatment in order to improve the resistance of rubberwood against moulds. This present work also aimed to apply MA in wooden toys. Children may obtain MA by saliva during chewing and leachable MA may be toxic to human cells, so the cytotoxicity of leachates from MA treated wood was investigated.

MATERIAL AND METHODS

Rubberwood material. Kiln-dried rubberwood samples (25 × 20 × 11 mm) for wooden toy preparation, untreated with wood preservatives and prepared in the tangential direction, were obtained from Plan Creations Co., Ltd., Trang, Thailand. The toy manufacturer prepared wood from natural rubber trees that no longer produce latex after 25 years and was obtained from Nakhon Si Thammarat. Rubberwood samples were kept in plastic bags until use and were oven dried at 103°C for 18 h before test.

Fungal cultures. *Aspergillus niger* van Tieghem PSU1, *Aspergillus flavus* PSU2 and *Penicillium citrinum* Thom PSU3 were isolated from the contaminated rubberwood samples, collected from Plan Creations Co., Ltd., Trang, Thailand. Fungal cultures were kept on potato dextrose agar (PDA; HiMedia Laboratories, Mumbai, India) slants at 4°C for further use and also kept in 20% glycerol at –20°C for long-term preservation. Fungal spores were collected from the mould grown on PDA plates at 25°C for 7 days by flooding the surface of the plates with sterile Tween-80 solution (0.1% v/v; LabChem, Zelienople, USA) and counted using a haemocytometer (Celeromics, Valencia, Spain). The spore suspension was standardized to 10^6 spores·ml^{-1} before use as an inoculum.

Preparation of MA solutions and wood treatment. Maleic anhydride was dissolved in water as concentrations of 0.5, 1.0, 1.5, 2, 2.5, 5 and 10% (w/v). Rubberwood samples (n = 5 for each concentration) were dipped in MA solutions for 5 min, immediately wrapped in foil and placed in an oven at 90°C for 2 h. To remove non-reacted MA, samples were soaked in water for 10 min and then dried at 90°C for 2 h.

Growth of fungi on rubberwood and viable count. Maleic anhydride treated wood samples and untreated samples were inoculated with 50 µl of the spore suspension of *A. niger* PSU1, *A. flavus* PSU2 or

P. citrinum PSU3 on surface and incubated at 25°C and 100% relative humidity (RH). The fungal growth on the surface of the wood samples was observed weekly for 52 weeks. The fungal growth on each sample was rated on a scale of 0–5, with 0 denoting a clean specimen and 5 representing heavy fungal growth: 0 = clean, 1 = 20%, 2 = 40%, 3 = 60%, 4 = 80%, 5 = 100% of fungal growth (American Society for Testing and Materials 1998). The percentages of fungal growth were calculated based on these ratings as Eq. 1:

$$\text{Percentages of fungal growth} = (A/B) \times 100 \quad (1)$$

where:

A – score sum for fungal growth with actual treatment,

B – score sum for fungal growth over control samples.

The lowest concentration showing total protection against moulds was selected for further studies.

For a viable count, *A. niger* PSU1 was selected as a model fungus. Maleic anhydride treated and untreated rubberwood samples were inoculated with 50 µl of the *A. niger* PSU1 spore suspension and kept in the humidity chamber at 25°C and 100% RH. At predetermined time points (3 h and 1 to 7 days), rubberwood samples (n = 3) were removed from the chambers and placed in a sterile flask containing 5 ml of 0.1% (w/v) Tween-80 solution and shaken for 30 min at room temperature. A sample (1 ml) was removed from each flask and serially diluted with sterile 0.1% (w/v) Tween-80 solution and a 100 µl aliquot was spread on PDA containing ampicillin (50 mg·l^{-1} of PDA) plate and incubated at 25°C for 72 h. The percentage of viable count of *A. niger* PSU1 was compared with the viable count on day 0 as follows (Eq. 2):

$$\text{Percentage of viable count} = (D_n \times 100)/D_0 \quad (2)$$

where:

D_0 – number of fungi counted on day 0,

D_n – number of fungi counted on day 1 to 7.

Determination of wood moisture content. Both maleic anhydride treated and untreated wood samples were dried at 103°C for 18 h and their oven-dried weights were measured. Wood samples were incubated in the humidity chamber maintained at 25°C and 100% RH. The weight of the wood samples was determined periodically until they reached constant mass and the equilibrium moisture content of the materials was calculated as follows (Eq. 3):

$$\text{EMC} = ((M_{\text{final}} - M_{\text{initial}})/M_{\text{initial}}) \times 100 \quad (3)$$

where:

EMC – equilibrium moisture content (%),

M_{initial} – initial mass of dry samples (g),

M_{final} – mass of samples at equilibrium with the water vapour in the chamber (g).

All measurements were done in triplicate.

Leaching study. 2.5% maleic anhydride treated and untreated wood samples were leached in a mammalian cell culture medium (Dulbecco's Modified Eagle's Medium (DMEM); Thermo Fisher Scientific, Waltham, USA). Samples of MA treated and untreated wood were sterilized by autoclaving. The wood samples (n = 3) were separately placed in 100 ml sterile laboratory glass bottle containing 20 ml of sterile DMEM. The samples were then shaken at 200 rpm and the leachates (2 ml) were collected at 5, 15, 30 and 60 min and 2 ml of DMEM was added to make up the volume each time. The leachates were used further in antifungal and cytotoxicity tests. The contents of released MA in the leachates were analysed by a high-performance liquid chromatography (HPLC).

HPLC analysis. The leachates of MA treated and untreated wood samples were analysed in comparison with the standard solution of the MA (1.25, 2.5, 5, 10 mg·ml^{-1}). The analyses were performed using an Agilent 1200 series liquid chromatography (Agilent Technologies, Waldbronn, Germany) coupled with a diode array detector (DAD; Agilent Technologies, Waldbronn, Germany). The C18 analytical column (Agilent 5 µm, 250 × 4.6 mm) was thermostatically controlled at 25°C. The mobile phase was dicyclohexylamine/formic acid/methanol/water (0.5:0.5:25:74). The UV detector wavelength was set at 254 nm. The flow rate was 1.5 ml·min^{-1}, the injection volume was 20 µl and the run time was 10 min.

Antifungal activity of leachates and diffusion test. The antifungal activity of leachates was tested against *A. niger* PSU1 by an agar diffusion method. An amount of 0.4 ml of fungal spore suspension (10^8 spores·ml^{-1}) was added to 3.6 ml of sterile molten PDA, then overlaid onto a PDA plate (16 ml) and allowed to solidify. Wells of 5 mm in diameter were aseptically bored into the agar and 50 µl of leachates were added to the wells.

The diffusion test was performed to measure whether there was any inhibition of the fungal growth when the MA treated wood samples were placed together with the tested fungus. The MA treated and untreated round wood slice (20 mm diameter × 5 mm thick) samples were placed in the centre of inoculated agar plates. The plates were incubated at 25°C for 72 h and the diameter of the inhibition zone was measured. Values are given as mean and standard deviation of tests performed in triplicate.

Microscopic examination. A scanning electron microscope (SEM) was used to examine the surface of the rubberwood samples. Three samples of each MA treated and untreated wood were used. Thin surface (around 2 mm) of each sample was cut into small pieces (5 × 5 mm). Each small piece was fixed in 3% glutaraldehyde, dehydrated in a graded series of alcohol, air-dried and then coated using a gold sputter coater. The coated specimens were examined with a SEM (Quanta400; FEI, Brno, Czech Republic) at the Scientific Equipment Centre, Prince of Songkla University. Three positions of each sample were scanned.

Cytotoxicity assay. The cytotoxic activity of leachates of both MA treated and untreated wood samples was studied against human keratinocyte cells using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983). In this method, the optical density of the solution containing the formazan produced by metabolically active cells is measured spectrophotometrically. Briefly, the cells (1×10^4 cells per well, cultured in 96-well microplates) were incubated at 37°C and 5% CO_2 for 3 days. The leachates (n = 3) collected at 60 min of leaching were sterilized by a filtration method (0.2 µm syringe filter) and added to seeded wells in triplicate and incubated at 37°C for 1 h. Control wells contained DMEM. At the end of the incubation time, cultured plates were washed with a sterile phosphate buffer saline (PBS, pH 7) solution. The MTT solution (5 mg·ml^{-1} in PBS) was subsequently added to each well and plates were incubated at 37°C for another 3 h. Supernatants were then discarded and 100 µl of dimethyl sulfoxide was added to the cultures and mixed thoroughly. Formazan quantification was performed using an automatic plate reader (Multiskan™ GO; Thermo Fisher Scientific, Waltham, USA) at 562 nm. The percentage of viability was determined using the following formula (Eq. 4):

$$\text{Viability (\%)} = \frac{\text{treatment absorbance} \times 100}{\text{control absorbance}} \quad (4)$$

Statistical analysis. Data were expressed as mean ± SD. Between-group differences were evaluated using one-way analysis of variance. Differences were considered as statistically significant at $P < 0.05$.

RESULTS

Growth of fungi on rubberwood

The growth of *A. niger* PSU1, *A. flavus* PSU2 and *P. citrinum* PSU3 on MA treated rubberwood at 25°C and 100% RH is displayed in Fig. 2. Growth of moulds on 0.5–1.5% MA treated wood reached 100% in the first week. By contrast, when 2% MA was used, *A. flavus* PSU2 and *P. citrinum* PSU3 were inhibited but the growth of *A. niger* PSU1 was 100%. However, the rubberwood treated with 2.5–10% MA showed no fungal growth at least up to 52 weeks, at which time observations were discontinued. Based on this result, wood samples treated with 2.5% MA were used in further studies. Moreover, after leaching, the 2.5% MA treated rubberwood samples had no fungal growth for 52 weeks either.

Viable count

The spores of *A. niger* PSU1 were inoculated on the wood surface treated with 2.5% MA and the inoculated samples were kept at 25°C and 100%. The

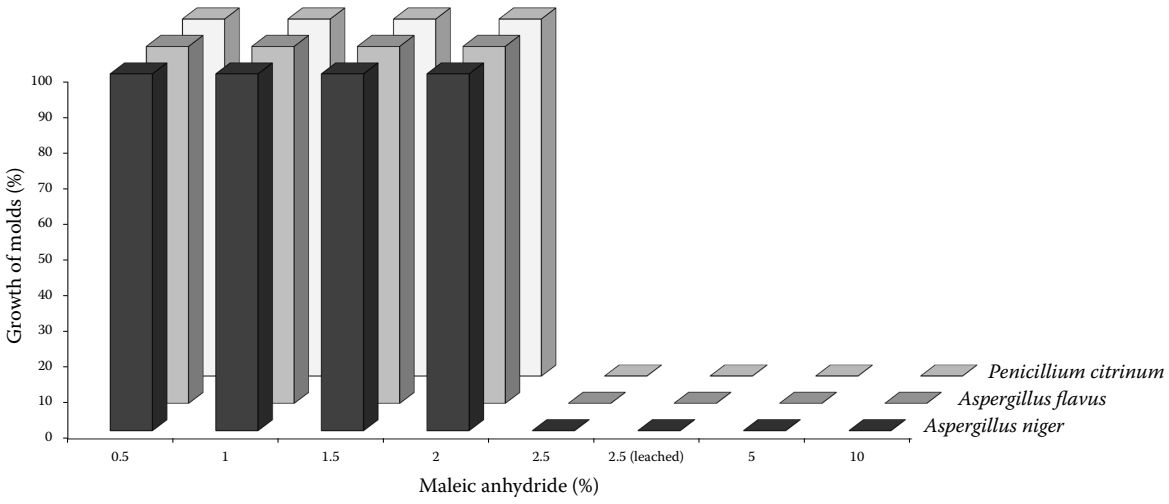

Fig. 2. Growth of inoculated fungi on maleic anhydride treated rubberwood (n = 5) after 52 weeks of incubation at 25°C and 100% relative humidity

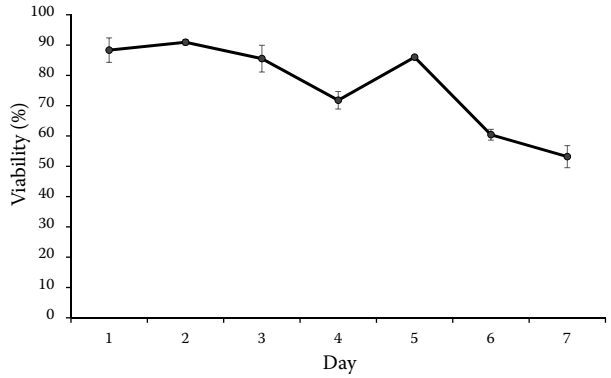

Fig. 3. Plots of the percentage of the viable count of *Aspergillus niger* van Tieghem PSU1 on maleic anhydride treated rubberwood at 100% relative humidity, 25°C after inoculation

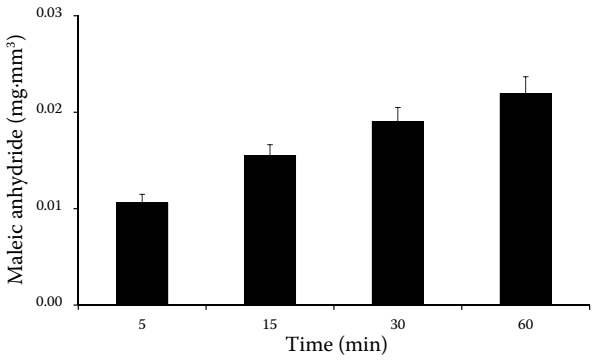

Fig. 4. Effect of the leaching time on maleic anhydride leached out from the treated wood

viable count was monitored for 7 days. The results are presented in Fig. 3. After 24 h, the percent of the viable count of *A. niger* PSU1 was about 90% and subsequently it was maintained at 85 to 60% within 5 days. On day seven, 53% of the viable count was observed.

Wood diffusion test

Maleic anhydride treated and untreated wood slices were placed on the centre of the inoculated plate and the inhibition zone was measured. After 72 h of incubation, no inhibition zone was observed on agar plates with both types of wood samples. The fungus grew on the agar plate normally. It is obvious that there was no inhibition of the fungal growth when inoculated together with MA treated wood samples.

Leaching of MA treated wood

Rubberwood samples treated with MA at 2.5% were leached in DMEM. The concentrations of leached MA are shown in Fig. 4. MA was leached out from treated wood. When the leaching duration increased, the concentrations of released MA increased gradually. Total concentration of MA leached from rubberwood for 60 min was 0.02 mg·mm^{-3}.

Antifungal activity of leachates

The result of well diffusion for the antifungal activity of leachates from MA treated and untreated wood collected after 60 min of leaching showed that there was no fungal inhibition zone against *A. niger* PSU1. Both kinds of leachates affected mould growth at the same rate. Although the leachate from MA treated wood contained MA, it did not show any antifungal activity.

Moisture content of rubberwood

The moisture contents of MA treated and untreated rubberwood samples kept at 25°C and 100% RH after 8 days were 16 and 17.5%, respectively (Fig. 5). The moisture content of MA treated wood was not significantly different from that of untreated wood ($P < 0.05$).

Microscopic examination

The rubberwood treated with MA results in significant changes of the wood surface morphology. The scanning electron microscope result showed that the treated wood had a smooth surface while the untreated wood had a rough surface (Figs 6a, b), suggesting that the rough surface of wood is physically coated.

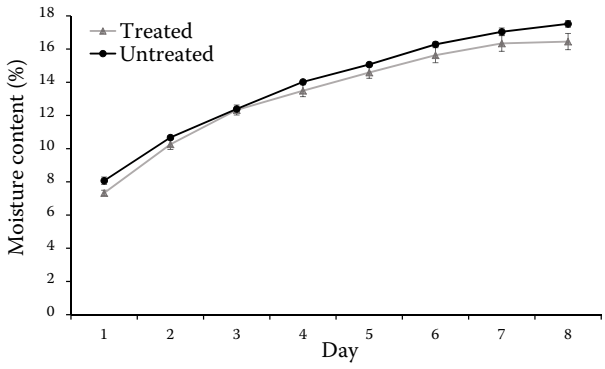

Fig. 5. Moisture contents of maleic anhydride treated and untreated rubberwood samples incubated at 100% relative humidity, 25°C

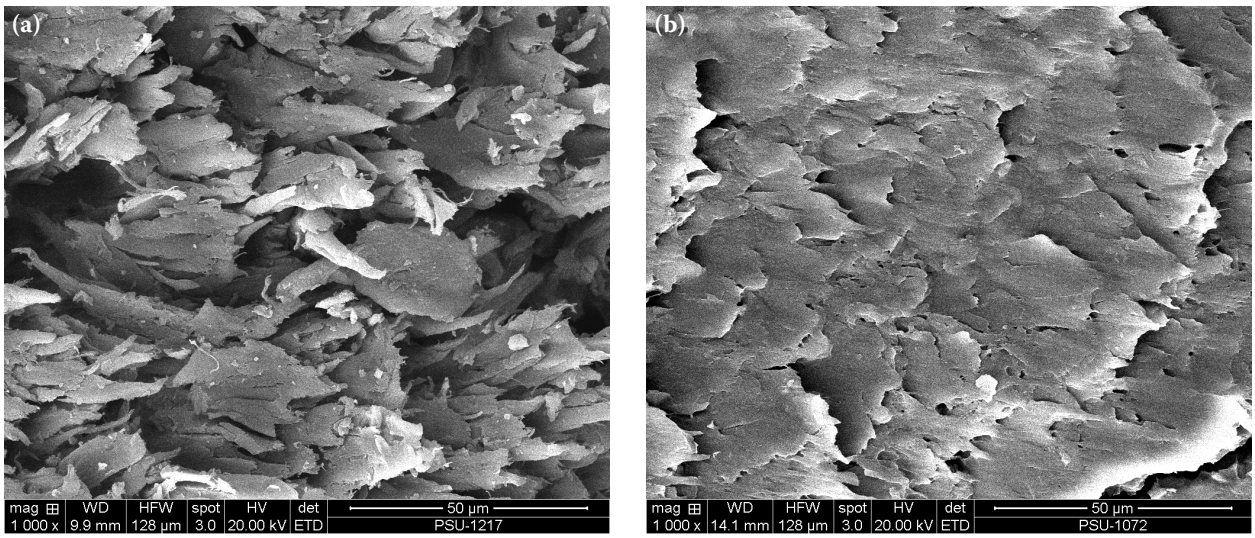

Fig. 6. Scanning electron micrographs of untreated rubberwood (a), and maleic anhydride treated rubberwood (b) mag – magnification, WD – working distance, HFW – horizontal field width, HV – high voltage, det ETD – Everhart-Thornley detector

Cytotoxicity assay

The results of the cytotoxicity test of leachates on keratinocyte cells are shown in Fig. 7. The leachates from both treated wood and untreated wood showed cell viability about 50% and were not significantly different ($P < 0.05$).

DISCUSSION

Chemical modification with MA afforded biological protection of rubberwood against moulds. Rubberwood samples treated with 2.5 to 10% MA had no fungal growth for 1 year although they were inoculated with fungal spores and kept at 100% RH. A concentration of MA at 2.5% was adequate for total protection of wood against the tested fungi. The treatment of wood with MA could be used to treat rubberwood for long-term prevention of mould growth. Many research studies have been carried out on the chemical modification of wood using anhydrides and focused on decay resistance of modified wood. Iwamoto and Itoh (2005) reported that the MA treated sugi sapwood (*Cryptomeria japonica* D. Don) carried in a vapour phase reaction system at concentrations of vapour phase MA at 4 and 8 × 10^{-3} mol·l^{-1} had strong resistance against fungal decay. The MA studied here is known to form covalent bonds with OH groups of wood (Iwamoto, Itoh 2005). By reducing free OH groups in wood, the sorption of water in the cell walls is prevented, the wood moisture content is reduced and the resistance of wood to fungal attack is increased. The present study showed that the moisture content of MA treated wood was slightly reduced compared to the control indicating that MA might not prevent water absorption by wood. This result is not correlated with the results previously reported on wood modified with MA (Chauhan et al. 2001). At least three mechanisms have been presented to explain the protection provided by wood modification: (*i*) changes of the cell wall polymers that become unrecognizable for fungal enzymes, (*ii*) reduction of the moisture content, and (*iii*) smaller micropore size in the wood cell wall (Li et al. 2011). It is obvious that in this study, the fungal growth resistance of MA treated wood was not provided by a reduction of moisture content but it is feasible that the cell walls of treated rubberwood became unrecognizable for fungal enzyme and the pore size of the wood cell wall was smaller. In addition, one possible explanation is that the resistance to the mould growth of MA treated wood might be due to the smoothness of

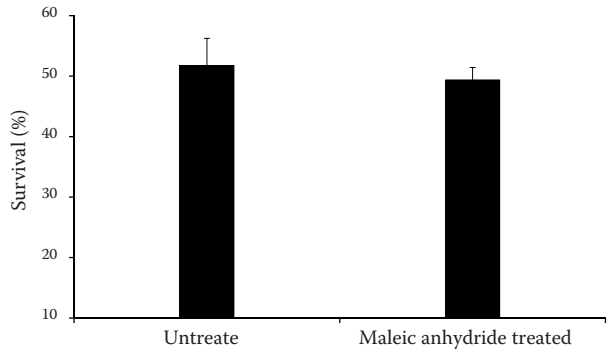

Fig. 7. Cytotoxic activity on keratinocyte cells of leachates from untreated wood samples and maleic anhydride treated wood samples collected at 60 min of leaching

the surface that was clearly seen from SEM images. A study by Bardage and Bjurman (1998) showed that surface roughness contributed to the adhesion of fungal spores. An increase in substratum surface roughness increases the retention of microorganisms when dealing with food contact surfaces, since spores are more easily attached to damaged or uneven surfaces than to smooth ones (Verran et al. 2000). Lugauskas et al. (2003) suggested that rough surfaces or surfaces with cracks might concentrate nutrients and moisture more easily and provide favourable conditions for fungal attachment and growth. Furthermore, physical treatment of rubberwood with MA might regulate the permeability of O_2 and water vapour, thereby preventing the fungal growth.

The unreacted MA quantity present in wood was determined by a leaching test and the result showed that 0.02 mg·mm^{-3} MA was leached from treated wood. The high concentrations of released MA at the initial period of leaching may be because of a high content of MA on the surface or in the pores of treated wood that is subject to very early loss. Once wood preservatives fix well during the reactions with wood, they should resist to leaching. This means that the protective agent will not leach. Although the results from the chemical analysis of leachates showed that MA was leached from the treated wood, the wood samples still had high fungal resistance. The concentration of non-reacted leachable MA from MA treated wood was 0.02 mg·mm^{-3} but did not contribute to fungal growth resistance. The leaching test indicated that MA was leached but the fixed MA in wood was enough for the prevention of mould growth.

The result of the viable count of *A. niger* PSU1 on MA treated wood showed that there was about 90% viable count of *A. niger* PSU1 after inoculation for 24 h. This result indicated that fungal spores were not largely reduced on MA treated wood. Fungicidal activity was defined as a reduction in fungal growth of ≥ 3 $\log_{10}$ CFU·ml^{-1} (CFU – colony-forming unit), resulting in about 99.9% reduction in CFU·ml^{-1} relative to the initial inoculum (Ernst et al. 2002). Certainly, MA did not exhibit any fungicidal activity against mould on wood. Additionally, the result of wood diffusion test showed similar antifungal activity for untreated and MA treated wood. In addition, the agar well diffusion showed that the amount of released MA in leachates did not contribute to any antifungal effects. These results suggested that MA used in this study did not act as a fungicide. This was clearly seen from both the antifungal study and the measurement of the growth inhibition test on mould.

Although there was 0.02 mg·m^{-3} released MA in the leachate from MA treated wood, it had the same cytotoxic effect as the leachate from untreated wood. The survivals of cells incubated with leachates from MA treated and untreated wood showed that both types of leachates had high toxicity to cell lines. One reason for the high leachate toxicity could be that the wood contained a high natural extractive content such as phenolic compounds and esters (Simatupang et al. 1994; Vetter et al. 2008). These results indicate that the MA treated rubberwood had a similar cytotoxic effect as the natural rubberwood. This study showed that the treatment of rubberwood with MA is an environmentally friendly alternative to prevent moulds instead of using highly toxic preservatives.

CONCLUSIONS

In this study, MA was tested for efficacy on wood protection against moulds isolated from rubberwood. 2.5% MA was effective to prevent mould growth on rubberwood for 52 weeks. The complete protection was not provided by a reduction of moisture content but possibly by a change of the recognizable site for fungal enzyme and a smaller pore size of the wood cell wall. After leaching, MA treated rubberwood still resisted to mould growth. Maleic anhydride used in the present study is not a fungicide, it is environmentally friendly and its cytotoxic effect against human cells is similar to untreated wood. Maleic anhydride has a potential to be used as a low toxic anhydride for wood modification, thereby increasing the wood utility for various applications related to humans such as wooden toys, kitchen wares and furniture.

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