Determining the Effects of Some Bacteria on Wooden Toys Treated with Antibacterial Protective Coatings

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Several protective coatings enhanced by antimicrobial agents and/or pigments were considered for the wooden toy market: water-based matte varnish, an ultra-hygiene water-based matte varnish (WBV-UH), a polyurethane matte varnish (PUV), and an ultra-hygiene antiviral polyurethane matte varnish (PUV-UH), as well as a water-based dye (WBV 5%K), an ultra-hygiene water-based dye (WBV-UH 5%K), a polyurethane dye (PUV 5%K), and an ultra-hygiene polyurethane dye (PUV-UH 5%K), which contain 5% red nano-pigment (K). By utilizing 7 kinds of bacteria and 2 types of yeast that are commonly detected in routine, daily settings, the efficacy of the different protective coatings on wooden toy surface was investigated. The antibacterial and antimicrobial activities of the tested dye samples were based on the agar-well diffusion method. Ultimately, the study found that the addition of antimicrobial agents to several different protective coatings and dyes resulted in the presence of antimicrobial activity *vs*. the lack thereof with protective coatings and dyes alone. Additionally, some of the dyes with added antimicrobial agents were found to be effective against biofilm formation. Overall, the addition of pigment into the coating, alongside the addition of antimicrobial agents, proved to be highly effective in inhibiting growth and spread of microorganisms on wooden toy surface.

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INTRODUCTION

Wood has played a pivotal role in social economic construction and human basic living environments as a natural renewable resource. It has been widely used as a construction material and for making furniture, decorative woodwork, tools, and paper. Wood has unique properties, one of which is hygroscopicity because of the presence of a large number of hydrophilic hydroxyl groups in its main components. Wood displays certain defects, including deterioration, dimensional deformation, biodeterioration, and degradation following absorption of water. These deficiencies may considerably damage service life and usage area of wood. For this reason, in order to protect the wood from various forms of degradation, it is necessary to protect it with varnish and paints, which are called wood preservatives. In addition to protective features, the wood coatings also transform the appearance of wood, making it look elegant.

The varnishes used in this study were colored to gain attractive appearance, as they are applied to the surface of the wooden toys. The market scale of this sector producing colorful toys reaches billions of dollars worldwide (PAGEV 2019). The largest scale producer of the products offered to this market is the Far East countries. The uncertainty of the origin of the SARS-CoV-2 virus and some national regulations (for example, banning imports) have led people to avoiding products from this region. During the Covid-19 pandemic, the demand on health, hygiene, and personal care products has risen (Özbay and Özcan 2021). For this reason, hygiene of furniture has gained increasing importance in today's society. Particularly, the furniture used in universities and schools augments running the risk of infection (Bilgiç 2020) (Kamel 2021).

It is possible to transmit SARS-CoV-2 to humans through contact with contaminated surfaces or objects (fomites), but the risk is generally considered low (CDC n.d.). According to the research conducted by Australia's national science agency CSRIO, the SARS-CoV-2 virus can stay alive for 28 days on rubber surfaces (CSRIO 2020). Unlike rubber (or another material), wood has naturally antimicrobial properties (Kamel 2021). The SARS-CoV-2 pandemic alerted parents to be more selective in terms of safety of toys and tighten their inspection through official sources (Koskunoğlu 2021).

Over the past few years, researchers have been rushing to develop antimicrobial coatings that can substantially reduce the pathogens' stabilization on various high-traffic wooden objects, including doors, window handles, office furniture, school desks, and toys. Protection in high-traffic wooden surfaces is of particular interest because they are constantly in contact with a large number of people, and because of the rapid recontamination, frequent cleaning of these surfaces is required, which is energy and cash consuming, as well as having negative impacts on human health and the environment. Antimicrobial surfaces that impede microbe development over time, either by killing them or limiting their capacity to reproduce and spread, might be a long-term cost-effective option (Luciana *et al.* 1997; Calogero and Di Marco 2008).

Antimicrobial protection – efficacy against viruses, bacteria, mold, and fungi (while antibacterial protection is only effective against bacteria) can be achieved by several methods, these include direct, indirect, and bioinspired antimicrobial surfaces (Sun and Ostrikov 2020). Among those, incorporation of inorganic materials possessing antimicrobial properties into coating formulations is a facile technology (Nasri *et al.* 2021). Silver is a naturally occurring inorganic element with high efficacy at low concentrations and a well-known broad-spectrum antibacterial property, as well as being universally accepted as safe for human contact. The interaction of ionic silver with thiol groups is considered to be the mechanism of action. Ionic silver binds to thiol groups in enzymes, disrupting folic acid production as well as protein synthesis, DNA synthesis, electron transport, and cell wall formation (Knetsch and Koole 2011).

Based on the development of naturally occurring aspergillic acid, zinc pyrithione (ZPT) is another well-known antimicrobial agent that has been used over 50 years to effectively control seborrheic dermatitis, acne, and scalp psoriasis. The ZPT inhibits fungal reproduction by causing copper import and damage to iron–sulphur proteins (Schwartz 2016). Furthermore, it exhibits broad spectrum of antimicrobial efficacy ranging from fungi to Gram–positive and –negative bacteria, making it an ideal candidate for use in coating formulations as an antimicrobial additive. The terms antimicrobial, antibacterial, antifungal, antiviral, and antiparasitic refer to groups of drugs used to prevent and treat infections. For example, the term 'antibacterial' is used only for drugs used for bacteria, while 'antimicrobials' include antibiotics, antivirals, antiprotozoals, and antifungals

(European Medicines Agency 2022). Biofilms are structured communities of microbial cells encased in a self-secreting extracellular matrix composed of exopolysaccharides, proteinaceous adhesion factors, and nucleic acids. The bacterial biofilm development process consists of four stages: initial attachment, microcolony formation, maturation, and dispersal (Liu *et al*. 2022). Biofilms develop not only on surfaces, but also in environments such as solid-liquid, solid-gas, liquid-liquid, and liquid-gas (Qian *et al*. 2022).

This study aims to identify activities related to some bacteria that could often be encountered in daily life as well as on polyurethane- and water-based varnishes surfaces used in wooden toys. For example, Aleksejeva *et al.* (2021) identified *Staphylococcus aureus* bacteria on the toys used in the children's hospitals. (Ibfelt *et al.* 2015) studied on effect of cleaning and disinfection of toys. They found respiratory virus DNA/RNA and very few pathogenic bacteria in the daycare nurseries. In this study polyurethane, water-based varnishes and developed varnishes added with 5% red nano-pigment were used. The study is evaluating the antimicrobial activity of varnishes against the microorganisms listed. *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 51299, *Bacillus cereus* 7064, *Escherichia coli* ATCC 11293, *Klebsiella pneumonia* ATCC 27889, *Shigella flexneri* ATCC 12022, *Pseudomonas aeruginosa* ATCC 27853, *Candida parapsilosis* ATCC 22019, and *Candida krusei* ATCC 6258. According to results, it is thought that antimicrobial features of developed varnishes and modified dyes can be used for wooden toys.

EXPERIMENTAL

Materials

Microorganisms

The following microorganisms were collected as pure cultures in the Microbiology Laboratory (Sinop, Turkey): The Gram-positive bacteria: *Staphylococcus aureus* ATCC 6538 (St-au), *Enterococcus faecalis* ATCC 51299 (En-fe), *Bacillus cereus* 7064 (Ba-ce); Gram-negative bacteria: *Escherichia coli* ATCC 11293 (Es-co), *Klebsiella pneumonia* ATCC 27889 (Kl-pn), *Shigella flexneri* ATCC 12022 (Sh-fl), *Pseudomonas aeruginosa* ATCC 27853 (Ps-ae); and yeast species *Candida parapsilosis* ATCC 22019 (Ca-pa) *and Candida krusei* ATCC 6258 (Ca-kr).

Nano-Pigment (Red Color)

In this study, red color pigment (K) used for coloring to varnishes was obtained from Kimetsan Chemistry Mining Metallurgy Industry Consulting Engineering Domestic and Foreign Trade Limited Company (Ankara, Turkey).

Protective coatings (water-based and polyurethane varnish-dye)

Water-based matte varnish (WBV) produced by Kubilay Boya Ltd. Company (İzmir, Turkey) for wooden surfaces, ultra-hygiene water-based matte varnish (WBV-UH), polyurethane matte varnish (PUV), and ultra-hygiene antiviral polyurethane matte varnish (PUV-UH) were utilized. The varnishes (ultra-hygiene) were modified with zinc pyrithione (Sigma Aldrich) to achieve antimicrobial activity. Further, water-based dye (WBV 5%K), ultra-hygiene water-based dye (WBV-UH 5%K), polyurethane dye (PUV 5%K), and ultra-hygiene polyurethane dye (PUV-UH 5%K) were prepared by adding 5% nano-pigment (red color) into these varnishes at the laboratories of Kastamonu University (Kastamonu,

Turkey). The reason for using this color is that the varnish manufacturer company does not produce colored dye. In this study, the goal was to produce colored dye. The reason for preferring the color red is that it is one of the most preferred colors in the toy industry. In order to obtain the desired red color tone, pigment was added starting from 1% and the desired image was obtained at a rate of 5% (more additions may deteriorate the properties of the antimicrobial varnish).

The antiviral performance of PUV-UH was tested and approved at London-based Virology Research Services Laboratory (VRS), and its antibacterial and antifungal activity were tested and approved at Kazlıçeşme Research and Development Center and Test Laboratory (KCL) accredited by TÜRKAK. Results of antiviral, antibacterial, and antifungal activities are presented in Table 1 (Bilgiç 2020).

Table 1. Results of Antiviral, Antibacterial, and Antifungal Activities of PUV-UH

Standard	Microorganism	Strains	% Decrease (Lab.)
ISO 21702 (2019)	Virus	Coronavirus NL-63	> 90 (VRS)
ISO 22196 (2011)	Bacterium	*Escherichia coli*	99.99
ISO 22196 (2011)	Bacterium	*Staphylococcus aureus*	99.99
ISO 22196 (2011)	Bacterium	*Salmonella typhimurium*	98.04
ISO 22196 (2011)	Bacterium	*Listeria monocytogenes*	99.99
ISO 22196 (2011)	Bacterium	*Klebsiella pneumoniae*	99.94
ISO 22196 (2011)	Bacterium	*Pseudomonas aeruginosa*	99.95
ISO 22196 (2011)	Fungal	*Candida albicans*	95.65 (KCL)
ISO 22196 (2011)	Fungal	*Aspergillus brasiliensis*	90.97

Methods

Preparation of dyes

Approximately 20 g red nano-pigment (5%) was added into 400 g samples of PUV, WBV, PUV-UH, and WBV-UH, which had been first weighed in a beaker using a precision balance (Fig. 1). The mixture was then mixed by blending with nano-pigment periodically rightward and leftward for 30 min (Fig. 2).

Antimicrobial activity measurement

The wet dye samples were used directly. The antimicrobial activities of the samples were evaluated using the agar well-diffusion method (Magaldi *et al.* 2004), which is suitable for wet dye sample testing. Before testing, the microorganisms were transferred into Muller Hinton Broth (MHB) medium for bacteria and Sabouraud Dextrose Broth (SDB) medium for fungus and cultured overnight at 37 °C and 28 °C, respectively. The turbidity was adjusted to 0.5 McFarland equivalent standards. Wells with a diameter of 6 mm were opened in the medium with a sterile piercing. The microorganisms were spread over the surface of an agar plate. 100 µL of the dye sample was poured into the wells. The media were incubated for 18 to 24 h at 35 ± 0.5 °C (for bacteria) and 24 to 72 h at 25 ± 1

°C (for fungus). Antibiotic susceptibility discs including erythromycin (E15), ampicillin (AM10), and cycloheximide (Cyc) were used as control, and the negative control used was *d*H_2O. The antimicrobial activity was evaluated by measuring the diameter of the inhibition zone. The tests were performed in triplicate.

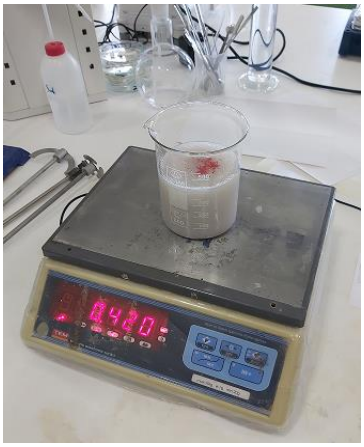


Fig. 1. Addition of red nano-pigment into varnishes



Fig. 2. Blending of red color nano-pigment into varnish *via* a mixer

Antimicrobial activity measurement

Biofilm activity was measured according to the method of Stepanovic *et al.* (2000). For this, five bacterial samples that were confirmed to form biofilms by the tube method, were selected, and the biofilm formation inhibition activity of the dye samples was studied by the 96-well plate method. The method is briefly as follows; Tryptic Soy Broth (TSB) medium containing 0.25% glucose was prepared sterile, and it was transferred to each well at a volume of 185 or 170 µL. Again, 5 or 20 µL of dye sample and 10 µL of fresh bacterial culture were added to each well. These plates were then mixed gently. After closing the lid, the plates were incubated for 24 h at 37 °C. At the end of the period, the wells were washed three times with 300 µL of phosphate buffer saline (PBS). After the washing process was finished, the plate was turned upside down and left to dry at room temperature. After drying, the plates were incubated for 60 min at 60 °C for temperature fixation. After fixation, 150 µL of methanol was added and left for 20 min. At the end of the period,

methanol was completely removed by pouring it out, and the plate was inverted and left at room temperature overnight. At the end of the period, 150 µL of 2% crystal violet was added to each well and stained for 15 min. After staining, it was washed with distilled water and 150 µL of ethanol was poured into each well, and the covers of the plates were closed and kept at room temperature for 30 min. After the ethanol was removed, readings were made using the Thermo Scientific Multiskan Go (Version 1.01.10, Thermo Scientific, Bedford, MA, USA) device at 570 nm, and calculations were made according to the formulas shown below. Only TSB was added as a negative control. Experimental studies were conducted in triplicate.

The method used to determine the biofilm production capacity of microorganisms according to their mean values (OD) was as follows:

*OD*c (Cut-off value) = Mean of negative controls + 3 (Standard deviation of negative controls)

OD (Mean value) = *OD* Mean of samples – *OD*c

When *OD* ≤ *ODc*; No biofilm production:
ODc < *OD* ≤ 2 x *OD*c; Poor biofilm production (+)
2 x *OD*c < *OD* ≤ 4 x *OD*c; Medium biofilm production (++)
4 x *OD*c < *OD*; Strong biofilm production (+++)

RESULTS AND DISCUSSION

The antimicrobial activities of the dye samples measured by the well diffusion method are shown in Table 2 and Fig. 3. When the antimicrobial effect of the PUV and PUV-%5K group was evaluated, it was seen that PUV and PUV 5%K showed minimal activities against Gram-negative bacteria *E. coli* and had no effect against other microorganisms. The PUV-UH and PUV-UH 5%K dyes were effective against all microorganisms. It can be stated that PUV-UH 5%K was more effective when compared to other PUV members. The high activities of these two dye groups without distinguishing between Gram-positive, Gram-negative, and fungi were remarkable. When the WBV group was evaluated among themselves, it was determined that WBV and WBV 5%K had low activity, except for Gram-negative *S. flexneri* and both fungi. It was observed that SUB-UH and WBV-UH 5%K showed high activities against all microorganisms and generally WBV-UH 5%K was more effective than WBV-UH. It is quite noteworthy that these two dye samples showed high activities especially against Gram-positive *S. aureus*, Gram-negative *S. flexneri*, and both fungi. They were more effective than some commercial antibiotics used (Table 2). When PUV and WBV dye groups were compared, WBV was much more effective.

Inhibition activity of the mixture of walnut shell color and copper sulphate against the microorganism, *E. coli* ATCC 25922 was measured as 9 mm in a study by Yeniocak *et al.* (2015). In this study, inhibition activities of all varnishes and dyes with ultra-hygiene additive against the microorganism, *E. coli* ATCC 11293 was measured as 20 mm to 26 mm. The ultra-hygiene utilized in this study had higher antimicrobial activity when compared to some commercially selected antibiotics (E15, AM10 ve Cyc). The antimicrobial ingredients that provide the antimicrobial feature is not announced by the manufacturer.

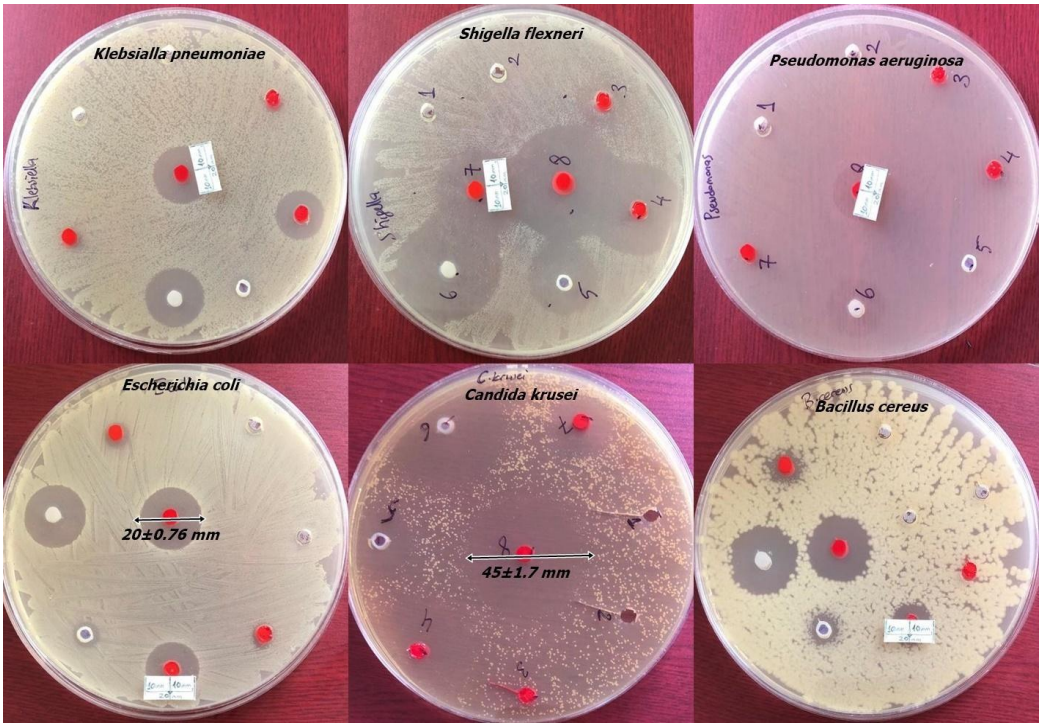


Fig. 3. Petri photos showing the antimicrobial activities of some dyes by well diffusion method

Biofilm formation can be expressed as the attachment of microbial community to abiotic surfaces by producing extracellular polysaccharides. Microbes within the biofilm formation are further protected from external conditions such as environmental, host, or antibacterial agents. Moreover, it has been reported that they exhibit up to a thousand-fold resistance to many antibacterial agents (Ghannoum and O'Toole 2004; Dosler *et al.* 2015).

A total of 80% of infectious diseases in humans are related to biofilm formation. Biofilm-forming bacteria are more resistant to antibiotics compared to other non-biofilm-forming bacteria. For this reason, it is difficult to destroy biofilm-forming bacteria with antibiotics (Jorge *et al.* 2012).

Biofilm formation inhibition activity of dye samples against some bacteria capable of producing biofilm are shown in Table 3 and Fig. 4. Accordingly, when Table 2 is examined, as the amount of dye was increased against all tested bacteria, the biofilm formation inhibition activity also increased. When the PUV group samples were evaluated among themselves, it was found that PUV-UH had the most effective biofilm inhibitory effect. Additionally, PUV-UH inhibited *E. coli* and *K. pneumonia* at both concentrations. It was observed that PUV alone could not completely stop biofilm formation. When the WBV group samples were evaluated among themselves, the most effective sample was WBV. In particular, it inhibited the biofilm production of *S. aureus* and *E. coli* at both concentrations. In addition, it was observed that this group (no results were obtained in some samples) had a lower biofilm formation inhibition activity effect than the PUV group. The fact that the PUV group was more effective than the WBV group in terms of biofilm formation inhibition activity was the opposite of the antimicrobial activity experiment of both groups.

Table 2. Inhibition Zones (mm) of the Dye Samples Against Tested Microorganisms Using Well Diffusion Method

Sample	*S. aureus* ATCC 6538	*B. cereus* ATTC 7064	*E. faecalis* ATCC 51299	*E. coli* ATCC 11293	*P. aeruginosa* ATCC 27853	*K. pneumonia* ATCC 27889	*S. flexneri* ATCC 12022	*C. parapsilosis* ATCC 22019	*C. krusei* ATCC 6258
PUV	-	-	-	8 ± 0.05	-	-	-	-	-
PUV-UH	18 ± 0.36	14 ± 1	13 ± 0.5	20 ± 0.76	10 ± 0.64	20 ± 0.5	34 ± 1.5	20 ± 0.15	9 ± 0.3
PUV 5%K	-	-	-	8 ± 0.28	-	-	-	-	-
PUV-UH 5%K	20 ± 0.76	15 ± 0.5	14 ± 0.5	20 ± 0.76	10 ± 0.25	20 ± 0.76	37 ± 1	36 ± 2	35 ± 1.7
WBV	9 ± 0.75	10 ± 0.5	-	10 ± 0.5	-	-	26 ± 0.5	20 ± 1	22 ± 1.5
WBV-UH	32 ± 2	26 ± 1	25 ± 0.36	26 ± 0.5	22 ± 0.28	24 ± 0.76	42 ± 0.76	38 ± 2.5	42 ± 2.1
WBV 5%K	12 ± 1.5	8 ± 1	-	8 ± 0.76	-	-	35 ± 1.5	14 ± 1.3	22 ± 2
WBV-UH 5%K	34 ± 0.87	25 ± 0.5	24 ± 0.7	25 ± 0.7	23 ± 0.26	24 ± 0.8	46 ± 1.4	42 ± 2.6	45 ± 1.7
AM10	19	*	18	15	23	*	*	*	*
E15	24	9	12	*	*	10	12	*	*
Cyc	*	*	*	*	*	*	*	41	40

(-) No effect; (*) Not tested; Negative control: dH_2O; Positive control: E15, AM10, Cyc; (±) Standard deviation

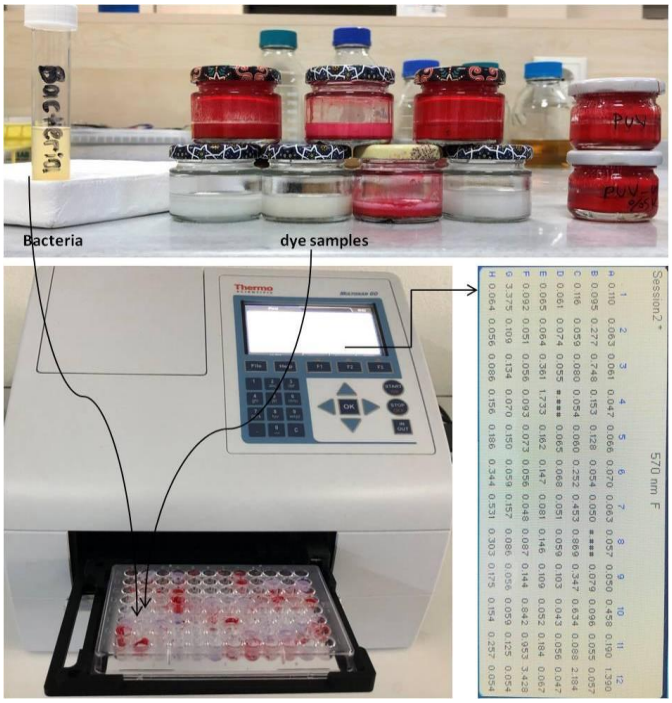


Fig. 4. Image showing the path followed for biofilm formation inhibition activity

Table 3. Biofilm Formation İnhibition Activity of Dye Samples Against Tested Bacteria

	S. aureus **ATCC 6538**		***E. faecalis*** **ATCC 51299**		***E. coli*** **ATCC 11293**		***P. aeruginosa*** **ATCC 27853**		***K. pneumonia*** **ATCC 27889**	
Bacterial Biofilm Activity	++		++		++		+++		++	
	5 µL	20 µL	5 µL	20 µL	5 µL	20 µL	5 µL	20 µL	5 µL	20 µL
PUV	++	+	+	+	+	+	++	+	+	+
PUV-UH	+	-	+	+	-	-	+	+	-	-
PUV 5%K	+	+	++	+	++	-	++	-	++	+
PUV-UH 5%K	+	+	++	+	+	-	+	-	+	-
WBV	-	-	+	+	-	-	+	+	+	+
WBV-UH	+	+	++	++	++	*	+	+	++	+
WBV 5%K	+	+	*	*	*	*	*	*	*	*
WBV-UH 5%K	++	++	+	*	+	+	*	*	++	++

(-) No effect; and (*) Not detected

CONCLUSIONS

1. Findings from the experiment indicated that protective coatings without antimicrobial agents showed no activity against all microorganisms, whereas protective coatings modified with antimicrobial agents generally showed activity against microorganism at a good level. One of the protective coatings, WBV-UH 5%K, showed high activity against all microorganisms, and generally WBV-UH 5%K was more effective than WBV-UH. It was noteworthy that some dyes inhibited the biofilm forming capacity of some bacteria as well as their antimicrobial properties.

2. It was also found that addition of antimicrobial agent and pigment color into water-based and polyurethane protective coatings inhibited the spread of microorganisms more when compared to non-antimicrobial agent and pigment added varnishes. For further research in this topic, the authors plan to determine quality features – *e.g.*, adhesion, hardness, color, brilliancy- of ultra-hygiene varnishes and dyes applied to the surfaces of wooden products and investigate the feasibility of applying them to wooden toys.

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