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RESEARCH ON THE USAGE OF ANTIFUNGAL AND ANTIBACTERIAL PROPERTIES OF INDIGO (*INDIGOFERA TINCTORIA* L.) COLORANT USED AS A WOOD PRESERVATIVE

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ABSTRACT

In this study the antifungal and antimicrobial property of plant extracts from indigo was determined when used as a wood preservative. The extract was dissolved from *Indigofera tinctoria* L. by using an ultrasound assisted method and then applied to Turkish oriental beech and Scots pine wood blocks with the immersion (classic) and immersion+ultrasonic assisted methods. Aluminum sulfate, copper sulfate and vinegar were used for mordants. Results were compared with synthetic dye. Treated blocks were exposed to *Rhodonia placenta* and *Trametes versicolor* attacks for 16 weeks, according the standard TS 5563-EN 113, 1996 method. Antibacterial activity and yeast inhibition of the extracts were determined by the agar dilution method by using the disk diffusion method for bacteria. As a result of substance indigo dye mordant mixtures of copper sulphate and vinegar based synthetic dyes fungis rots more resistant against which that have emerged. Copper mixes showed better antimicrobial activity against all types of microorganisms. In conclusion, it was found that indigo extracts and mordant mixes could be used as wood preservatives.

KEYWORDS: Ultrasonic method, indigo, *Rhodonia placenta*, *Trametes versicolor*, antimicrobial activity, wood preservation.

INTRODUCTION

Wood is a renewable natural resources but must be protected by impregnation material against some biotic and abiotic factors. This impregnation material has emerged damaging to humans and environment with toxic properties of chemicals. Some wood preservatives containing chromated copper arsenate (CCA) (Kartal et al. 2004) and pentachlorophenol (PCP) are banned or limited in some European countries, the United States and Japan. For this reason, the development of environmentally acceptable preservatives has led to a renewed interest in natural–based wood preservatives.

Recent studies were done on the use of natural dyes to protect wood. For example, Ozen et al. (2014) reported antimicrobial and antifungal properties of extracts from madder root (*Rubia tinctorium*) Goktas et al. (2010; 2007a, b) investigated the antifungal properties of extracts from *Muscari neglectum, Gynandriris sisyrinchium, Sternbergia candidum* and *Nerium oleander.*

Indigo is one of the oldest dyes known to humans. In ancient times, it was obtained as a plant extract from several species of the genus *Indigofera* and to a lesser extent from European wood plants. According to research by archaeologists almost all of Egyptian mummies were wrapped in indigo dyed hemp fabric that has emerged (Anonymous 2012). The natural indigo dye homeland is Africa, India, Indonesia, China and North America. In recent years a revival of commercial *Indigofera* cultivation has occurred, notably in India, Bangladesh Mexico and El Salvador (Cardon 2007). Of the various *Indigofera species*, *Indigofera tinctoria* and *Indigofera suffruticosum* are especially used to produce the dye indigo (Leite et al. 2003). Several species of this group are used in anticancer therapy (Vieira et al. 2007). The herbs are generally regarded as an analgesic with anti-inflammatory activity.

Unlike other naturally sourced dyes, indigo does not occur in the plants themselves, but is made during the extraction process from precursors accumulated by the plants. The precursors are compounds containing the indoxyl group. During extraction, the indoxyl is released and is spontaneously oxidized by atmospheric oxygen to indigo (Bechtold and Mussak 2009). Today large amounts of indigo are used for dyeing cotton and wool fabrics.

The objective of this study was to determine colorability the wood material by indigo and mordant mixes and to observe theantifungal and antibacterial properties. Another aim is to utilize the potential of natural sources as an alternative colorant to synthetic wood dyes.

MATERIAL AND METHODS

Wood material

Specimens derived from wood free from cracks, stain, decay, insect damage or other defects had not been water-stored, floated, chemically treated or steamed. Specimens were prepared from Scots pine (*Pinus sylvestris* L.) sapwood and oriental beech (*Fagus orientalis* L.) according to the TS 5563 EN 113, 1996 standards with a sample size of 50 x 25 x 15 mm (longitudinal x radial x tangential directions). All specimens were conditioned at 20 ±2°C and 65 ± 3 % RH for 3 weeks before the subsequent treatment.

Plant materials and preparing dye extracts

The extracts used were obtained from an herbalist located in Karaman, Turkey. A weighed amount of dry plant material was extracted with distilled water in an ultrasonic bath (Elmasonic X-tra H). Ultrasound-assited extraction is an expensive or inexpensive, simple and efficient alternative to conventional extraction techniques. The main benefits of using ultrasound in solid-liquid extraction include the increase of extractionyield and faster kinetics. Ultrasound can also reduce the operating temperature, allowing the extraction of thermolabile compounds. Compared with other novel extraction techniques such as microwave–assisted extraction, the ultrasound apparatus is cheaper and its operation is easier (Wang and Weller 2006).

Dyeing test amples

In the standard procedure the ratio of mass of plant material to the volume of liquid was 1/20. Extraction was performed for 180 min., at 45°C and under 180 W of sonic power in a stainless ultrasonic bath. Due to the rather high liquor ratio, some manual stirring was sufficient to distribute the plant material in the liquid during the extraction period. Volume loss due to evaporation was compensated by the addition of water at the end of the extraction period to obtain the initial volume.

Aluminum sulphate ($Al_2(SO_4)_3 18H_2O$), and copper sulphate ($CuSO_2 5H_2O$) were provided from Kimetsan Co. and vinegar was purchased from Fersan Co. Mordants were prepared by adding the following to aqueous solutions: 5 % aluminum sulphate, 5 % copper sulphate, and 10 % and grape vinegar. This was done in order to stabilize the color of dyes extracted, to ensure it would remain on the applied material (to increase retention amount), and to create color options.

The ultrasound method was used in extracting colorants from plants and applying these colors to food, wool, cotton and leather materials. Some studies have suggested that this method has various advantages in extracting colors and applying them to materials. These advantages consist of a conservation of energy as the process of coloring is performed at a lower temperature, a shortening of the duration of the process, a decrease in the consumption of auxiliary chemical products and thus a reduction in waste load, control of the color tone, a lower transaction cost and consequently an increased competitive capacity (Perincek et al. 2009; Sivakumar et al. 2009; Kamel 2007; Kamel et al. 2005). In this regard the colorants also penetrate more deeply into the materials to which they are applied (Tavman et al. 2009). Considering the porous structure of wooden materials, the use of this method was expected to enable the penetration of colorants into the fibers and lead to a greater resistance to the colorants being washed away compared to the classic (immersion) method.

Fungi, bacteria and antibiotics

As test organisms, the white rot fungus *Trametes versicolor* (L: Fr.) Pilat (FFPRI 1030) and the brown rot fungus *Rhodonia placenta* (Fr.) Niemelä, K.H. Larss. & Schigel (syn. *Postia placenta*) (Mad-698-R), the yeast *Candida utilis* CCTM and the bacteria *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 6538/P were used. They were obtained from the culture collection of Mugla Sitki Kocman University, Faculty of Science, Department of Mushroom Research Center. The standard antibiotic discs used for comparison, such as Penicillin G, Ampicillin, Chloramphenicol, Gentamycin, Erythromycin, Nystatin, and Tetracyclin, were purchased from OXOID Co.

Methods

Treatment

After oven-drying (maintained at 103 ± 2°C) wood specimens were cooled in a desiccator to determine the initial dry mass and stored there in order to keep them dry until dying. The air-dried wood specimens were placed into an ultrasonic bath container according to the intended treatments. Two different methods (immersion and ultrasound-assisted immersion) of dyeing were used. Treatment procedures are given in Tab. 1. The weight percent gain (WPG) (%, w/w) due to chemical load was calculated from the following equation:

$$WPG = \frac{W_{of} - W_{oi}}{W_{oi}} \times 100 \quad (\%) \qquad (1)$$

where: W_{oi} - oven-dry weight (g) of a wood specimen before impregnation,
W_{of} - oven-dry weight (g) of a wood specimen after impregnation.

The treated wood blocks were stored for four weeks according to standard TS 5563 EN 113, 1996 in a conditioning room at 20 ± 2°C and 65 ± 3 % relative humidity until they reached a stable weight before the decay resistance tests.

Tab. 1: Parameters of treatment procedures.

Dye	Treatment method	Sonic power (W)	Temperature (°C)	Time (min)
Natural dye	Control (immersion)	---	45	60
Synthetic wood dye*	Ultrasound-assisted immersion	300	45	60

(*) Genc Fulltex Wood Colourant Properties: Cellulosic alkyd based tining by thinner, Solid Content (%) 5-25, Density 0.79- 0.85 kg.m^{-3}.

Decay resistance test

Untreated and treated wood specimens were exposed to *Trametes versicolor* and *Rhodonia placenta* according to standart EN 113 (1996). *R. placenta* was maintained on 0.39 % potato dextrose agar (PDA) medium, while *T. versicolor* was grown on 0.48 % malt extract agar (MEA) medium. The media were sterilized at 120 ± 2°C for 15 min and transferred to pre-sterilized Petri dishes. After inoculation, the dishes were kept at 26 ± 2°C and 70 ± 2 % RH until the media surfaces were completely colonized by the fungi. The treated and untreated wood blocks were sterilized at 120°C for 15 min after their oven-dried reference weights were determined. Five specimens per group were placed in pre-inoculated Kolle flaks on solid maple feeder strips to minimize direct contact with nutritional media surfaces. Following fungal exposure for 16 weeks at 26 ± 2°C and 70 ± 2 % RH in an incubator, the exposed wood specimens were weighed immediately after the surface mycelium was removed. Percent mass losses were calculated from the difference according to standard EN 113 (1996) in the 103± 2°C oven-dried weight of each specimen before and after the decay test.

$$\text{Mass losses} = [100 \times (W_1 - W_2) / W_1)] \quad (\%) \qquad (2)$$

where: W_1 - weight of wood specimen in over dry state plus remaining preservative after conditioning and before exposure to the test fungus,
W_2 - weight of the wood specimen in over dry state after test and after final conditioning.

Disc-diffusion assay

Extracts were sterilized by filtration with a 0.45 µm millipore filter for antimicrobial tests. Antimicrobial tests were then carried out by disc-diffusion method (Murray et al. 1995) using 100 µL of a suspension containing 108 CFU/mL of bacteria and 106 CFU/mL of yeast, and 104 spores/ml of fungi spread on nutrient agar (NA), sabourand dextrose agar (SDA), and potato dextrose agar (PDA) medium, respectively. Empty sterilized discs of 6 mm were each impregnated with 50 µl indigo and its mordant mixes. The inoculated plates were incubated at 28°C for 12 hours for clinical bacterial strains and 48 hours for the yeast. Plant-associated microorganisms were incubated at 28°C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms at 24 and 48 hours in milimeters. Each assay in this experiment was repeated twice.

RESULTS AND DISCUSSION

Mean extracts retentions calculated from the data fort he gross solution uptake and concentration of treated wood specimens are given in Tab. 2. When wood species treated by a natural extract were compared. The highest retentions were recorded for Scots pine sapwood, (3.78 % indigo+copper mixing treated with the classic method), and the lowest retentions were recorded for beech (1.70 % of synthetic group with the classic method). The particle size of the extracts is responsible for the penetration efficiency of the retention rate.

Tab. 2: Mean extract retentions of wood species treated with different extracts.

Retention (%)			
Extracts	**Treatment method**	**Beech R (St.d.)**	**Scots pine R (St.d.)**
Control (Non mordant)	Ultrasonic	2.79 (0.05)	3.66 (0.04)
	Classic	2.93 (0.02)	3.09 (0.05)
Indigo + Aluminium	Ultrasonic	2.58 (0.09)	2.91 (0.16)
	Classic	2.70 (0.02)	3.23 (0.15)
Indigo + Copper	Ultrasonic	2.39 (0.31)	3.24 (0.31)
	Classic	2.10 (0.06)	3.78 (0.19)
Indigo + Vinegar	Ultrasonic	2.47 (0.28)	2.91 (0.25)
	Classic	2.44 (0.25)	2.86 (0.35)
Synthetic	Ultrasonic	1.72(0.07)	2.46(0.21)
	Classic	1.70(0.08)	2.42(0.17)

Note: Mean of 5 replicates, numbers in parenthesis are standard deviations.

Mass losses

Trametes versicolor

Mass losses of the wood species treated with extract solutions and exposed to *Trametes versicolor* white rot fungus for 16 weeks are given in Tab. 3.

Tab. 3: Mass losses of wood species treated with indigo and mordant mixes after 16 week exposure to Trametes versicolor.

Mass losses (%)			
Extracts	**Treatment method**	**Beech R (St.d.)**	**Scots pine R (St.d.)**
Control (Non mordant)	Ultrasound	14.1 (1,.3)	20.3(2.5)
	Classic-immersion	15.9 (1.5)	22.8 (2.0)
Indigo + Aluminium	Ultrasound	10.2 (1.0)	13.1 (1.5)
	Classic-immersion	11.2 (0.6)	14.8 (0.3)
Indigo + Copper	Ultrasound	10.8 (0.6)	11.9 (2.0)
	Classic-immersion	11.9 (1.6)	13.2 (2.0)
Indigo + Vinegar	Ultrasound	9.4 (0.6)	15.9 (2.1)
	Classic-immersion	9.5 (0.4)	15.2 (2.7)
Synthetic wood dye	Ultrasound	18.3 (2.6)	19.7 (1.6)
	Classic-immersion	19.8 (2.1)	21.7 (1.9)

Note: Mean of 5 replicates, numbers in parenthesis are standard deviations.

The effects of the wood species, mordant agent, treatment method andtheir interaction on mass loss data were evaluated for *T. versicolor* and found to be statistically significant (Tab. 4).

Tab. 4: Multiple variance analysis for mass losses of Trametes versicolor.

Factors	Degrees of freedom	Mean squares	F-value	P-value* (P<0.05)
A: Wood species	2	475.57	111.34	0.000*
B: Mordant agent	3	1168.82	273.66	0.000*
C: Treatment method	2	637.31	149.21	0.000*
Interaction A*B	6	252.67	59.15	0.000*
Interaction A*C	2	97,29	22.77	0.000*
Interaction B*C	6	202.39	47.38	0.000*
Interaction A*B*C	6	216.56	50.70	0.000*
Error	128	4.27		
Total	160			

Rhodonia placenta

Mass losses of the wood species treated with extract solutions and exposed to *Rhodonia placenta* are given in Tab. 5. Untreated control specimens of Scots pine and oriental beech wood were severely decayed by *Rhodonia placenta*. Mass losses of indigo (*Indigofera tinctoria*) (non-mordant) treated wood species showed better resistance to the brown rot fungus compared to the untreated specimen. On the other hand, Scots pine species mass losses for Indigo (*Indigofera tinctoria*) and mordant mixtures showed better resistance against *Rhodonia placenta* when compared to synthetic specimens.

Tab. 5: Mass losses of wood species treated with indigo and mordant mixes after 16 week exposure to Rhodonia placenta.

Weight losses (%)			
Extracts	Treatment method	Beech R (St.d.)	Scots pine R (St.d.)
Control (Non mordant)	Ultrasonic	23.6 (1.4)	10.3 (1.3)
	Classic-immersion	25.0 (1.5)	11.6 (1.7)
Indigo + Aluminium	Ultrasonic	22.3 (1.7)	8.3 (0.7)
	Classic-immersion	23.0 (1.8)	8.5 (0.6)
Indigo + Copper	Ultrasonic	20.5 (1.9)	5.8(0.6)
	Classic-immersion	23.1 (1.6)	6.6 (0.8)
Indigo + Vinegar	Ultrasonic	25.7 (0.8)	12.4 (2.8)
	Classic-immersion	27.7 (0.9)	11.1 (2.9)
Synthetic wood dye	Ultrasonic	23.4 (1.4)	27.2 (3.1)
	Classic-immersion	24.9 (2.5)	29.1 (2.5)

Note: Mean of 5 replicates, numbers in parenthesis are standard deviations.

The effects of the wood species and mordant agent, the interaction of treatment method and mordant agent, the interaction of wood species and mordant agent, and the interaction of treatment method, wood species, and mordant agent on mass loss data were evaluated for *Rhodonia placenta* and found to be statistically significant (Tab. 6).

Tab. 6: Multiple variance analysis for mass losses of Rhodonia placenta.

Factors	Degrees of freedom	Mean squares	F-value	P-value* (P<0.05)
A: Wood species	1	4226.05	1038.80	0.000
B: Mordant agent	3	62.49	15.36	0.000
C: Treatment method	1	2.28	0.56	0.457
Interaction A*B	3	62.73	15.41	0.000
Interaction A*C	1	106.32	26.13	0.000
Interaction B*C	3	45.22	11.11	0.000
Interaction A*B*C	3	10.82	2.66	0.056
Error	64	4.06		
Total	80			

The resistance to decay of Scots pine and beech wood impregnated with indigo (*Indigofera tinctoria*) extract against *Rhodonia placenta* and *T. versicolor* was studied. Results showed that mass losses varied among both fungi. The mass losses of wood specimens were the lowest when impregnated with indigo + vinegear extract after attack by *Rhodonia placenta* and *T. versicolor*. The mordant agents have a complex chemical bend binding with the (-OH) groups of the wood components. This is thought to be an effects of the fungus attack (Vasisth 1996).

Antimicrobial activity

Tab. 7 shows the antimicrobial activities of indigo and its mordant mixes screened against bacteria (*Staphylococcus aureus* and *Escherichia coli*) and the yeast *Candida albicans*.

According to the results, the extracts of indigo mixed with copper had an antibacterial effectiveness against the bacteria and inhibited the yeast (Tab. 7). On the other hand, control, indigo + aluminum sulphate mixes, indigo + vinegar mixes and syntheticdye did not show any antimicrobial activity.

Tab. 7: Antimicrobial activity of indigo-mordant mixes and some standard antibiotics.

Extracts	Tests		
	Staphylococcus aureus	***Candida albicans***	***Bacillus megaterium***
Control (Non mordant)	0	0	0
Indigo + Aluminium	0	0	0
Indigo + Copper	7	12	7
Indigo + Vinegar	0	0	0
Synthetic wood dye	0	0	0

In the recent time; studies focused on the biological activity of *Indigofera* species with antimicrobial activity *Indigofera oblongifolia* (Dahot 1999), *I. sedgewickiana* (Alasbahi et al. 1999), *I. suffruticosa* (Leite et al. 2006) and *I. longeracemosa* (Thangadurai et al. 2002). The antimicrobial properties of different extracts from the leaves of I. dendroides exhibited significant activity against pathogenic microbial populations (Esimone et al. 1999). In this study the results for indigo (*I. tinctoria*) extract and copper mixes showed antimicrobial activity against *S. aureus*, *Candida albicans* and *Bacillus megaterium* 7, 12, and 7 mm, respectively. Recent studies indicated that bacteria, including certain harmful strains of *E. coli* and *S. aureus*, can cause serious nosocomial or hospital acquired infections, but will simply die in a few hours when placed on copper alloy surfaces at room temperature (Lewis 2005).

CONCLUSIONS

This study evaluated the decay resistance of wood treated with indigo and its mordant mixes against brown rot and white rot decay fungi and antimicrobial activities of dyes tuffs. According to the test results beech wood samples colored with indigo + vinegar mixes (9.4 %) and for the Scots pine samples colored with indigo + copper sulfate mixes (11.9 %) were showed minimum mass losses by white rot fungus *Trametes versicolor*. Generally indigo andits mordant mixes were more effective than commercial synthetic dye against both fungi. The indigo + copper mixes showed minimum mass losses by *Rhodonia placenta* for beech wood (20.5 %) and Scots pine samples (5.8 %), respectively. The samples colored by ultrasonic were showed better mass losses results compared to classic method.

Indigo and copper mixes showed antimicrobial activity against all types of microorganisms. The other dyestuff and synthetic dye did not demonstrate any antimicrobial activity.

The plant extracts can offer substantial advantages for wood protection and aesthetics aspects at low cost and ease of handling and treatment. On the other side for the assessment of plants, which are naturally grown in Turkey and are potential rich sources, studies will be very efficacious from economic and ecological standpoints.

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