EVALUATION AND IDENTIFICATION OF WALNUT HEARTWOOD EXTRACTIVES FOR PROTECTION OF POPLAR WOOD

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Walnut (*Juglans regia L.*) heartwood extractives were identified and their potential for protection of poplar wood was evaluated. Test specimens were prepared from poplar wood (*Populus nigra L.*) to meet BS 838:1961 requirements. Samples were impregnated with heartwood extractive solution (1.5, 2.5, and 3.5% w/w in ethanol-toluene), followed by 5 hours vacuum desiccator technique to reach complete saturation. Impregnated specimens were exposed to white-rot fungus (*Trametes versicolor*) for 14 weeks according to BS 838:1961 applying the kolle-flask method. The weight loss of samples was determined after exposure to white-rot fungus. The highest weight loss (36.96%) was observed for untreated control samples and the lowest weight loss (30.40%) was measured in samples treated with 1.5% extractives solution. The analyses of the extracts using GC/MS indicated that major constituents are benzoic acid,3,4,5-tri(hydroxyl) and gallic acid (44.57 %). The two toxic components in the heartwood are juglone (5.15 %) and 2,7-dimethylphenantheren (5.81 %).

Keywords: Walnut heart wood extractives; Trametes versicolor; Weight loss; Gallic acid; Juglone; 2,7-dimethylphenantheren

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INTRODUCTION

One of the promising strategies to slow down the decay and deterioration of wood structure is to rely upon durable wood species. Usually, heartwood of most species contains higher levels of extractives compared to sapwood, and some natural durability exists against microorganism in some timbers. The heartwood of species, such as western red cedar (*Thuja plicata*), redwood (*Sequoia sempervirens*), teak (*Tectona grandis*), black locust (*Robinia pseudoacacia*), cypress (*Cupressus sempervirens*), walnut (*Juglans nigra*), osage orange (*Maclura pomifera*), and ebony (*Diospyros lotus*), are very resistant to decay (Reyes-Chilpa et al. 1998; Gottlieb and Yoshida 1989; Rudman 1963; Thevenon et al. 2001; Waterman 1946). In this respect, natural compounds extracted from various woods especially heartwood have been proven to impart antifungal properties in some woods. Examples of such compounds are cinnamaldehyde (Wang et al. 2005), α-cadinol (Chang et al. 1999), carvacrol (Kai 1991), Tmuurolol, T-cadinol, c-cadinene (Kondo and Imamura 1986), cryptomeridiol (Morita et al. 1997), tropolones (Hart 1989), pinosylvin (Schultz et al. 1990), oxyresveratrol, dihydromorin (Schultz et al. 1995), gallic acid (Kishino et al. 1995) and ferruginol (Rudman 1965). Therefore, in recent years, the wood preservation industry has preferred natural and vegetable-based chemicals for wood treatments. Some extractives contain tannin or exhibit toxic effects against biotic agents,

and are preferred for protection of wood or wood-based materials against microorganisms (Schultz and Nicholas 2000).

However, these species are not widely available, and their utilization is limited and even in some cases prohibited (Thomasson et al. 2002). Consequently, consumers ought to utilize wood from other species, applying impregnation by an effective preservative that is environmentally acceptable (Thevenon et al. 2001).

Chemical preservatives impart a negative impact on our environment, including malabsorption, unsuitable fixation, and leaching of arsenic containing compounds from CCA treated wood, as well as transfer to human beings from playground equipment, thus presenting risks to children's health (Lebow and Tippie 2001; Stevanovic-Janezic et al. 2001), etc. Also, the application of some treated wood may be excluded in housing and other venues due to the emission of toxic vapors, difficulty in handling, allergic effects for animals and people, as well as inadequacies in dealing with related environment issues (Thomasson et al. 2002).

The genus *Juglans* of the family Juglandaceae contains more than 25 species distributed throughout many regions of the world. *Juglans regia* L., which is the native of Central Asia extending from Xinjiang province, western China, Kazakhstan, Uzbekistan, and southern Kirghizia to Nepal, Tibet, northern India, and Pakistan through Afghanistan, Turkmenistan, and Iran to portions of Azerbaijan, Armenia, Georgia, and eastern Turkey (McGranahan and Leslie 1991), produces durable wood which has lasted for centuries in ancient buildings and in common application such as home furniture. It is envisioned that the extracts of walnut wood powder exhibits potential as an environmentally friendly and natural wood preservative. It has been acknowledged that the extraction yield of this wood is high, producing low cost material for wood preservation (Thevenon et al. 2001; Steber 2000; Da Costa et al. 1958; Rudman and Da Costa 1959).

Walnut (*Juglans regia L.*) is a commercial hardwood species growing in the north of Iran. Walnut heartwood is dark brown and often veined with excellent drying and shrinkage characteristics and strength properties. As a result it has found a wide range of applications from rustic furniture, sculpture, fine joinery, carving, and decorative veneering, to outdoor use such as decking and structural applications. Such processing generates vast quantities of residues. Walnut heartwood is classified as a durable wood (Scheffer and Cowling 1966; Scheffer and Morrell 1998) which has been related to the presence of phenolic compounds such as flavonoids, naphtoquinones, and hydrolysable tannins (Gupta et al 1972).

Detailed phytochemical and pharmacological studies have shown that the extractive is mostly juglone, which can cause blackening, blistering, and peeling of the skin. It also acts as a tranquilizer and sedative (Bhargava 1967; Westfall et al. 1961), shows antitumor activity (Bhargava and Westfall 1968), and it is fungi toxic, antibiotic, and allelopathic (Soderquist 1973). The wood of black walnut contains appreciable amounts of gallic acid as well as ellagic acid, glucose, and a dark violet polymer (Gupta et al. 1972; Seshadri 1973). Ellagic acid is a sedative and tranquilizer and has antitumor activity (Bhargava and Westfall 1968).

The objective of this study was to evaluate the toxicity potential of walnut heartwood extractives, identify the components present in the extracts, and investigate whether such extract could be successfully used as preservative to protect non-durable

poplar wood against white-rot fungal (*Trametes versicolor*). Also it was of interest to examine the effect of heartwood extractives on the weight loss of poplar wood, a common wood for rural construction to improve its performance for applications such as decking, carrier and roof poles, pile and truss, joinery and furniture, and box making.

EXPERIMENTAL

Materials

Poplar wood (*Populus nigra* L.) was selected according to TS 2476, as defect-free, clear, and normally grown (without zone lines, reaction wood, decay, and insect damage, or fungal infection) wood from a plantation in northern city of Tonekabon, Iran located on fertile lands at the elevation of 20 meters above sea level. The annual precipitation of this area is usually about 1100-1500 mm. Walnut wood (*Juglans regia* L.) was collected from a forest in the northern city of Ramsar, Iran located at an elevation of 640-1000 meters above sea level with annual precipitation of 934-1616 mm.

Wood Extraction

Walnut wood powder (flour) was soxhlet extracted according to ASTM D 1107-96. Wood powder was filled in the extraction apparatus and successively extracted with ethanol-toluene (2:1 mL, v/v) until a colorless solution was obtained. Usually 6-8 hours was required to complete the extraction. The solvents (ethanol-toluene) were evaporated at 40^{o}C in a rotary evaporator to reach a viscous solution in the flask, and then dried under nitrogen gas to produce powder.

Toxicity Screening

To evaluate the toxicity potential of the extract, 25 mg (dry powder) sample of extract was dissolved in either 2 mL mixture of ethanol-toluene or toluene individually and then filtered using a syringe filter and poured into a glass vial. The media containing malt extract agar (MEA) was sterilized in an autoclave at 120 ^{o}C for 20 minutes, and then the solution containing sample of extract was poured into the 75 mL of sterile media using micro-sampler. Then the media was poured into three petri plates. Five concentrations of extract solution, ranging between 0 to 0.15 mg/mL (Table 1) were added to petri plates and thoroughly mixed with media. Each test was duplicated in three replicates. The plates were cooled in a sterile hood and inoculated with 0.5 cm round plugs of *Trametes versicolor* (*TV*) fungus mycelia, which were introduced into the center of the petri plate using the tip of pasture pipet. Inoculated plates were placed in 23^{o}C and 75% relative humidity without light. Fungal growth was monitored daily measuring the radial growth and calculating the percentage of area that was covered by fungus in each plate. The toxicity level was determined as the extract concentration which exhibited the lowest fungal growth as specified in modified method by Nzokou and Kamdem (2002).

Preparation of Test Specimens

Wood specimens were randomly selected. Rough boards at 30 mm thickness were tangentially sawn and then stored at 20°C and 65% relative humidity for 2 months to

reach 12% final moisture content. Then test specimens were cut from these rough boards (Fig. 1) having dimensions of 50 x 25 x 15 mm for weight loss according to BS 838:1961. Specimens were randomly used in our experiments.

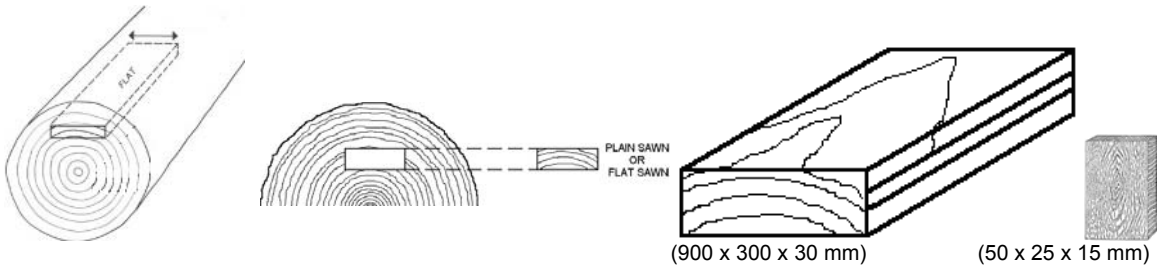

Figure 1. Specimen preparation from the log

Test Specimens Impregnation

Specimens were treated applying solutions of walnut heartwood extracts in ethanol-toluene at concentrations of 1.5, 2.5, and 3.5%. These had shown higher toxicity in previous tests, and they were above the relative threshold. To reach uniform distribution and absorption of the solution, specimens were submerged in the treatment solution applying 0.78 bar (11.3 psi) vacuum in vacuum desiccators for 5 hours. Specimens were then wiped to remove the excess solution from the surface and weighed to determine the amount of treating solution absorbed.

Treated specimens were stored under 65% relative humidity and 20 ^{o}C temperature for 4 weeks to reach the equilibrium moisture content of 12%. Before and after impregnation, samples were oven dried to 0% moisture content. After cooling in desiccators, the oven dry weights of the specimens were measured. The retention ratios of chemicals (*R %*) were calculated as follows,

$$R(\%) = \frac{Mdi - Md}{Md} \times 100 \quad (1)$$

In this equation, *Mdi* is the oven-dry mass after impregnation (*grams*) and *Md* is the oven-dry mass before impregnation (Table 3).

Decay Test

Decay tests were conducted in accordance with BS 838:1961 as applied by the kolle-flask method for 14 weeks exposure to *Trametes versicolor*. The fungus was grown and maintained on malt extract agar (MEA). The medium was sterilized for 30 min. at 125^{o}C and cooled to room temperature before inoculation. Test kolle flasks were prepared with 60 mL of MEA and closed with a cotton cap. The filled kolle flasks were then loosely capped and autoclaved for 30 min. at 105 kPa and 125^{o}C. After cooling the kolle flasks, the plug was cut from the actively growing edge of a 7-day old MEA culture of white-rot fungus and placed into the kolle flask. After the next seven days the treated and control specimens (*Populus nigra L.*) were placed on the top of the two small glass legs in each kolle flask (Fig. 2), parallel to opposite corners of the mycelia plug.

Each inoculated kolle flask was then incubated at 23 ^{o}C and 75% relative humidity until the specimens were heavily colonized by fungus. All decay tests were performed on specimens from each group of treated specimens impregnated with different concentrations of extractives solution. For either of three concentrations of extractives solution (1.5, 2.5, and 3.5%) three replicates were tested. We realize that three replications are not sufficient for this case. However, our limitation on the availability of extract necessitated lesser number of specimens than usually required. The labeled test specimens were placed on a screen tray and oven dried to 0% MC. The specimens were weighed to the nearest 0.01 gram and recorded as un-decayed weight (*W1)*. At the end of the exposure period, the exposed test specimens were removed from the kolle flasks, their surfaces were carefully brushed, and then they were oven-dried to 0% MC. The specimens were weighed to the nearest 0.01 g to determine the decayed weight (*W2*). Weight loss was calculated as percentage of the initial weight.

$$\text{weight loss (\%)} = [(W1 - W2)/W1] \times 100) \quad (2)$$

Identification of Extract Components

The pure walnut heartwood extract was separated and dried under nitrogen gas to give pale red colored extracts at 12% yield, based on oven dried weight of the heartwood. In order to identify the components present in the extracts, about 1 mg (powder) extract was selected, mixed with 30 micro liter N,O-bis (trimethylsilyl) triflouroacetamide (BSTFA) + 1% timethylchlorosilane (TMCS) reagent and about 15 micro liter pyridine in test tube, then the test tube was placed in a water bath at 70 °C for one hour.

Extracts were analyzed using GC/MS on an Agilent 6890 Gas Chromatograph, equipped with a split/splitless injector and a 7963 Mass Selective Detector (MSD). The chromatography was performed on a HP-5MS capillary column (SGE, 30 m, 0.25 mm) with helium as carrier gas, at 1mL/min flow. The temperature change was programmed between 60 and 260 °C, increasing at the rate of 6 °C/min.

Statistical Procedure

To evaluate changes in measured property of untreated decayed, treated decayed, treated un-decayed, and untreated (un-decayed or control) specimens, one-way ANOVA analyses of variance was used. The effects of different concentrations of extractives on weight loss, density, and retention ratio are determined. Duncan's test was used for ranking of the average values of measured property (Table 2, and 3).

RESULTS AND DISCUSSION

The results from toxicity screening tests revealed that the walnut heartwood extract exhibits preservative potential toward non durable wood such as poplar wood in the experiment (Table 1). The fungal, *Trametes versicolor* (*Tv*), growth on untreated wood reached almost 100%. However, applying increasing concentrations of walnut extracts reduced the growth. Applying 0.15 mg/mL solution, *Tv* showed a growth reduction of at least 27.91%. Therefore, the relative toxicity threshold was estimated to

be about 0.15 mg/mL (150 ppm) for *Tv* in ethanol-toluene solvent. Of course, this was not unexpected, because it has been reported that the concentration of 200 ppm juglone has been reported to cause 72.1% inhibition of white-rot fungus (*Pleurotus sajor-caju*) (Currelli et al. 2001).

Table1. The Fungal Growth under Various Concentrations of Walnut Heartwood Extracts

Extract Concentration (mg/mL solvent)	Growth Rate (% of Specimen Area)	
	Ethanol-Toluene	Toluene
0	100	100
0.0015	100	100
0.003	100	97
0.075	56.46	82
0.15	27.91	65

Since the toxicity screening demonstrated that the concentration of 1.5% extract in ethanol-toluene resulted in the lowest growth, this concentration and higher levels were selected for further decay tests on wood. The average and standard deviation of weight loss measurements are summarized in Table 2. Statistical analysis showed that the effect of extract solution concentration on weight loss was significant at the 5% level. Consequently, the ranking of the average values using Duncan test is provided in Table 2 by lower case letters.

Table 2. The Average and Standard Deviation Values of Weight Loss and Extractive Retention Ratio of Poplar Wood

Specimen Condition	Weight Loss (%)	Retention Ratio (%)
Decayed (Untreated)	$36.96^{c*} \pm 1.64$	-
Decayed (Treated, 1.5%)	$30.40^{a} \pm 2.11$	$1.99^{a*} \pm 0.33$
Decayed (Treated, 2.5%)	$34.50^{bc} \pm 1.06$	$3.67^{ab} \pm 1.02$
Decayed (Treated, 3.5%)	$32.44^{ab} \pm 2.96$	$5.32^{b} \pm 1.90$

* Superscripts, lower case letters, indicate Duncan ranking of the average value of measured weight loss

Table 3. The Average and Standard Deviation Values of Dry Density of Poplar Wood Specimens

Specimens Condition	Dry Density (gr/cm^3)	
	untreated	Treated
Untreated and un-decayed (Control)	$0.387^{abc*} \pm 0.006$	-
Decayed (Untreated)	$0.367^{a} \pm 0.006$	-
Decayed (Treated, 1.5%)	$0.417^{bc} \pm 0.035$	$0.420^{c} \pm 0.030$
Decayed (Treated, 2.5%)	$0.377^{a} \pm 0.021$	$0.380^{ab} \pm 0.020$
Decayed (Treated, 3.5%)	$0.377^{a} \pm 0.006$	$0.387^{abc} \pm 0.006$

*Superscripts, in lower case letters, indicate Duncan ranking of the average value of measured dry density

The mean weight loss values obtained from the decay test were 36.96% for untreated specimens and 30.40%, 34.50%, and 32.44% for treated poplar wood specimens impregnated with 1.5%, 2.5%, and 3.5% extractives solutions, respectively (Table 2). It is interesting to note that specimens impregnated with the solution containing 1.5% extract showed the lowest weight loss, even though the retention ratio of the extract was lowest at this concentration (Table 2). Nevertheless, we tried to use randomly selected specimens with similar density, but the density of samples treated with 1.5% solution was higher and therefore more resistant toward fungus. The dry density gain after treatment of these specimens treated with solutions containing either 1.5% or 2.5% extract is almost the same and for the other specimens is almost four times higher (Table 3). Surprisingly, the average untreated dry density of specimens impregnated with solution containing 1.5% extract was higher than other specimens, which inhibited the penetration of fungus mycelium and consequently lower deterioration and weight loss.

The Willeitner scale showed distinct evidence of fungal colonization (100%) on the specimens surfaces (Willeitner 1984). However, extractives treatment reduced the fungal growth (67% colonization) to a noticeable extent (Fig. 2).

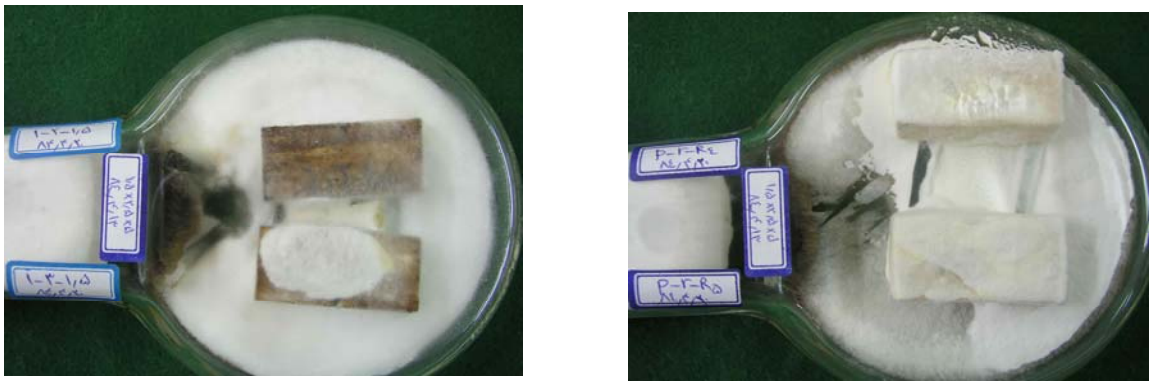

Figure 2. Mycelium growth of *Trametes versicolor* fungus on treated specimens with 1.5% extractives solution and decayed (left), and the untreated decayed specimens (right)

Even though decay by white-rot fungus was evident on untreated specimens and those treated with 3 concentrations of extractives, the penetration of extractives into the treated poplar wood specimens provided some protection against white-rot fungus.

The result of the analysis of walnut heartwood extractives is presented in Table 4. Gallic acid, D- fructose,1,3,4,5,6-pentakis-o-(hydroxyl), D-glucose,2,3,4,5,6-pentakis-o-(hydroxyl), and xylitol 5-TMS are present. In addition, 2,7-dimethylphenantheren as an important component of the extractives was detected, and its presence in walnut heartwood extract has not been reported previously. 2,7-dimethylphenantheren-like compounds (naphthalene) have been reported in the fruit extracts of *Juglans regia L.* (Talapatra et al. 1988; Muller and Leistner 1978). The heartwood extractives also contained minor quantities of α-pinene and juglone.

Table 4. Composition (area %) of the Walnut Heartwood (*Juglans regia* L.*)* Extractives

Components	RT (min)	Heartwood
α-pinene	5.69	5.28
Juglone	6.02	5.15
propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethyl	8.69	1.07
silane, trimethyle (phenylmethoxy)	10.66	1.33
3,7-dioxa-2,8-disilanonane, 2,2,8,8-tetramethyl	13.34	2.40
3,8-dioxa-2,9-disiladecane, 2,2,9,9-tetramethyl	17.81	2.15
2,7-dimethylphenantheren	19.99	5.81
xylitol 5-TMS	21.37	3.26
benzoic acid,3,4-bis(hydroxyl)	22.67	2.06
d-fructose,1,3,4,5,6-pentakis-o-(hydroxyl)	22.87	0.79
d-glucose,2,3,4,5,6-pentakis-o-(hydroxyl)	23.94	4
Benzoic acid,3,4,5-tri(hydroxyl)/Gallic acid	24.72	44.57

CONCLUSIONS

1. Analysis of variance and Duncan's test (Table 3) indicated that the dry density of treated specimens impregnated with a solution containing 1.5% extract was significantly different from specimens before treatment. This difference was attributed to lower weight loss of these specimens, which was significantly different from other decayed specimens. On the other hand, the dry density of specimens treated with a solution of 3.5% extract was not significantly different from other decayed specimens, except for those treated with 1.5% concentration. Furthermore, the weight loss of specimens treated with solution of 3.5% extract was significantly different from the control decayed specimens. Thus it can be concluded that at higher concentration of extractives, fungal resistance of treated poplar wood specimen is higher. This can be attributed to the antimicrobial potential of extractive components such as gallic acid, 2,7-dimethylphenantheren, and juglone. Such compounds have anti-fungal toxicity and can serve as wood preservatives. The effect of extractives to impose lower weight loss was maximized using solution of 3.5% extract.

2. The precipitation of extractives in the cell lumens and the cell wall introduces weight gain (density gain) in poplar wood specimens, and consequently reduces the weight loss of the wood.

3. Gallic acid probably can be extracted from any walnut wood. Analysis of the walnut heartwood extractives indicated that these extractives can provide a major natural source of gallic acid and 3,7-dioxa-2,8-disilanonane,2,2,8,8-tetramethyl. The juglone and 2,7-dimethylphenantheren were found to occur as minor constituents in the walnut heartwood extractives.

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