Chemical Contents and Antifungal Activity of Some Durable Wood Extractives *vs. Pleurotus ostreatus*

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The total phenolic content (TPC), total flavonoid content (TFC), phenolic compounds, and antifungal activity of olive (*Olea europaea* var. *sylvestris*) and juniper (*Juniperus foetidissima*) sapwood and heartwood extractives were examined. The extractives were obtained using methanol solvents. The different compounds in extractives were identified and quantified. The antifungal activities of different parts of the olive and juniper wood extractives were determined *in vitro*. *Pleurotus ostreatus* mycelium was used for the antifungal activity experiment. Extractive compounds obtained from olive and juniper woods were found to be effective, natural antifungal agents.

Keywords: Antifungal activity; Juniper; Olive; Total flavonoid contents; Total phenolic contents

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INTRODUCTION

Wood is used in many applications on account of its excellent mechanical strength, low thermal conductivity, and aesthetic properties. However, wood also has some disadvantages and is biodegradable, especially when used in certain areas (Ates *et al*. 2009; Srinivas and Pandey 2012; Temiz *et al*. 2014). Fungus easily degrades wood in certain conditions, but some more durable wood species can be used in outdoor environments. Heartwood contains many extractive compounds, which may effectively protect it against harmful microorganisms (Onuorah 2000; Schultz and Nicholas 2000; Mun and Prewitt 2011). Wood can also be protected against fungi and other degradation using certain methods. Chemical treatments to protect wood against fungi have some disadvantages and often involve toxic chemicals. Wang *et al.* (2005) stated that obtaining environmentally friendly wood preservatives called "non-toxic preservatives" from durable tree species could protect wood without polluting the environment. Chang *et al.* (1999) reported that, as regard for environmental preservation increases, determining the bioactive constituents in durable wood species and understanding the mechanisms by which they function are the best ways to protect wood. Thus, phenolic and flavonoid compounds obtained from natural resources could solve some of the problems of the wood protection industry.

Phenolic compounds are small molecules with one or more phenolic groups. They display remarkable antioxidant activity *in vitro*, and play a significant role in the protection of plants against UV radiation, pathogens, and predators (Strack 1997). Phenolic compounds are significant plant constituents because of their ability to scavenge radicals and active oxygen species such as singlet oxygen, free radicals, and hydroxyl

radicals (Hall and Cuppett 1997; Güder and Korkmaz 2012a). Flavonoids are important plant components because of their active hydroxyl groups and antioxidative properties (Güder *et al.* 2014). Flavonoids are secondary metabolites that provide UV protection and color to almost all terrestrial plants and fruits. They consist of fused aromatic and benzopyran rings with phenyl substituents. Their bioactivities have an impact on human health and they are target molecules for the development of new medicines (Esmaeili *et al*. 2013).

A future advance of the wood preservatives industry would be to understand why the heartwood of some tree species has considerable natural resistance to degradation (Schultz and Nicholas 2000). Some juniper species have strong anti-termite, anti-bacterial, and anti-fungal properties (Mun and Prewitt 2011). Olive tree wood is classified as a moderately durable wood (Govorčin *et al.* 2010). Gupta *et al.* (1972) stated that the durability of wood is related to its phenolic and flavonoid contents. Clark *et al.* (1990) explained that methanol extracts from *Juniperus virginiana* heartwood and needles exhibited anti-fungal and anti-bacterial activity. Kwon *et al.* 2010 stated that widdrol compound isolated from *Juniperus chinensis* has anti-cancer activities *in vitro*. One of the active constituents of olive leaves and fruits, oleuropein, has exhibited antioxidant, antimicrobial, antiviral, and antitumor activities (Bisignano *et al.* 1999; Benavente-Garcia *et al.* 2000; Hamdi and Castellon 2005; Micol *et al.* 2005). Tumen *et al.* (2013) hypothesized that some durable woods (for example, juniper) can act as non-toxic, natural wood preservatives derived from a renewable source. Extractives from these durable wood species may be used to protect against wood decay and termite attack. The purpose of this study was to investigate the total phenolic content (TPC) and total flavonoid content (TFC) of olive and juniper sapwood and heartwood extracts, and determine their antifungal activities against *Pleurotus ostreatus* wood decay fungi. A further purpose was to investigate some of the most commonly known chemical constituents of TPC.

EXPERIMENTAL

Materials

Chemicals

Agar powder was purchased from Sisco Research Lab., Pvt., Ltd. (Mumbai, India). Other chemicals were of analytical grade and obtained either from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany) or from E. Merck (Darmstadt, Germany). *Pleurotus ostreatus* (HK-35) mycelium was purchased from Agroma (Denizli, Turkey).

Preparation of Wood Samples

Olive (*Olea europaea* var. *sylvestris*) and juniper (*Juniperus foetidissima*) wood samples were obtained from the Balıkesir province of Turkey in June of 2012, according to the TAPPI standard T257 cm-85 (1985). Trees of each species were randomly selected at different locations. Wood samples were prepared both from freshly cut wood and from dried wood. Wood samples were left to dry under ambient conditions. The dried wood samples (moisture content: 8%) were chopped into small parts with a special knife and powdered with a hammermill. Sapwood and heartwood were separated and powdered for extraction. The wood powder (particle size between 0.05 and 0.4 mm) was stored in closed glass jars at room temperature.

Extraction

The extraction test was carried out using Soxhlet extractors in accordance with TAPPI standard T204 om-88 (1988). Wood samples (10 g) were extracted using methanol (150 mL) for 6 h. The methanol extracts were evaporated and dried extractive samples were kept in glass bottles at approximately -18 °C until future experiments.

Methods

High performance liquid chromatography (HPLC)

To identify the phenolic compounds present in the samples, the extracts were analysed using HPLC (Shimadzu, Kyoto, Japan) with a photodiode array detector (DAD, λmax=278). The HPLC analysis was performed with an Agilent Eclipse XDB-C18 (250 x 4.60 mm) with a 5 micron column (Agilent Technologies Inc., USA) at 30 °C and 0.8 mL/min flow.

Determination of Total Phenolic Content (TPC)

The total phenolic content was analyzed using Folin and Ciocalteu's (FC) colorimetric method as described by Dewanto *et al.* (2002) with minor modifications. Stock solutions (1000 µg/mL) were prepared from the dried extractives. Each solution (500 µL) was mixed with distilled water (7 mL) and FC reagent (500 µL). After 6 min, Na_2CO_3 solution (2 mL, 7%) was added to the mixture. The observed color during a 90 min period and the absorbance at 760 nm was measured by a OPTIZEN POP UV/Vis Single Beam Spectrophotometer (Shimadzu, Kyoto, Japan). The total phenolic contents were expressed as micrograms of gallic acid equivalent (GAE) using an equation (Eq. 1) obtained from a standard gallic acid graph (0 to 1000 µg/mL) (R^2 = 0.9960).

$$\text{Absorbance}=0.1463\times\text{Concentration (µg/µL)} - 0.0080 \quad (1)$$

Determination of Total Flavonoid Content (TFC)

The total flavonoid contents of the extracts were determined according to the colorimetric method described by Chang *et al.* (2002) with some modifications. Extract solutions (0.5 mL) at different concentrations (10 to 100 µg/mL) were added to a tube containing 1.5 mL of methanol. Solutions of $AlCl_3 \cdot 6H_2O$ (0.1 mL, 10%) and potassium acetate (0.1 mL, 1 M) were subsequently added. Distilled water was added to bring the total volume to 5 mL, and the absorbance at 415 nm was read after 30 min. The total flavonoid contents were expressed as micrograms of catechin equivalent and were obtained from a standard graph (R^2 = 0.9983).

Antifungal Assays

Antifungal assays were carried out using methods described in previous studies (Chang *et al.* 1999; Wang *et al.* 2005) with some modifications. Malt agar media was prepared with 50 g of malt, 30 g of agar, and 1000 mL of pure water. The media was sterilized in an autoclave at 121 °C for 15 min. To evaluate the antifungal activity of olive and juniper sapwood and heartwood, four different dosages of extractive sample (50, 100, 300, and 500 µL; 20 µg/µL) were prepared by dissolution the dried extractives with methanol solvent. After, 20 mL of hot sterilized liquid malt agar (60 °C) and prepared extractives dosages were mixture in Petri dishes. Final concentrations of extractives and malt agar mixtures were of 0.05, 0.1, 0.3, and 0.5 µg/µL (50, 100, 300, and 500 ppm), respectively. The Petri dishes were cooled in a sterile environment at room

temperature for 24 h, and methanol was removed by vaporization. Each mycelium plug (4-mm diameter) was inoculated at the centre of the Petri dishes that were filled with nutrient media. Test plates were placed in a dark, conditioned, sterilized room at 23 ± 2 °C and 70 ± 5% relative humidity. Antifungal assays were terminated when the mycelium of *Pleurotus ostreatus* reached the border of the control petri dish on the 8th day, and the antifungal activity was calculated according to the formula (Eq. 2),

$$(1-(Dt/Dc)) \times 100 \quad (2)$$

where *Dt* is the diameter of the mycelia growth zone in the test plate and *Dc* is the diameter of the mycelia growth zone in the control plate. Control and test groups were performed at least three times and the final data reported as the arithmetic mean. The half maximal inhibitory concentration (IC_{50}) and minimal inhibitory concentration (IC_{100}) were calculated using Microsoft Excel.

Statistical Analysis

Experimental results were reported as the mean ± standard deviation of the three parallel measurements. Analysis of variance (ANOVA) was performed. Both operations were carried out with SPSS 15 software (IBM, New York, USA).

RESULTS AND DISCUSSION

The HPLC analyses indicated the presence of 7 phenolic compounds in olive heartwood, 5 phenolic compounds in olive sapwood, and 3 phenolic compounds in juniper heartwood and sapwood, as shown in Table 1.

Table 1. Compounds of the Methanol Extract from Juniper, Olive Sapwood, and Heartwood (ppm)

Olea europaea* var. *sylvestris	**Protocatechic acid**	**Vanilin**	**p-coumaric acid**	**Benzoic acid**	**Hydroxy tyrosol**	**Tyrosol**	**Oleuropein**
Sapwood	1.4	1.5	0.4	9.9	8.2	3.3	703.2
Heartwood	1.5	0.9	*	*	29.5	10.7	746
Juniperus foetidissima	**Protocatechic acid**	**Vanilin**	**Catechin**				
Sapwood	0.2	0.4	3.7				
Heartwood	0.3	0.5	3.7				
* not detected							

These phenolic compounds were determined using an HPLC database. Twenty-five different previously determined phenolic compounds were recognized. The HPLC analyses showed that the main phenolic compounds of olive heartwood were oleuropein, hydroxytyrosol, tyrosol, vanillin, and protocatechic acid and of olive sapwood were oleuropein, hydroxytyrosol, tyrosol, vanillin, protocatechic acid, benzoic acid, and p-coumaric acid. Tasioula-Margari and Okogeri (2001) identified similar phenolic compounds in olive oil. Phenolic compounds of juniper heartwood and sapwood

contained only protocatechic acid, catechin, and vanillin. Lesjak *et al.* (2013) detected similar phenolic compounds in *Juniperus foetidissima* seeds and leaves. Figure 1 shows the structures of the detected phenolic compounds.

Plants containing various phenolic compounds important due to their efficient radical scavenging effects. These properties may be related to hydroxyl groups on benzene rings. These compounds offer electron or hydrogen radicals to scavenge free radicals (Güder and Korkmaz 2012b). This counteracts and reverses the degradation effects of oxidative stress damage (Ames *et al.* 1993).

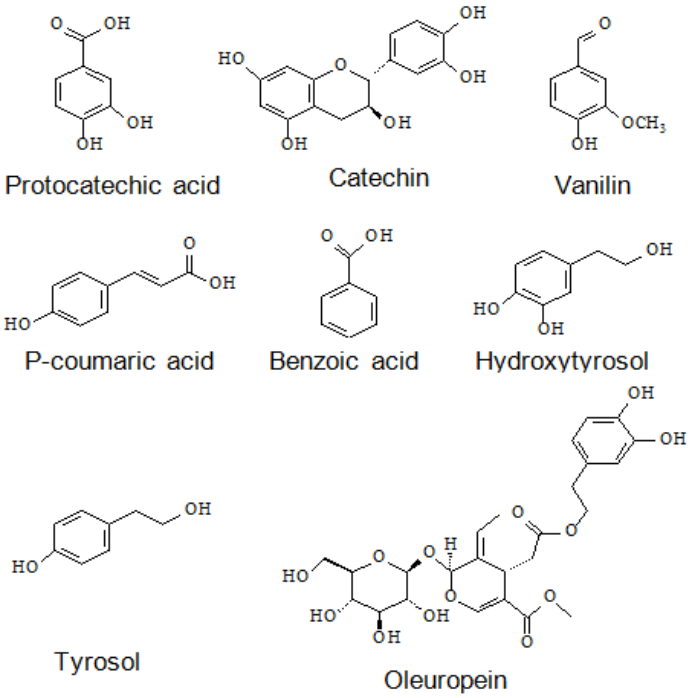

Fig. 1. Chemical structures of phenolic compounds in *Olea europaea* var. *sylvestris* and *Juniperus foetidissima* wood

Figure 2 shows the total phenol contents expressed as gallic acid equivalents (GAE) for all extracts. The confidence level was %95 ($P< 0.05$), and the phenolic content of MEJF-H was 717.07 ± 2.12 µg GAE/g dried extracts. In addition the other extracts such as MEJF-S, MEOE-S, and MEOE-H had 239.60 ± 1.44, 122.36 ± 0.87, and 139.79 ± 0.69 µg GAE in 1 g of the dried extracts, respectively. As can be seen in the results, MEJF-H was greater than the others for this confidence level. Miceli *et al.* (2009) and Taviano *et al.* (2013) reported that antimicrobial effects of *Juniperus communis* and *Juniperus oxycedrus* berries extractives are not only dependent on the phenolic content (TPC) but also on other secondary metabolites.

Flavonoids give fruits and vegetables various red, blue, or violet colors and are related to the group of bioactive compounds called stilbenes (Güder and Korkmaz 2012b). Flavonoids are significant plant constituents because they exhibit antioxidant activity (Kumar *et al.* 2008). The total flavonoid content was expressed as µg of catechin equivalents (CE)/g dried extracts. The data presented in Fig. 2 indicate that the highest flavonoid content, 91.00 ± 0.76 µg CE/g dried extracts, was observed in MEOE-S. The lowest flavonoid content, 41.19 ± 0.55 µg CE/g dried extracts, was observed in MEOE-

H. The MEJF-S and MEJF-H contained 89.03 ± 1.03 and 44.62 ± 0.92 µg CE/g dried extracts, respectively at 99% confidence level (P< 0.01).

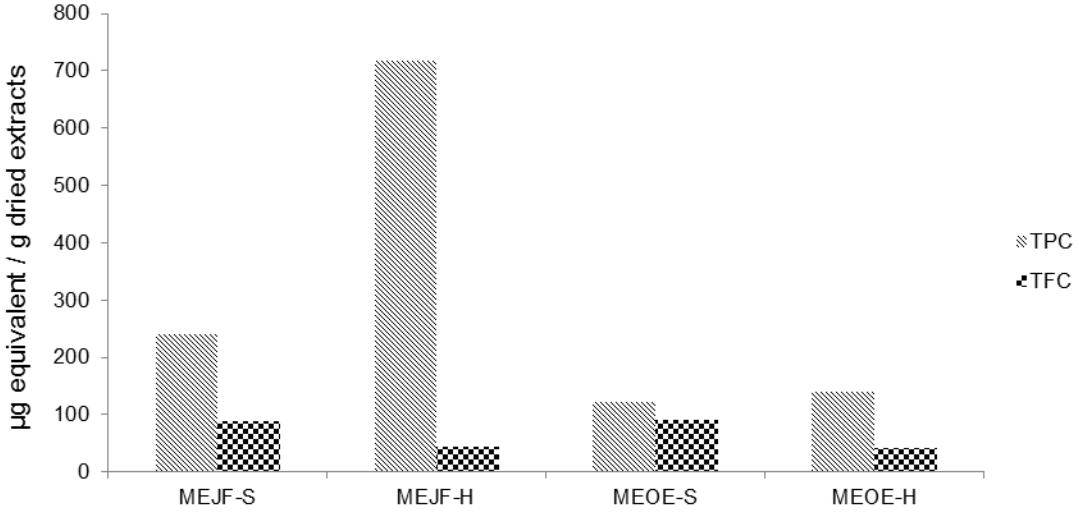

Fig. 2. Comparison of the total phenolic contents (TPC) and total flavonoid contents (TFC) of wood extracts. MEJF-S, Methanol Extract of *Juniperus foetidissima* Sapwood; MEJF-H, Methanol Extract of *Juniperus foetidissima* Heartwood; MEOE-S, Methanol Extract of *Olea europaea* Sapwood; and MEOE-H, Methanol Extract of *Olea europaea* Heartwood

The anti-fungal activities of *O. europaea* var. *sylvestris* and *Juniperus foetidissima* sapwood and heartwood extracts and their total phenolic and flavonoid contents were determined. To evaluate the antifungal activity of *O. europaea* var. *sylvestris* and *Juniperus foetidissima* sapwood and heartwood, *Pleurotus ostreatus* fungi was used and different doses of extractives were applied in nutrient media. Table 2 shows the antifungal activity of *O. europaea* var. *sylvestris* and *Juniperus foetidissima* wood extractives.

Table 2. Antifungal Activity

Wood Extractives	Final Concentration (ppm)	Antifungal Activity (%)
Juniperus foetidissima **Sapwood**	500	83
	300	61
	100	6
	50	0
Juniperus foetidissima **Heartwood**	500	78
	300	56
	100	11
	50	6
Olea europaea* var. *sylvestris **Sapwood**	500	83
	300	67
	100	11
	50	6
Olea europaea* var. *sylvestris **Heartwood**	500	83
	300	61
	100	11
	50	6

Pleurotus ostreatus growth was inhibited (approximately 80%) with a final concentration of 500 ppm from all extractive samples. Previous studies have shown that oleuropein, hydroxytyrosol, tyrosol, benzoic acid, p-coumaric acid, and catechin exhibit good antifungal or antibacterial activity (Daayf *et al.* 1997; Bisignano *et al.* 1999; Amborabe *et al.* 2002; Friedman 2007; Yangui *et al.* 2010). Protocatechic acid is also an antioxidant (Li *et al.* 2011).

The IC (inhibitory concentrations) assay is used for early stage research of drugs performance. The IC assay protocols have been developed to find out whether tested compounds have desired properties such as antifungal activity (Sebaugh 2010). So, the IC assay will help to determine the antifungal activity of new natural wood preservatives. Figure 3 also shows that the lowest IC_{50} value, 0.28 µg/µL, was observed in MEOE-S. The IC_{50} values for MEOE-H, MEJF-S, and MEJF-H were 0.29, 0.30, and 0.31 µg/µL, respectively. No significant correlations between the TFC, TPC, and IC_{50} of sapwood or heartwood extracts were seen. However, these extracts may be effective antifungal agents against wood pathogens. Also, the IC_{100} (minimal inhibitory concentration of fungus) values for MEOE-S, MEOE-H, MEJF-S, and MEJF-H were 0.55, 0.57, 0.56, and 0.61 µg/µL, respectively, as shown in Fig. 3. In this study, IC values of sapwood extracts were less than heartwood extracts. However, heartwood was found to be more resistant to fungi than sapwood. This is because heartwood contains more extractives than sapwood (Fengel and Wegener 1984). Also, other factors are related to natural resistance, water permeability, and the idea that hygroscopicity of heartwood is smaller than those of sapwood, and this situation may increase the resistance against fungal growth in heartwood (Kollman and Cote 1968).

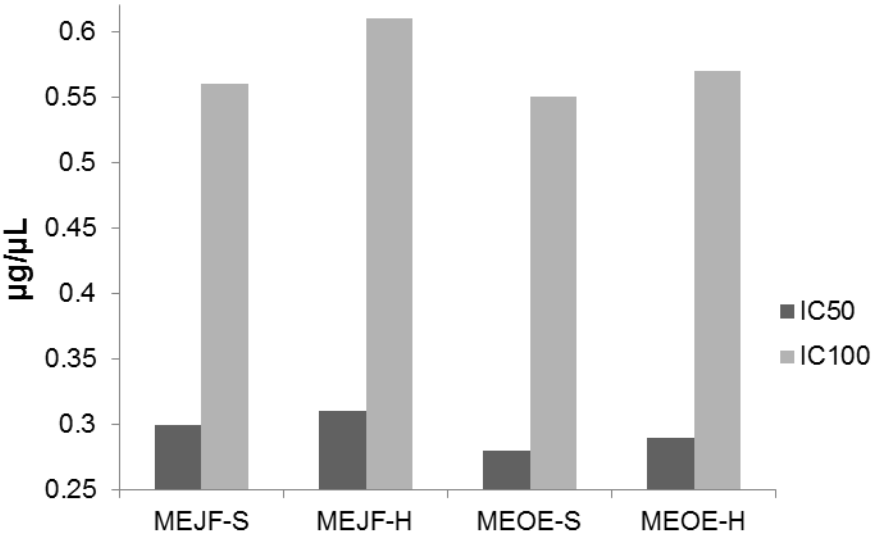

Fig. 3. Comparison of MIC values (µg/µL) of wood extracts. MEJF-S (methanol extract of *Juniperus foetidissima* sapwood); MEJF-H (methanol extract of *Juniperus foetidissima* heartwood); MEOE-S (methanol extract of *Olea europaea* sapwood); and MEOE-H (methanol extract of *Olea europaea* heartwood)

Kirker *et al.* (2013) stated that extractive compounds are primarily responsible for the durability of wood. However, the extractives content was not directly related to durability in this study. It is probable that certain individual extractive compounds are

more effective in facilitating wood durability than others. Kirker *et al.* (2013) also suggested that the problem of micro-distribution of wood extractives that are localized within the heartwood and sapwood, should not be disregarded. In some studies, the location of extractives in wood and how they impact fungal mycelia are important to properly understanding their real functions.

The phenolic and flavonoid compounds of both sapwood and heartwood extract inhibited wood decay fungi as in previous similar studies (Onuorah 2000; Yen *et al.* 2008; Morikawa *et al.* 2012; Maoz *et al.* 2012; Tumen *et al.* 2013; Li *et al.* 2014).

CONCLUSIONS

1. When the both sapwood and heartwood extractives content increased in nutrient media, the antifungal activity of the wood extractives increased.
2. Methanol extract from *Olea europaea* sapwood yielded the minimum IC_{50} value, followed by MEOE-H, MEJF-S, and MEJF-H.
3. Methanol extract from *Olea europaea* sapwood yielded the minimum IC_{100} value, followed by MEJF-S, MEOE-H, and MEJF-H.
4. The MEJF-H had greater phenolic content than other extractives, while MEOE-S had greater flavonoid content than other extractives.
5. Results showed that olive and juniper wood extractives are potential antifungal agents protecting against wood pathogens.

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