Activity of Spent Coffee Ground Cinnamates against Wood-decaying Fungi *in vitro*

Aitor Barbero-López,[a,*] Antonio Ochoa-Retamero,[a] Yeray López-Gómez,[a] Teemu Vilppo,[b] Martti Venäläinen,[c] Anu Lavola,[d] Riitta Julkunen-Tiitto,[d] and Antti Haapala [a,b]

Fungi and microbes can remarkably degrade the appearance and durability of organic materials, such as wood. The inhibitory effects of natural phenolics may offer more sustainable alternatives to preserve wood than the toxic biocides that are currently used. Although pure caffeine has been proven to have antibacterial properties, the applicability of spent coffee in wood preservation has not been determined. This work conducted *in vitro* tests with three brown rot and one white rot fungi and demonstrated the potential of spent coffee-derived cinnamates, analyzed with high-performance liquid chromatography, as antimicrobial agents. Spent coffee at concentrations of 1% and above in the growing media caused significant growth suppression of all of the fungi. This was not only because of the caffeine, but also the other chemicals present in the residue extracts, which demonstrated that spent coffee could be used as a source of green chemicals in wood preservative formulations.

Keywords: Biorefining; Preservatives; Secondary metabolites; Wood decay; Wood preservation

Contact information: a: School of Forest Sciences, University of Eastern Finland, FI-80101, Finland; b: Department of Applied Physics, University of Eastern Finland, FI-70211, Finland; c: Natural Resources Institute Finland, FI-58450 Punkaharju, Finland; d: Department of Environmental and Biological Sciences, University of Eastern Finland, FI-80101, Finland;
** Corresponding author: Aitor.Barberolopez@uef.fi*

INTRODUCTION

Increasingly strict legislation on toxic wood preservatives is driving the development of green chemicals for wood. The use of many wood preservatives is limited or banned, as chromated copper arsenate (Mohajerani *et al.* 2018). Other types of metal salt, borate, and creosote products are still used, although constant pressure is applied by the national chemicals agencies for less toxic substitutes. More limitations for wood preservatives can be expected, as they belong to a class of so called emerging pollutants, together with several pesticides and pharmaceuticals (Geissen *et al.* 2015).

Green chemicals that protect wood from decay and pathogens, while also maintaining a low environmental impact (Ding *et al.* 2017), are sought from plant extracts. Examples of these chemicals are the polyphenols, such as condensed tannins (Anttila *et al.* 2013) and stilbenes (Lu *et al.* 2016), known antifungals with some commercial products already on the market. Novel and interesting sources for potential antifungals can be found in industrial side-streams, such as coffee refining (Arora and Ohlan 1997; Acevedo *et al.* 2013). The phenolics and alkaloids (*e.g.* caffeine) in the side-streams of coffee refining have no competing industrial use. Mazela *et al.* (2016) recently verified the antifungal activity of purified caffeine in a wood preservation setting, while Lekounougou *et al.* (2007) demonstrated its antifungal properties against some wood-decaying fungi. Contradicting results have been found for bacteria; Sant'Anna *et al.* (2017) found that spent

coffee grounds (SCGs) does not inhibit the growth of different foodborne pathogenic bacteria and phytopathogenic fungi, while Sousa *et al.* (2015) suppressed different disease-causing bacteria and yeasts using spent coffee.

Despite several interesting functionalities of SCGs, the limited availability of the raw material often renders their valorization economically unattractive. This has been shown in the case of biodiesel production from SCGs (Kookos 2018), but obviously a different outcome can be achieved if the end product has significantly higher market value or lower cost of production. In most of the soluble coffee producing industries, the waste is collected by specialized agencies, which sell the residues for different purposes (*i.e.* composting, gardening, bioenergy production, mushroom growth) but for commercial or household SCGs there is no established recovery and recycling system. Present studies focus in different aspects of fresh and spent coffee. Their components have been extensively analyzed (Mullen *et al*. 2013; Monente *et al*. 2015). Contemporary studies concerning coffee have focused mostly on health implications (Koloverou *et al*. 2015), land use, and climate issues (Ricketts *et al.* 2004). Studies on the revalorization of spent coffee grounds have focused on energy applications (Santos *et al.* 2017) and food supplements (Panzella *et al.* 2017). These studies and the recent reviews of this topic (Kourmentza *et al.* 2018; Mata *et al.* 2018) do not address the potential of spent coffee-derived chemical components as a potential resource for antifungal chemicals with applications, *e.g.* in wood preservation.

In this paper we present for the first time the capacity of spent coffee as a green alternative to reduce wood-decaying fungi growth. We also characterized fresh and spent coffee grounds in terms of the chemical composition and assessed the efficiency of the extracted chemical mixes against common wood-decaying fungi. Our aim was to determine if the waste generated from coffee brewing has potential for use as a feedstock in chemical extraction and if its functionality can be utilized in wood preservation or as an antifungal in a more general context.

EXPERIMENTAL

Fresh and spent medium-roast coffee grounds (100% *Coffea arabica*) were tested by extracting 50-g batches of coffee grounds in 1 L of boiling Milli-Q water (Merck KGaA, Darmstadt, Germany) for 45 min to obtain a concentrated hot water extract with a low number of volatile components. After extraction, the solids were removed with either a fine sieve or 30-µm filter. The growth media included 4% malt powder and 2% agar, along with 1%, 2%, or 5% sieved spent coffee extracts (SCEs) (w/w), 1% filtered SCEs, or 1% fresh coffee. Reference media were made with 1.6% copper-based preservative (Celcure C4, Koppers Inc., Pittsburgh, PA, USA), 4% malt, and 2% agar to have an industrial NTR (Nordiska Träskyddsrådet) AB-class standard capable preservative as reference. The commercial copper-based wood preservative contained copper(II) carbonate (17%), ethanolamine (< 35%), benzalkonium chloride (4.75%), cyproconazol (0.096%), sodium nitrite (< 5%), and polyethoxylated tallow amine (< 5%).

The chemical composition of the SCEs was analyzed in detail with an HP 1100 Series LC-system (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector, where the reverse-phase separation was performed on a Hypersil ODS (Thermo Fisher Scientific, Waltham, MA, USA) with a RP C18 column (Thermo Fisher Scientific, Waltham, MA, USA) with the dimensions 75 mm × 4.6 mm. Phenols were separated by

gradient elution using aqueous 1.5% tetrahydrofuran with 0.25% orthophosphoric acid (A) and methanol (B) as eluents. The following elution gradient was used: 0 min to 5 min with 100% A, 5 min to 10 min with 85% A and 15% B, 10 min to 20 min with 70% A and 30% B, 20 min to 60 min with 50% A and 50% B, and 60 min to 65 min with 100% B. The flow rate was 2 mL/min, the column temperature was 30 °C, and the injection volume was 20 µL. The compounds were detected at a wavelength of 270 nm, and the tentative identification was based on the retention time, UV spectra of the compounds, and the literature. The quantification of the compounds was based on the response factors specific to the standard compounds. Standards provided with the chemicals purchased from Sigma-Aldrich (Sigma-Aldrich Finland Oy, Helsinki, Finland) were used in the identification and quantification of the components in the coffee samples: chlorogenic acid for all of the chlorogenic acid derivatives; ferulic acid; *p*-OH-cinnamic acid for the *p*-OH-cinnamic acid derivatives; cinnamic acid for the caffeic acid derivatives; protocatechuic acid for the protocatechuic acid derivative; and caffeine.

The growth reduction efficiency of the SCEs was tested *in vitro* with three brown rot fungal strains (*Coniophora puteana* BAM 112, *Gloeophyllum trabeum* BAM 115, and *Rhodonia* (*Poria*) *placenta* BAM 113) and a white rot fungal strain (*Trametes versicolor* BAM 116) obtained from the Federal Institute for Materials Research and Testing (BAM, Berlin, Germany). Fungal hyphae plug measuring 0.28 cm^2 were placed in a petri dish (Ø 90 mm) and incubated with no light at 22 °C ± 2 °C and 70% relative humidity between 9 and 11 days, when the mycelium of the control fungi reached the edge of the dish. Between six and ten replicates were prepared, depending on the species and its growth variability. The growth rate inhibition was measured by following the method from Chang *et al*. (1999). The inhibition rate of the fungal growth with the addition of extracts in the growth media was assessed according to Eq. 1,

$$Inhibition\ (\%) = (1 - (AT - IA) / (AC - IA)) \times 100 \quad (1)$$

where *AT* is the fungal area in the experimental sample (cm^2), *AC* is the fungal area in the control sample (cm^2), and *IA* is the size of the inoculated plug (cm^2). Pictures of the petri dish were taken with the same set up as explained in Ancin-Murguzur et al. (2018).

The statistical significance of the coffee treatments was determined using Fisher's least significant difference test with the least significant difference as the post-hoc for the analysis of variance using the software SPSS Statistics 23 (IBM, New York, USA).

RESULTS AND DISCUSSION

Most of the water-soluble, low molecular weight components, including sugars, caffeine, and other polar compounds like phenolic compounds, were removed during the first extraction (coffee brewing), which left less soluble components in the SCEs. Volatile components and large molecular weight components, like tannins and carbohydrates, were not detected. The amount of caffeine decreased significantly between the fresh coffee and SCEs, and the concentrations of other components were also noticeably lower.

The phytochemical profiles (high performance liquid chromatography (HPLC)-fingerprints) of the samples were comparable. Seventy and 75 different components above the detection limit at 270 nm were separated from the SCEs made from fresh coffee and the filtered fresh coffee sample, respectively. In the SCEs from the spent coffee grounds,

the number of components was consistent between the samples and was approximately half of that in the fresh coffee samples (Table 1).

Table 1. Concentrations of the Compounds in Different Liquid Coffee Samples Analyzed by HPLC

Compound	Retention Time (min)	Fresh Coffee (mg/L)	Filtered Fresh Coffee (mg/L)	SCEs (mg/L)	Filtered SCEs (mg/L)
Protocatechuic acid der.	4.0	-	-	2.7	2.1
Chlorogenic acid der. 1	4.1	39.3	33.6	-	-
Chlorogenic acid der. 2	4.6	26.9	22.9	3.9	4.0
Chlorogenic acid der. 3	5.3	7.5	6.3	-	-
Caffeine (alkaloid)	6.0	1104.1	934.3	31.7	31.0
Neochlorogenic acid	6.6	733.8	613.9	60.8	64.9
Chlorogenic acid der. 4	8.6	2.7	2.3	1.2	-
p-OH-Cinnamic acid der. 1	9.1	7.5	6.1	0.8	0.7
Chlorogenic acid der. 5	10.3	175.8	143.9	17.7	19.2
Chlorogenic acid	10.8	1138.7	949.6	97.9	102.3
Chlorogenic acid der. 6	11.5	638.7	526.1	58.2	58.2
p-OH-Cinnamic acid der. 2	13.3	5.0	4.9	0.6	-
p-OH-Cinnamic acid der. 3	13.6	7.5	5.0	0.5	0.3
Chlorogenic acid der. 7	14.0	18.6	14.9	0.8	0.8
Ferulic acid	14.4	120.1	99.1	11.4	11.4
Chlorogenic acid der. 8	15.3	67.6	56.0	7.2	8.9
Caffeic acid der. 1	16.7	1.4	1.4	-	-
Caffeic acid der. 2	16.9	3.7	3.1	-	-
Chlorogenic acid der. 9	18.6	4.3	3.0	-	-
Chlorogenic acid der. 10	20.4	2.1	2.4	-	-
Chlorogenic acid der. 11	20.6	1.5	1.8	-	-
Chlorogenic acid der. 12	20.8	1.5	1.6	-	-
Chlorogenic acid der. 13	21.3	4.2	1.8	1.0	0.7
Chlorogenic acid der. 14	21.7	7.4	3.3	0.6	0.9
Chlorogenic acid der. 15	22.2	76.3	59.5	18.7	19.2
Chlorogenic acid der. 16	22.6	49.1	36.4	9.2	9.8
Caffeic acid der. 3	24.9	1.5	1.0	0.4	0.3
Chlorogenic acid der. 17	25.2	9.6	7.9	2.0	2.2
Chlorogenic acid der. 18	25.9	72.8	58.0	13.8	15.7
Caffeic acid der. 4	28.4	1.0	1.0	0.1	0.2
Chlorogenic acid der. 19	29.5	9.6	8.9	1.4	2.0
Chlorogenic acid der. 20	32.5	4.1	4.7	-	-
Total of cinnamates	-	3240	2680	308	322

The coffee samples consisted of hydroxycinnamates, which were mainly different derivatives of chlorogenic acid (caffeoylquinic acids) and ferulic acid, but also the derivatives of caffeic and *p*-hydroxycinnamic acid. Additionally, one of the main components in the samples was the methylxanthine alkaloid, caffeine.

Recently, Martínez *et al.* (2017) established that chlorogenic acid inhibits mycelial growth and spore germination at doses of 15 μg/μL against phytopathogenic fungi relevant in horticulture and agriculture. Chlorogenic acid has been known to be abundantly available in industrial by-products, such as those derived from coffee (Murthy and Naidu 2012). The recovered phenolic components have mostly been considered for new value-added products, such as phenolic antioxidant adjunct for food processing, but not for wood preservation.

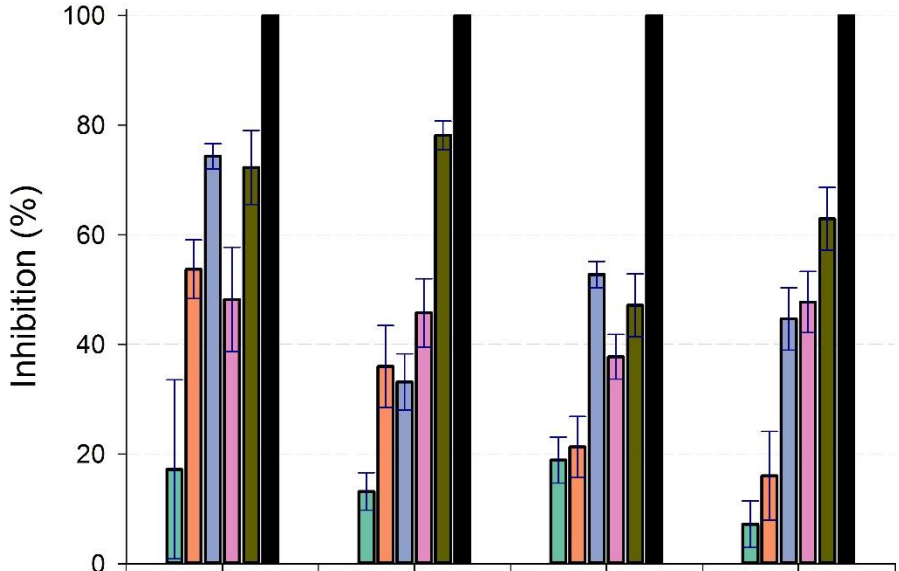
Caffeic and protocatechuic acids have been found to completely inhibit the growth of two *Aspergillus* spp. molds at concentrations of 0.2 mg/mL and 0.3 mg/mL (Aziz *et al.* 1998). Cinnamic acid and its derivatives have been shown to inhibit the growth of *Candida albicans* and *A. niger* at low doses (Narasimhan *et al.* 2004). Also, caffeic, chlorogenic, ferulic, and trans-cinnamic acids have recently been reported to prevent the activity of several *Colletotrichum* spp. isolates that cause anthracnose fruit rot (Roy *et al.* 2018).

The proportions of the compounds were similar between the two fresh coffee samples and between the two SCEs samples. The compound amounts were highest in the fresh coffee sample. Filtration of the fresh coffee reduced the concentrations of the compounds, and thus the amount of cinnamic acids and caffeine in the filtered fresh coffee sample was 17% and 15% lower, respectively, than in the unfiltered sample. In SCEs, the difference in the total cinnamates between the filtered and unfiltered samples was approximately 4%.

The SCEs inhibited the growth of the four species of decay fungi when applied at concentrations of 1% and higher (Fig. 1). Arora and Ohlan (1997) concluded that 0.5% caffeine fully inhibits fungal growth. The highest concentration (w/w) of SCEs in this study was 5%, which indicated the presence of less than 0.1% caffeine. These findings agreed with those of Arora and Ohlan (1997) but showed a stronger effect on *G. trabeum* compared with pure caffeine. This indicated that there are synergistic effects between the chemicals in spent coffee grounds that prevent the growth of wood-decaying fungi.

Filtering the SCEs caused a significant increase in the inhibition of *C. puteana*, *G. trabeum*, and *T. versicolor* (see Fig. 2 for a practical example). Arora and Ohlan (1997) found that filtering the coffee decreased its antifungal effect by 53%. This decrease in effectiveness of the spent coffee when filtered may have been caused by the presence of other chemicals, such as carbohydrates, that promote fungal growth and hinder the antifungal activity of other chemicals.

A preliminary test was done based on the same method, but with 0.2% spent coffee in the media. This test promoted the growth of *G. trabeum* (data not shown), which supported the conclusion that there are chemicals present in the SCEs that promote the growth of fungi. For *C. puteana* and *R. placenta*, the inhibition caused by 1% fresh coffee did not differ significantly from that of the spent 5% SCEs. However, compared with the other species, 1% fresh coffee caused the highest inhibition, which differed significantly from that of the other concentrations. These differences indicated that chemicals present in fresh coffee and not spent coffee can play a role in fungal inhibition. The medium with 1.6% copper-based preservative completely inhibited the growth of all of the fungi.



	Inhibition (%)			
1% SCE	17.2 ± 8.2	13.2 ± 1.7	18.9 ± 2.1	7.2 ± 2.1
1% filtered SCE	53.7 ± 2.7	36.0 ± 3.7	21.3 ± 2.8	16.1 ± 4.0
1% fresh SCE	74.3 ± 1.1	33.2 ± 2.6	52.7 ± 1.2	44.7 ± 2.9
2% SCE	48.2 ± 4.8	45.8 ± 3.1	37.7 ± 2.0	47.7 ± 2.8
5% SCE	72.3 ± 3.4	78.1 ± 1.3	47.1 ± 2.9	62.9 ± 2.9
Cu preservative	100 ± 0	100 ± 0	100 ± 0	100 ± 0

Fig. 1. Inhibition of wood-decaying fungi by fresh and spent coffee ground extracts: *C. puteana*, *G. trabeum*, and *R. placenta* (N = 10), and *T. versicolor* (N = 6)

The statistical analysis confirmed that all the SCEs inhibited significantly (P=0.000) the growth of all species of fungi when compared to reference media. Fresh coffee caused a significantly higher inhibition than spent coffee in all fungi (P=0.000) and filtering of 1% SCEs caused significant growth inhibition in all fungi (P<0.05) except *R. placenta* when compared to 1% SCE.

Fig. 2. Growth of *C. puteana* (BAM 112) after 13 d in malt-agar media petri dish with the control on the left and the 5% non-filtered spent coffee ground extract infused media on the right

During the experiment, a decolorized halo around the mycelia of *C. puteana* was observed after 4 d to 5 d, which could have indicated that compounds were released by the fungi that detoxified the growth media. This was similar to the results reported by Kovačec *et al.* (2017).

The minimum inhibitory concentration (MIC) to completely inhibit the growth of fungi was not determined, but the results showed that the MIC value was beyond 5% for all of the fungi tested. This meant that spent coffee is not a feasible wood preservative, but it could be considered a green source of chemicals that can contribute to preservative formulations against fungi after selective separation and purification. Recent studies have also found a high antioxidant activity in SCEs (Panusa *et al.* 2013) that is often related to the natural durability of wood (Binbuga *et al.* 2008) and may enhance the potential for the use of these extracts in wood preservative formulations.

The antifungal activity of the SCEs against wood-decaying fungi was thus because of the caffeine, as well as several identified antifungal chemicals, such as chlorogenic acid and ferulic acid derivatives and caffeic and *p*-hydroxycinnamic acids. These two sets of chemicals suggested that there is a joint interaction with microbes and that those components contribute to the decay resistance. Furthermore, previous studies have found that low doses of some of the constituents of spent coffee inhibit different kinds of fungi (Aziz *et al.* 1998; Narasimhan *et al.* 2004; Lekounougou *et al.* 2007). Alternative extraction methods of spent coffee ground constituents, their selective separation, and further use in wood preservative formulations may lead to novel methods for the use of bio-based chemicals that can substitute current fungicides.

Recent investigations have presented spent coffee grounds as a useful substrate for the cultivation of wood-degrading fungi that form edible mushrooms (Leifa *et al.* 2001). In contrast, the findings in this paper showed that several extractives with antifungal activities against wood-decaying fungi remain in coffee grounds after they are used for making coffee. Fan *et al.* (2006) found that even if the fungus *Pleurotus ostreatus* still fructified when grown with tannins and caffeine, increasing the concentrations of these chemicals caused reductions in the mycelial growth of the fungus. Their study also found that the tannins were completely degraded, while the caffeine was only partially degraded and accumulated in the mycelium and fruiting bodies of *P. ostreatus*. Oh *et al.* (2018) found that the mycelial growth of edible mushrooms decreased when coffee hydrolysates were present in the media, but the antioxidant properties were improved and the number of polyphenols of the mycelia increased. Extraction and selective removal of green chemicals with an antifungal activity as a first step could lead to a better performance of the solid fraction of spent coffee as a media for mushroom cultivation. For this application, both the chemical recovery and mushroom cultivation need to be studied further.

Until recently, SCG has been discarded to landfills and considered solid waste with low value. The commercial viability spent coffee ground chemicals will strongly depend on availability of spent coffee grounds. The coffee industry generates large volumes of wet SCGs from the manufacture of instant coffee and caffeinated drinks (Pfluger 1975), and utilization of this resource is still a rather original and environmentally friendly approach of international and societal interest, avoiding the disposal of such residue in landfills (Stylianou et al. 2018). Collecting the waste from private users may not be a feasible option, but collecting the SCGs from the coffee industry is a clear opportunity for its valorization.

CONCLUSIONS

1. We demonstrated that spent coffee ground extracts contain chemicals that inhibit the growth of wood-decaying fungi at low concentrations. To extract and isolate antifungal

components from spent coffee grounds has high potential for developing green preservatives and promoting the valorization of organic residues.

2. It was found that the antifungal activity was not solely derived from the presence of caffeine, but also from the synergistic reactivity of the cinnamates and alkaloids that were present in the residue at reasonable concentrations.

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