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Antimicrobial properties of American Hazelnut oil and extracts

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Abstract
While European hazelnuts are a globally traded commodity, American hazelnut and its hybrids are grown by a relatively small number of growers and are marketed regionally. In recent years, plantings have expanded in the Upper Midwest. With this expansion has come a desire to develop markets for co-products, such as the husks. The objectives of this study were to 1) characterize American hazelnut phenolic extracts and 2) to investigate the antimicrobial properties of American hazelnut oil and phenolic extracts. Phenolics were extracted from the husks of cultivated American hazelnuts and were tested with HPLC. Phenolic extracts and pressed hazelnut oil were tested for antimicrobial properties against five Gram-positive bacteria, seven Gram-negative bacteria and two fungi. Husk extract showed inhibition against eight of these organisms. Overall, this exploratory project suggests potential for hazelnut husk extract to be used as an antimicrobial compound against some bacteria.

Keywords: Filbert, phenolic, involucre, Corylus

1. Introduction
Hazelnut (*Corylus spp.*) is a perennial crop grown for its use as an edible nut and cooking oil. A project to select high-productivity genotypes of American hazelnut (*Corylus americana* Marshall) began in 2008 in Wisconsin [1]. The nuts from these selections were shown to be of high quality with good processing characteristics; however, mild bitterness of the nuts was noted with some selections [2]. For this reason, hazelnut nutmeats, shells, leaves and involucres were assessed by Demchik *et al.* for levels of total phenolics which are a common source of bitterness [3]. They found rather high levels of total phenolics combined with strong antioxidant capacity in extracts of all of the tested parts of American hazelnut plants. The strong antioxidant capacity suggests the potential for these extracts to be used in industrial or food applications. A wide range of phytochemicals are present within tree nuts [4]. Oil generated from American hazelnut was shown to be similar to European hazelnut [5]. While distribution of total phenolics and antioxidant activity of American hazelnut was also shown to be similar to European hazelnut [3], the composition of the phenolics was not assessed in this project. Because both European hazelnut oils [6] and European hazelnut phenolics [7] have been shown to exhibit some antimicrobial properties as well as antioxidant potential [8-9], the potential for use of American hazelnut oil and phenolic extracts for industrial or food uses seems plausible. The objectives of this study were to 1) characterize American hazelnut phenolic extracts and 2) to investigate the antimicrobial properties of American hazelnut oil and phenolic extracts.

2. Materials and methods
2.1 Phenolics
Husks were collected from selections of wild American hazelnut grown under cultivated conditions in September 2016 (see Demchik, *et al.* for description of the selection procedure [1]). Husks from 6 selections were frozen from fresh harvested condition with nuts intact in a -20 °C freezer. Husks from 3 selections were air-dried in mesh bags and stored for 7 months in that condition. Samples were freeze dried (LABCONCO Freezone 4.5 at -48 °C/0.046 mbar/72 hours) and ground using a Wiley Mill. Samples were weighed into Soxhlet extraction thimble (approximately 1 g) and Soxhlet extract was completed using 80% ethanol for 6 hours. Final volume of ethanol extract was weighed. Extract was filtered through a 0.45 µm filter. Resulting sample was analyzed by HPLC (Agilent 1260 Infinity equipped with a DAD detector), with dilution used if needed.

Results were quantified against curve using standards for protocatechuic acid, chlorogenic acid, catechin, caffeic acid, epigallocatechin, vanillin, p-coumaric acid, rutin, ferulic acid, sinapinic acid, quercitrin, spiraeoside and resveratrol. Standards were obtained from either Fisher Scientific or Sigma Aldrich. Data was presented on a dry mass basis.

2.2 Bulk Phenolics for Use in Antimicrobial Screening

To generate phenolic extracts used in the antimicrobial screening, bulk collected samples of both fresh and air-dried husks were used. Samples were freeze dried (LABCONCO Freezone 4.5 at -48 °C/0.046 mbar/72 hours) and ground using a Wiley Mill. Samples were weighed into Soxhlet extraction thimble (40 ml) and Soxhlet extract was completed using 80% ethanol for 6 hours. Folin Ciocalteu assay was used to determine the total phenolics in the samples. The procedures of Shahidi *et al.* (2007) [9] were followed. Briefly, a 1:1 ratio of extract: Foli-Ciocalteu phenol reagent were added to centrifuge tubes and mixed. Saturated sodium carbonate was added and total volume of sample was corrected to 10 ml with deionized water. Tubes were mixed using a Scientific Industries Vortex Genie 2 vortex and then allowed to rest for 45 minutes. Samples were centrifuged for 5 minutes at 4000 g (Thermo Scientific Sorvall ST40R). Samples were read using a spectrophotometer (725nm; Thermo Scientific Evolution 60S) against a standard curve for gallic acid.

2.3 Bulk Oil for Use in Antimicrobial Screening

Hazelnuts clusters were hand-collected from wild American hazelnuts grown under cultivated conditions. The nuts were husked using a bucket husker and shelled using Drill Cracker (see Fischbach and Brasseur for design specifics [10]). Nuts were cold-pressed slowly (to avoid heating the oil) using a hand-powered oil press (Piteba, Scheermda, Netherlands). Samples were centrifuged at 4000 g for 5 minutes and supernatant oil was poured off for use. Samples were stored in -20 C freezer until needed.

2.4 Antimicrobial testing

Microbial strains and growth conditions. The bacterial and yeast strains used in this study were from the UW-Stevens Point culture collection and American Type Culture Collection (Table 2). Bacteria were maintained on Trypticase Soy Agar medium. Yeast were maintained on Sabouraud Dextrose Agar medium. Stock cultures of bacteria and yeast were kept on slants of media at 4 °C throughout the study. For testing, the oil was mixed 1:1 with 10% dimethyl sulfoxide with 0.5% Tween 80. The solutions were sterilized by filtration through a 0.45µm Purodisc® syringe filter. The oil and extracts were tested with two different methods. In both methods, the medium used for bacteria was Mueller Hinton Agar, and the medium used for testing yeast was Sabouraud's Dextrose Agar. A disk diffusion assay was conducted as described by Kalemba and Kunicka [11]. Bacteria were grown overnight on a shaker (180 rpm) at 30 °C in Mueller Hinton broth. Yeast was grown in Sabouraud Dextrose broth. Using a sterile swab, plates were inoculated with bacteria to achieve confluent growth. Twenty microliters of oil mixture or extract was added to sterile Taxo antibiotic disks (6.35 mm) and the disks were placed on the lawn of bacteria. A disk containing 10% DMSO was used as a negative control and a disk containing cinnamon oil mixed 1:1 with DMSO and 0.5% Tween 80 was used as a positive control. Plates were incubated overnight at 35 °C. Testing was also performed with a well assay based on the method of Cui *et al.* [12]. One-tenth ml of overnight culture was added to 10 ml of molten medium (50 °C). The inoculated medium was poured into a sterile petri dish. After the medium solidified, wells were cut from the plate with a 9-mm diameter sterile cork borer. Sixty to seventy microliters of oil or phenolic extract was placed in each well. A control well was filled with either 10% DMSO (for the oil) or 80% ethanol for the extract. Plates were incubated for 1 to 2 days at 35 °C. The Minimum Inhibitory Concentration (MIC) of phenolic extracts was determined using a similar well assay. *Escherichia coli* (ATCC 11775) was grown overnight at in Mueller Hinton broth at 30 °C on a shaker. One hundred microliters of culture were added to 10 ml of molten Mueller Hinton agar medium. After the medium was solidified, wells were created with a sterile 5 mm diameter cork borer. Ten-fold and two-fold and dilutions were made in 80% ethanol of the phenolic extract. Thirty microliters of each dilution were placed individually in wells in the *E. coli* inoculated plate. A solution of 80% ethanol was used as a negative control. Plates were incubated overnight at 35 °C. Zones of inhibition were measured to the nearest 0.5 mm. A zone that was larger than any zone observed in the negative control was interpreted as positive inhibition.

3. Results & Discussion

Fresh husks had significantly higher levels of phenolics than dried (5061±411 and 2058±382, respectively). The main phenolic was rutin (over 40% for both dry and fresh husks; Table 1). The bulk of the remaining phenolics were composed of catechin, quercitrin and spiraeoside. Indeed, for our samples, the glycosides of quercetin (rutin, quercitrin and spiraeoside) represent greater than 60% of the total phenolics identified. Del Rio *et al.* found rutin to be one of the main phenolics in hazelnut skins [12] and Amaral *et al.* found quercitrin (quercetin 3-rhamnoside) to be one of the two main phenolic in European hazelnut leaves [13]. Peev *et al.* showed the majority of phenolics within hazelnut buds to be glycosides of quercetin [15]. While the phenolic profile is somewhat different than European hazelnut, the dominance of glycosides of quercetin was similar between those published for European hazelnuts and our findings for American hazelnuts. No zones of inhibition were observed in the disk diffusion assays with bacteria and yeast tested with hazelnut oil, while the disks with cinnamon oil had large zones of inhibition. No zones of inhibition were observed using the well assay. Several plant oils have been shown to have antibacterial properties in *in vitro* assays [16]. Contrary to a previous report [6], we observed no evidence of antimicrobial activity of American hazelnut oil. It is possible that there are antimicrobial compounds in the oil, but that they are below the detection ability of the assays used. There also could be unidentified differences in the genotypes of the European and American hazelnut cultivars or in the cultivation methods used to produce these crops. This merits further research. By comparison, the extracts showed moderate to strong inhibition of 10 of the 16 bacterial specimens tested (Figure 1, Table 3). Two species (*Bacillis subtilis* and the ATCC strain of *Staphylococcus aureus*) were slightly inhibited. The MIC was determined for *Escherichia coli* and was found to be 1074 mg/L as gallic acid equivalent (GAE). Many studies have focused on the antimicrobial effects of phenolic compounds from plant and fungal sources [17-21]. Our results show that hazelnut husk extracts have the ability to inhibit bacteria. Our data indicate that Gram-negative bacteria were more sensitive to the husk extract than the Gram-positive bacteria, as

demonstrated by larger zones of inhibition. These findings were consistent with results of a study of phenolic extracts of olives [18]. However, a paper by Rauha *et al.* [20] tested various phenolic compounds individually and found that Gram-positive bacteria (species of *Staphylococcus* and *Bacillus*) were more sensitive to quercetin than the Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). Glycosides of quercetin were the prominent type of phenolic compound in the hazel extracts we investigated. We noted that *Staphylococcus aureus* showed a 2-phase zone with an inner ring that was clearer than that of the zone seen in Gram-negative bacteria. This could suggest more than one mechanism by which phenolic compounds in the extract inhibited the bacteria. The extracts did not appear to inhibit yeast (Table 2). The antimicrobial properties of husk extracts shown by this study could provide growers with a use for hazelnut crop residues and could be developed into potential products with additional research.

3.1 Tables and Figures

Table 1: Polyphenol extraction of dry and fresh American hazelnut husks. Note: caffeic, epigallocatechin, vanillin and resveratrol were not identified in these samples

Sample	Classification	Dried	Fresh
n		3	6
Protocatechuic	hydroxybenzoic acid	42±19	0
Catechin	flavanol	220±46	927±221
p-Coumaric	hydroxycinnamic acid	0	23±23
Chlorogenic	hydroxycinnamic acid	60±18	338±137
Ferulic	hydroxycinnamic acid	79±29	81±81
Sinapinic	hydroxycinnamic acid	0	470±24
Rutin	glycoside of quercetin	838±321	2237±347
Quercitrin	glycoside of quercetin	354±116	553±269
Spiraeoside	glycoside of quercetin	507±65	431±63
Total		2058±382	5061±411

Table 2: Microorganisms used in this study

Microorganism	Source
Gram-positive bacteria	
Bacillus subtilis	UWSP teaching collection
Enterococcus faecalis	UWSP teaching collection
Staphylococcus aureus	UWSP; ATCC 12600
Staphylococcus epidermidis	UWSP; ATCC 12228
Staphylococcus saprophyticus	ATCC 15305
Gram-negative bacteria:	
Alkaligenesfaecalis	UWSP
Escherichia coli	ATCC 11303; 11775
Klebsiella pneumoniae	UWSP; ATCC 13883
Proteus vulgaris	UWSP
Pseudomonas aeruginosa	UWSP
Salmonella cholerasuis	UWSP
Serratia marcescens	UWSP
Fungi (yeast)	ATCC 18804
Candida albicans	UWSP teaching collection;
Saccharomyces cereviseae	ATCC 18804
	ATCC 9763, ATCC 9080

Table 3. Inhibition of bacteria by hazelnut extracts. Zones were measured to the nearest 0.5 mm and included the diameter of the well (9 mm). The difference in diameter between the extract zone and the ethanol control solution zone is shown in parentheses. A "o" indicates no zone of inhibition or a zone not larger than the ethanol control (0.5 mm). All results are based on at least 2 replicates except where noted (*)

Microorganism	Inhibition
Gram-positive bacteria	
Bacillus subtilis UWSP	+/– (1-1.5)
Enterococcus faecalis UWSP	o
Staphylococcus aureus UWSP	+ (11-12)
Staphylococcus aureus 12600	+/– (1.5-2)
Staphylococcus epidermidis UWSP*	++ (21)
Staphylococcus epidermidis ATCC 12228	+ (6-7.5)
Staphylococcus saprophyticus ATCC 15305	+ (3.5-4)
Gram-negative bacteria	
*Alkaligenesfaecalis*UWSP	++ (19.5)
Escherichia coli UWSP	+ (10.0)
Escherichia coli ATCC 11775	+ (4-5)
Klebsiella pneumoniae UWSP	++ (10.5-15)
Klebsiella pneumoniae ATCC13883	++ (19-19.5)
Proteus vulgaris UWSP	+ (8)
Pseudomonas aeruginosa	o
Salmonella cholerasuis	o
Serratia marcescens	o
Yeast	
Candida albicans	o
Saccharomyces cerevisiae	o

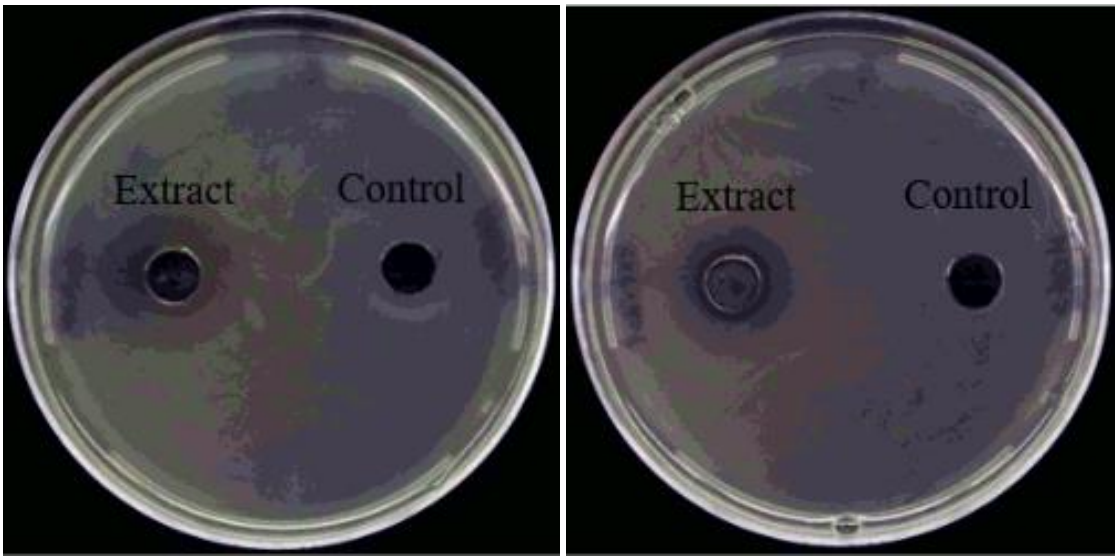

Fig 1: Representative results from the phenolic extract well assay

A) *Escherichia coli* ATCC strain 11303, B) *Staphylococcus aureus* UWSP strain

4. Conclusions

American hazelnut oil showed no zones of inhibition for the organisms tested. The phenolic extracts produced zones of inhibition for eight of the organisms tested. Overall, those Gram-negative bacteria were more sensitive to the husk extract than the Gram-positive bacteria. This exploratory project suggests potential for hazelnut husk extract to be used as an antimicrobial compound against some bacteria.

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