Agriculture and Food

AIMS Agriculture and Food, 5(2): 218–232.
DOI: 10.3934/agrfood.2020.2.218
Received: 26 December 2019
Accepted: 23 April 2020
Published: 18 May 2020

http://www.aimspress.com/journal/agriculture

Research article

Pecan shell by-products—phenolic compound contents and antimicrobial properties

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Abstract: Pecans are a popular nut throughout the world. The USA produces several million kg/yr of pecan kernels and shells. Pecan kernels have high phenolic compound content and pecan shells have even higher phenolic concentrations than the kernels. High phenolic contents in biological materials have been linked to high antioxidant and antimicrobial activity. If pecan shells could be shown to have good antimicrobial potential, then it would demonstrate possible alternative uses for this by-product of pecan production. The total phenolics, flavonoids, and phenolic acid contents were determined for native pecans from Central Texas. Then, the *in vitro* antimicrobial activity of pecan shell water extracts was determined for four microbes (*Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus mutans*) and general oral cavity bacteria. The total phenolic content of the shells was 60% higher and the total flavonoid content of the shells was five times higher than the kernels. The pecan shells contained gallic, vanillic and caffeic acid. Water extracts from pecan shells inhibited the growth of the bacteria studied, and inhibited the growth of oral cavity specimens. Overall, the pecan shell water extracts showed good potential for antimicrobial activity.

Keywords: pecans; shells; phenolic compounds; antimicrobial activity

1. Introduction

Pecan (*Carya illinoinensis*) nuts are one of the most consumed tree nuts in the USA, and they are becoming increasingly popular throughout the world. In 2014, the USA production of pecans was reported to be 463 million kg of pecan meat plus 119 million kg of unshelled whole pecans [1]. The shell-out percentage is the weight of nut meat yield relative to the weight of the whole pecan, and whole pecan nuts typically have a shell-out percentage of around 57% [2]. At a 57% shell-out percentage, the USA production of pecan shells would be around 400 million kg/yr, and this abundant resource currently has relatively low value.

Nuts contain many phytochemicals, including phenolic compounds that promote health including cardiovascular health [3,4]. Pecan kernels contain the highest antioxidant content from tree nuts [5]. Nut by-products also have high levels of phenolic compounds and antioxidant activity [6]. Pecan shell acetone extracts were shown to have phenolic and flavonoid concentrations that were 5 to 20 times higher than the pecan kernel [7]. Phenolic compounds contribute to the total antioxidant capacity of plant materials [8], plus antifungal, antimutagenic, and antiglycemic properties [9]. Pecan shell extracts with high levels of beneficial compounds, such as phenolics and flavonoids, have been shown to also have high antioxidant activity [7,10]. Human consumption of phenolic compounds has also been reported to have antimicrobial benefits [11]. Therefore, further study of the antimicrobial activity of pecan shell extracts is warranted.

Escherichia coli, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus mutans* are common pathogens that create problems for human health, so they were selected as the targeted microbes to investigate in this study. *E. coli* is an important cause of urinary tract, bloodstream, and surgical site infections, as well as pneumonia [12]. *P. aeruginosa* is frequently found in hospital patients, and it causes a variety of diseases within healthcare facilities [13]. *S. aureus* is a major concern because it causes airborne infections in medical facilities [14]. *S. mutans* is a primary source of periodontal disease. Poor oral health is usually a result of either periodontal disease or dental caries, and these are affected by the foods we eat [15]. Almost all adults have at least one of these oral diseases.

The objectives were to determine total phenolic, flavonoid, and some phenolic acid contents of the pecan kernels and shells used in this study, and determine the *in vitro* antimicrobial activity of water extracts from pecan shells on four targeted microbes (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus mutans*), and on general oral cavity bacteria.

2. Materials and methods

2.1. Chemicals used

All the standards used in this study had a purity over 95%. *p*-Coumaric acid, ferulic acid, syringic acid, vanillic acid, caffeic acid, Folin-Ciocalteu's phenol reagent, quercetin, hydrochloric acid (37%), sodium carbonate, and aluminum chloride hexahydrate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Gallic acid, sodium hydroxide, and methanol were obtained from Merck Chemical Co. (Darmstadt, Germany). The *n*-hexane was obtained from ALPS Chemical Co. (Hsinchu, Taiwan). Sodium sulfate and diethyl ether were obtained from Nihon Shiyaku Reagent (Tokyo, Japan). Ethylene dinitrilo tetra-acetic acid (EDTA) and acetic acid were obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ, USA). Ascorbic acid was obtained from Wako

Pure Chemical Industries, Ltd. (Osaka, Japan). Ethyl acetate was obtained from Lab-Scan Chemical Co. (Patumwan, Bangkok, Thailand).

The positive comparison standards for antibiotics, Tetracycline hydrochloride and Polymyxin B (purity over 95%), were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ethanol absolute, Agar-agar, Nutrient broth, and Tryptic soy broth were obtained from Merck Chemical Co. (Darmstadt, Germany). Difco™ Plate Count Agar (PCA), Bacto™ Brain Heart Infusion (BHI), and Mueller Hinton Broth were obtained from Becton, Dickinson and Co. (Sparks, MD USA).

2.2. Test materials

The test materials were pecan kernels and shells from pecans harvested in 2015 from eight mature native pecan trees (*Carya illinoinensis*) in central Texas. No fertilizers, pesticides or other chemicals were applied to the trees. All the samples were prepared in triplicate. The harvested pecans were immediately air-dried indoors and shelled. The pecan shells and kernels were ground into a powder form. The powder was passed through an 18-mesh (1000 μm) sieve. Before extracting phenolic compounds, the fatty acids were removed from the pecan samples according to the procedures in Chang et al. [16].

2.3. Phenolic analysis

Phenolic compounds or flavonoid compounds are present in plant material in different forms and are extracted by different extraction methods. The total phenolic or flavonoid compound values are determined by summing the content values of these compounds that are determined by these different extraction methods. In this paper: (the total phenolic or flavonoid compound extraction values) = (water soluble, non-bounded compound extraction values) + (bound compound values extracted by alkaline hydrolysis) + (bound compound values extracted by acid hydrolysis). The extraction of the phenolic compounds was conducted according to the methods of Krygier et al. [17] and detailed procedures are in Chang et al. [16].

Removal of fatty acids: Prior to extractions of phenolic compounds, the fatty acids were removed from the samples. Samples of the powders were weighed (0.5 ± 0.0001 g each) and placed into 50 mL centrifuge tubes. Fifteen mL of n-hexane and 5 drops of 10 M sodium hydroxide were added to the tubes and subjected to ultrasound vibrations (Leo Ultrasonic, Model LEO-150, Taiwan) for 10 min. The top clear liquid (n-hexane with the fatty acids) was removed. The remaining precipitate (fatty acids were removed by the n-hexane) was the material used for all the following extractions. Each extraction method is described below.

Water soluble (non-bounded type) phenolic compounds extraction: First, 15 mL of 80% methanol was added into the precipitated test material and subjected to ultrasound vibrations for 30 min. Next, the mixture was centrifuged for 15 min at 2500 rpm and the clear solution at the top and the precipitate portions were separately collected. The precipitate portion was further processed as described in the next section. The clear solution was vacuum-concentrated at 35 ℃ until dry and 5 mL of pure methanol was poured into this dried material. Ultrasound vibration was used to dissolve the dried precipitate and it was filtered with 0.45 μm filter membrane (Minisart NML syringe filter). The remaining clear liquid (Test material "I") was placed in a sealed glass bottle (protected from light) and stored at 4 ℃, until it was analyzed for total phenolics, total flavonoid and specific phenolic acids.

Bound phenolic compounds extraction-via alkaline hydrolysis: The precipitate portion obtained from the previous section was the test material used in this extraction. A solution of 10 M sodium hydroxide, 1% ascorbic acid and 10 mL EDTA was premixed and 5 mL was added to the precipitated material in a centrifuge tube. The centrifuge tube head space was displaced with nitrogen gas by injecting it into the head space for 1 min. The centrifuge tube was immediately sealed with a lid coated with para film and it was stored 16 h to allow the alkaline hydrolysis to take place. Next, a 37% concentration of sulfuric acid was added to adjust this solution pH to 1–2, 15 mL of a solution of ether/ethyl acetate (1:1 v/v) was added to extract the phenolic acids. This mixture was centrifuged for 15 min at 2500 rpm and the top clear solution (the desired alkaline hydrolysis bounded phenolic compounds extraction) was placed into a beaker (Beaker A). This process was repeated twice for the bottom precipitate part, and the top clear solution from these two repeated procedures were added into Beaker A. The precipitated material from these three replications was used as the test material in the next section. The solution in Beaker A was vacuum-concentrated at 35 ℃, until dry and 5 mL of methanol was added. This material was dissolved by ultrasound vibration and the solution was filtered with a 0.45 μm membrane. The clear liquid (Test material "II") was sealed in a bottle and stored at 4 ℃ until it analyzed for total phenolic compound, total flavonoid and specific phenolic acids.

Bound phenolic compounds extraction-via acid hydrolysis: The precipitate portion obtained from the previous section was the test material used for this extraction. This material and 2.5 mL of hydrochloric acid (37% concentration) were added to a centrifuge tube and maintained at 85 ℃ for 30 min in a thermostatic water bath, so acid hydrolysis could take place. Then, 10 M of sodium hydroxide was added to titrate the solution to obtain a pH of 1–2. Next, 15 mL of a solution of ether/ethyl acetate (1:1 v/v) was added to extract the phenolic acids. This material was centrifuged for 15 min at 2500 rpm and the top clear solution (the desired acid hydrolysis bounded phenolic compounds extraction) was placed in a beaker (Beaker B). This process was repeated twice for the bottom precipitate and the top clear solution of this two times repeated procedure was added to Beaker B. The solution in Beaker B was vacuum-concentrated at 35 ℃ until dry, then 5 mL of methanol was added, and it was dissolved with ultrasound vibration and was filtered with a 0.45 μm membrane. The clear liquid (Test material "III") was sealed in a bottle and stored at 4 ℃, until it was analyzed for total phenolics, total flavonoid and specific phenolic acids.

Determination of total phenolic content: The total phenolic content was determined by the methods presented in Quettier-Deleu et al. [18] and Taga et al. [19], and detailed procedures are in Chang et al. [16].

Part A: First, 0.5 mL of test material "I" was mixed with 0.5 mL of folin-ciocalteu's phenol reagent and 5 mL of 20% sodium carbonate. It was then incubated at room temperature for 30 min. Next, the absorbance of the mixture was measured with a spectrophotometer (Metertech, Model SP-830, Taiwan) set at 750 nm. The total phenolic content values were determined from the regression curve developed in part C of this section. Total phenolic content results were presented with the standard expression of mg Gallic Acid Equivalent (GAE)/g dry weight of test material;

Part B: The same procedures given in Part A were followed for test material "II" and for Test material "III";

Part C: To obtain the gallic acid standard curve, readings were determined for five standard solutions of gallic acid (50, 100, 200, 400 and 500 ppm) using the procedure in Part A. Then, the linear regression standard curve was developed.

Determination of total flavonoid content: Total flavonoid content extraction was done according to the methods presented in Quettier-Deleu et al. [18], and detailed procedures are in Chang et al. [16].

Part A: To start, 0.5 mL of test material "I" was mixed with 0.5 mL of 2% aluminum chloride hexahydrate, then incubated at room temperature for 10 min. Next, a spectrophotometer (Metertech, Model SP-830, Taiwan) set at 430 nm measured the absorbance of the mixture and the total flavonoid values were determined from the regression curve developed in part C. A blank test (with methanol) was also done prior to sample measurements to zero the device. The flavonoid content results were presented using the standard expression of mg Quercetin Equivalent (QE)/g dry weight of test material;

Part B: The same procedures described in Part A were used for test material "II" and test material "III";

Part C: To obtain the quercetin standard curve, spectrophotometer readings were obtained for 6 standard solutions of quercetin (1, 5, 10, 25, 50, 100 ppm) by using the process in part A. The linear regression standard curve was determined from these values.

Determination of phenolic acids: The content of p-coumaric acid, gallic acid, syringic acid, caffeic acid, ferulic acid and vanillic acid were determined by HPLC analysis with the methods presented in Rao and Muralikrishna [20], and detailed procedures are in Chang et al. [16]. The HPLC had a C-18 column (4.6 H 250 mm, 5 mm; Phenomenex, Inc., USA) and column temperature was set at 50 ± 1 ℃. It had a UV-VIS detector (UV-2070 Plus, JASCO, Japan) with a chromatography data handling system (Peak-ABC Software). There were 2 mobile phases and the flow rate of each was set to 1 mL/min. The injection volume of each test sample was 20 mL and the ultraviolet detector wavelength was 280 nm. For mobile phase 1, the elution solvent was 4% acetic acid that was dissolved in water. For mobile phase 2, the elution solvent was a lab analytical methanol solution. A gradient elution was used and the initial gradient was 92% of the mobile phase 1 solution and 8% of the mobile phase 2 solution. These values were gradually changed to 75% of the mobile phase 1 solution and 25% of the mobile phase 2 solution, over a period of 0–15 min. Then, the gradients remained at 75% of mobile phase 1 and 25% of mobile phase 2 for the next period of 15–40 min. Next, the gradients gradually changed back to 92% of the mobile phase 1 and 8% of the mobile phase 2 for the last stage of 40–70 min.

2.4. Antimicrobial analysis

Extracts from the powdered (<1000 μm) pecan shells were prepared for the antimicrobial research as follows. For the hot water extraction (HWE), 15 g of the powdered pecan shells were macerated by placing them in separate containers with 90 mL of distilled water. These 16.7% (w/v) samples were sterilized in an autoclave at 121 ℃ for 30 min; then the mixture was filtered through Whatman filter paper (NO.1). The filtrate was concentrated with a freeze dryer, and stored at 4 ℃. For the ultrasonic-assisted water extract (USE), 15 g of pecan shell samples were placed in individual sealed flasks with 90 mL (16.7% w/v) of water at room temperature and soaked for 60 min. During this period, the flasks were placed in a Bransonic CPX8800H Ultrasonic bath (Emerson), that was operated at 40 kHz ± 6% and 280 W. Then, the mixture was filtered through Whatman filter paper (NO. 1). The filtrate was concentrated with a freeze dryer, and stored at 4 ℃. When each of these prepared samples were needed for the antimicrobial tests, the stock was diluted with distilled

water to a concentration of 200 mg/mL. These mixtures were analyzed for the comparison between the hot water extraction (HWE) and the ultrasound-assisted extraction (USE) treatments.

2.4.1. Bacterial strains and growth conditions

Four bacterial strains (two gram-negative and two gram-positive) were used for antibacterial testing. These microorganisms were acquired from the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (FIRDI, Hsinchu, Taiwan). The gram-negative bacterial strains were: *Escherichia coli* (BCRC 11634) and *Pseudomonas aeruginosa* (BCRC 11633). The gram-positive bacterial strains were: *Streptococcus mutans* (BCRC 10793) and *Staphylococcus aureus* (BCRC 10451). The cultivation and assay medium was nutrient broth/agar for *E. coli*, brain heart infusion broth/agar for *S. mutans*, and tryptic soy broth/agar for *P. aeruginosa* and *S. aureus*. The bacteria were cultured according to the procedures described in Huang et al. [21].

2.4.2. Antimicrobial analyses methods

Two types of antimicrobial activity tests were done on each of the four bacteria strains. The Agar Dilution Method determined the Minimal Inhibitory Concentration (MIC) of antimicrobial materials. MIC is the lowest concentration of the antimicrobial material that will inhibit the visible bacterial growth. The Disk Diffusion Method measured the ring diameter that forms around a colony after treatment. If no ring develops, then there was no antimicrobial activity. If a ring develops, then the size of the ring indicates the degree of antimicrobial activity. The Agar Dilution Method is more accurate and is considered to be a reference method for other antimicrobial tests [22]; however, the Disk Diffusion Method is more commonly used because it is easier.

Agar dilution method: This method was based on an international standard [23]. The procedures are described in detail in Huang et al. [21]. The MIC was defined as the lowest concentration of pecan shell extract or positive control, where no growth was observed in the solid media [24]. The MIC was determined at various concentrations of pecan shell extracts and positive controls, ranging from 0.391 to 200.00 mg/mL. The stock solution was used to get the desired extract concentrations of 200.0, 100.0, 50.0, 25.0, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391 mg/mL by using the two-fold serial dilutions method. The stock solutions were then mixed with 19 mL of agar to get 11 different levels of extract concentrations in the medium which had a range of 0–10 mg/mL. The agar plate number and the corresponding extract concentration in mg/mL were: 0 = 10, 1 = 5, 2 = 2.5, 3 = 1.25, 4 = 0.625, 5 = 0.313, 6 = 0.156, 7 = 0.078, 8 = 0.039, 9 = 0.019 and 10 = 0. The bacterial suspensions that were developed previously were diluted to 10^6 CFU/mL. Then, 0.1 mL of this diluted bacterial suspension was inoculated onto the solid MHA medium. The agar plates were incubated aerobically for 18–24 h at 37 ℃ for all tested bacterial cultures. The positive control used for the *S. aureus*, *E. coli* and *S. mutans* bacterial cultures was Tetracycline hydrochloride, and the positive control used for the *P. aeruginosa* bacteria culture was Polymyxin B.

Disk diffusion method: This method was based on an international standard [25]. The procedures are described in detail in Huang et al. [21]. The disk diffusion method was used to determine the antimicrobial activity of the two types of pecan shell extracts, two positive controls, and one negative control on the four bacteria strains. The test materials included the two types of

pecan shell water extracts, a positive control (either Tetracycline hydrochloride or Polymyxin B), and one negative control (sterile water) [26,27]. The positive control used for the *S. aureus, E. coli* and *S. mutans* bacterial cultures was Tetracycline hydrochloride, and the positive control used for the *P. aeruginosa* bacteria culture was Polymyxin B. The concentration of the pecan shell extracts was 200 mg/mL and the concentration of the positive controls was 200 μg/mL. The negative controls were paper disks loaded with 0.05 mL of sterile water.

The inoculum for each tested strain developed earlier was diluted around 100 times to obtain a bacterial suspension of around 1.5×10^6 CFU/mL. The inoculum suspensions were spread evenly over the nutrient agar surface (Mueller-Hinton Agar, MHA) and incubated for 48 h at 37 ℃ (Shaker Incubator, COCONO LM-590).

The 8 mm diameter disks were first sterilized at 121 ℃ for 15 min in an autoclave. The disks were loaded with 0.05 mL of one of the antimicrobial test materials. The concentration of the pecan shell extracts was 200 mg/mL and the concentration of positive controls were 20 mg/mL.

The disks were then dried for 5 min and placed onto the surface of the inoculated plates with sterile forceps. Each disk was pressed down to ensure complete contact with the agar surface. The disks were placed a suitable distance apart and not moved once making contact with the agar surface. The plates were incubated for 24 h at 37 ℃. The diameter of the Zone of Inhibition (ZI) of bacterial growth around each disk was measured.

2.4.3. Antimicrobial activity on oral cavity bacteria

The total colony count method was used to determine if the pecan shell extracts have any potential for treating general oral health problems. Bacterial samples were obtained from volunteers about 1~2 h after they ate a meal by swabbing the buccal mucosa with sterilized cotton. No human subjects review was required for this study. The samples were immediately placed into transport fluid (6 mL of Nutrient broth) for 5 min. Then 1.0 mL of the oral cavity specimens and 1.0 mL of either pecan shell extract sample or negative control (sterilized water) were placed in a tube and incubated at 37 ℃ and 100 rpm for 1, 20, 60, and 1440 min. After incubation, a set of serial dilutions was made with phosphate buffer saline (PBS), and a sample of each dilution was placed into a non-selective liquefied agar medium, which was immediately poured into Petri dishes. The plates were rotated to mix the dilution and agar, and the agar was allowed to solidify at room temperature. This produced a set of pour plates from the various dilutions to allow accurate counting of the microorganisms. Next, the plates were inverted and incubated for 48~72 h at 37 ℃. After incubation, all visible growth colonies were counted. Only the plates with 30 to 300 colonies were counted. CFU/mL was calculated by using the standard formula (a) presented in Lubrizol Advanced Materials, Inc. [28] and Reynolds [29]: CFU/mL = ((Colony Forming Units, CFUs, on an agar plate)/(Total dilution of tube)) x (Volume plated).

2.5. Statistical analysis

Samples were prepared in triplicate. For total phenolics, flavonoids, phenolic acids, and ZI there were three replicates of the analysis. The significant differences between the means of treatments were determined by one-way analysis of variance (ANOVA). They were further analyzed with Tukey HSD test and Duncan's multiple range test to determine significant differences between the

means. Analyses were done using SAS (SAS 9.3, SAS Institute Inc., Cary, NC). For the MIC and oral cavity tests, the standard tests were conducted on one sample so no statistical analysis was conducted.

3. Results

3.1 Phenolic contents of pecan shells and kernels

Table 1 presents the total phenolic content found for the pecan shells (10.2 ± 0.15 mg Gallic Acid Equivalent (GAE)/g dry weight (d.w.)) and kernels (6.4 ± 0.40 mg GAE/g d.w.). The pecan shells had significantly greater ($P < 0.05$) total phenolic content than did the pecan kernels. Table 1 also presents the total flavonoid content found for the pecan shells (12.6 ± 0.03 mg Quercetin Equivalent (QE)/g d.w.) and kernels (2.5 ± 0.12 mg QE/g d.w.). The total flavonoid content of the pecan shells was significantly greater ($P < 0.05$) than for the pecan kernels. Table 2 presents the results of the specific individual phenolic acids contents. The only measurable phenolic acids found in pecan shells were gallic acid at 5.5 mg/100 g d.w., vanillic acid at 2.6 mg/100 g d.w., and caffeic acid at 4.9 mg/100 g d.w.

Table 1. Total phenolic contents and flavonoids in four samples of pecan shells and kernels, as determined by different extraction methods.

	Total Phenolics (mg GAE/g d.w.)		Flavonoids (mg QE/g d.w.)	
	Pecan Shell	Pecan Kernel	Pecan Shell	Pecan Kernel
Water soluble	0.8 ± 0.09[a]	0.8 ± 0.04[a]	N.D.	N.D.
Insoluble-bound (alkaline hydrolysis)	8.0 ± 0.12[a]	5.2 ± 0.31[b]	12.6 ± 0.03[a]	2.5 ± 0.12[b]
Insoluble-bound (acid hydrolysis)	1.4 ± 0.09[a]	0.4 ± 0.07[b]	N.D.	N.D.
Total	10.2 ± 0.15[a]	6.4 ± 0.40[b]	12.6 ± 0.03[a]	2.5 ± 0.12[b]

Values are expressed as mean ± standard deviation, n = 3. [a–b]When comparing total phenolic and flavonoid values in the same row, different letter superscripts indicate significant differences between the means at $P < 0.05$ statistical level. GAE = gallic acid equivalent; d.w. = dry weight; QE = quercetin equivalent; N.D. = not detectable.

Table 2. Phenolic acid (gallic, vanillic and caffeic) contents in pecan shells, as determined by different extraction methods. Levels of syringic acid, p-coumaric acid and ferulic acid were undetectable.

Sample	Etraction Method	Phenolic acids contents (mg/100 g d.w.)		
		Gallic acid	Vanillic acid	Caffeic acid
Water soluble	Methanol extraction	N.D.	N.D.	N.D.
Insoluble-bound	Alkaline hydrolysis	N.D.	N.D.	N.D.
Insoluble-bound	Acid hydrolysis	5.5 ± 1.38	2.6 ± 0.43	4.9 ± 0.78
Total		5.5 ± 1.38	2.6 ± 0.43	4.9 ± 0.78

Values are expressed as mean ± standard deviation, n = 3; N.D. = not detectable.

3.2. Agar dilution method

Table 3 presents results from the Dilution Method as MIC values for each bacteria type and treatment. For *S. aureus*, the MIC for HWE and USE were both 0.625 mg/mL while the positive control (Tetracycline hydrochloride) was better because it controlled bacterial growth at a lower concentration (0.039 mg/mL). For *E. coli*, the MIC for HWE and USE were both 2.5 mg/mL and the positive control (Tetracycline hydrochloride) was better at 1.25 mg/mL. For *S. mutans*, the MIC for HWE, USE and the positive control (Tetracycline hydrochloride) were all 2.5 mg/mL. For *P. aeruginosa*, the MIC for USE was the best of the three treatments at 0.625 mg/mL and HWE was second best at 1.25 mg/mL, followed by the positive control (Polymyxin B) at 2.5 mg/mL. These results showed that the water extracts from pecan shells were effective in inhibiting the growth of all the bacteria studied, even at the lower extract concentrations studied. In fact, they were as good as the positive control for *S. mutans* and better than the positive control for *P. aeruginosa*.

Table 3. Antimicrobial activity for pecan shell extracts and controls as expressed by minimum inhibitory concentration (MIC) as determined by the agar dilution method and as expressed by zone of inhibition (ZI) as determined by the disk diffusion method.

	Bacteria type			
	S. aureus	*E. coli*	*S. mutans*	*P. aeruginosa*
BCRC:	10451	11634	10793	11633
Antimicrobial treatment				
	MIC[1], mg/mL			
Positive control[2]	0.039	1.25	2.50	2.50
HWE[4]	0.625	2.50	2.50	1.25
USE[5]	0.625	2.50	2.50	0.625
	ZI, mm (mean ± standard deviation, n = 3)			
Negative control[3]	--	--	--	--
Positive control[2]	27 ± 0.5[a]	25 ± 3.0	25 ± 1.0	9 ± 0.5[c]
HWE[4]	27 ± 1.0[a]	--	--	9 ± 0.5[c]
USE[5]	21 ± 1.0[b]	--	--	10 ± 1.0[c]

[a–c]The means in a column with different superscript letters are significantly different from each other ($P < 0.05$). [1]Three replications were conducted but all gave the same values, so standard deviation = 0 for all values; n = 3. [2]Positive control was tetracycline hydrochloride for *S. aureus, E. coli* and *S. mutans* and Polymyxin B for *P. aeruginosa.* [3]Negative control = sterile water. [4]HWE = hot water extract of pecan shells. [5]USE = ultrasound water extract of pecan shell. -- = no effect achieved.

3.3. Disk diffusion method

The zone of inhibition for each bacteria strain and treatment is presented in Table 3. The negative control (water) did not restrain any bacterial growth. For *S. aureus*, HWE and the positive control (tetracycline hydrochloride) both had ZI values of 27 ± 0.5 mm. USE was slightly less at 21 ± 1.0 mm. For the *E. coli* and *S. mutans*, the positive control (tetracycline hydrochloride) showed good inhibition with a ZI of 25 ± 3.0 mm, and 25 ± 1.0 mm, respectively. However, HWE and USE showed no inhibition of those two bacterial strains. This result seems to contradict the results from

the MIC tests, where HWE and USE showed a good level of inhibition of *E. coli* and *S. mutans*. One possible explanation is that the treatment concentration level may have been less in the disk diffusion method than for the agar dilution method. In the disk diffusion method, the concentration of the treatments decreases with distance from the disk; however, the gradation in concentration is not precise. For the agar dilution method, the exact dilutions provide precise concentrations in each batch which provides a more accurate assessment. For *P. aeruginosa*, the positive control (polymyxin B) showed inhibition with a ZI of 9 ± 0.5 mm, while HWE and USE were similar with ZI values of 9 ± 0.5 mm and 10 ± 1.0 mm, respectively. Overall, HWE and USE showed similar levels of bacterial growth inhibition as the positive control for *S. aureus* and *P. aeruginosa*, but the disk diffusion method didn't show any growth inhibition of *E. coli* and *S. mutans* from HWE and USE.

3.4. Total bacterium count from the oral cavity tests

Table 4 presents the total bacterium count in colony-forming units (Log CFU/mL) from the oral cavity tests. For the negative control (sterile water), the total bacterium count increased from 6.23 log(CFU/mL) to 7.41 log(CFU/mL) after 60 min of incubation, and eventually increased to 8.00 log(CFU/mL) after 1440 min of incubation. The total bacterium count for the HWE and USE treatments were similar to the negative control for the first 20 min, but they decreased over time until they reached a negligible level at 1440 min. Over time, the pecan shell extracts inhibited the growth of the oral cavity specimens.

Table 4. Total bacterium count from the oral cavity specimens after treatment with the pecan shell extracts and negative control.

Sample	Incubation time (min)	Cell number (CFU/mL)	Log of cell number Log(CFU/mL)
Negative Control[1]	1	1.70E + 06	6.23
	20	1.60E + 06	6.20
	60	2.60E + 07	7.41
	1440	9.90E + 07	8.00
HWE[2]	1	6.10E + 05	5.78
	20	2.99E + 06	6.48
	60	4.30E + 05	5.63
	1440	TFTC[4]	TFTC[4]
USE[3]	1	1.20E + 06	6.08
	20	2.20E + 06	6.34
	60	4.70E + 05	5.67
	1440	TFTC[4]	TFTC[4]

Negative control = sterile water; HWE = hot water extract of pecan shells; USE = ultrasound extract of pecan shells; TFTC = Too few to count.

4. Discussion

4.1. Phenolic contents of pecan shells and kernels

Other studies have reported total phenolic content for pecan shells and kernels for a variety of cultivars, locations, extraction methods and growth conditions [6,30,31]. Their values were expressed in a variety of forms which makes it difficult to make direct comparisons of values. Some studies reported the values in units of fresh weight without reporting moisture contents or defatted weight, or they used different methods for extracting the phenolic compounds. However, they all found pecan shells to have higher contents than the kernels, as did the current study. De La Rosa et al. [30] reported the total phenolic content of pecan shells and kernels for different regions of Mexico and found a range of 24.7 to 54.3 mg GAE/g for shells and 8.3 to 9.6 mg GAE/g for kernels. The southern regions had higher values than the northern regions. De La Rosa et al. [6] reported total phenolic compounds of pecan shells and kernels for different regions of the state of Chihuahua, Mexico and found a range of 65.3 to 92.5 mg GAE/g fresh weight (f.w.) for shells and 11.7 to 12.5 mg GAE/g f.w. for kernels. Villarreal-Lozoya et al. [31] reported total phenolic compounds of pecan shells and kernels for seven cultivars of pecans and reported an average of 76 ± 1.9 mg CAE (Chlorogenic Acid Equivalents)/g of defatted kernels and 448 ± 45 mg CAE/g of defatted shells. For direct comparison, one would need to make assumptions for the conversion of CAE to GAE and to convert defatted samples to samples based on dry weight.

De La Rosa et al. [30] reported total flavonoids of pecan shells and kernels for different regions of Mexico and found a range of 16.3 to 32.9 mg GAE/g sample for shells and 3.5 to 4.0 mg GAE/g sample for the kernels. In an earlier study, De La Rosa et al. [6] reported total flavonoids of pecan shells and kernels for different regions of the state of Chihuahua, Mexico and found a range of 26.3 to 36.1 mg GAE/g f.w. for shells and 5.8 to 6.4 mg GAE/g f.w. for the kernels. These studies, as well as the current study, found that pecan shells had much higher total flavonoids content than the kernels.

Pinheiro do Prado et al. [31] found gallic acid in pecan shells in the range of 125 to 829 μg/mL, depending on the type of extraction method used. De la Rosa et al. [6] detected the presence of gallic acid in pecan shells. The current study found vanillic acid and caffeic acid in addition to gallic acid in pecan shells. No other literature was found on other individual phenolic compounds in pecan shells or kernels.

4.2. Antimicrobial activity

The results of the current study indicate that there are good levels of many beneficial constituents in pecan shells. Phenolic compounds and flavonoids were reported to have many benefits for humans including antioxidant and antimicrobial benefits [11]. Villarreal-Lozoya et al. [32] showed a strong correlation between the total extractable phenolic content of pecan kernels and antioxidant capacity.

In general, the pecan shell water extracts from the current study indicated the potential for antimicrobial activity. The pecan shell extracts were shown to inhibit the growth of *S. aureus*, *E. coli*, *S. mutans*, and *P. aeruginosa*. *P. aeruginosa* and *S. aureus* are normal skin flora. *E. coli* is a normal

intestinal parasite. *S. mutans* is the most important bacterial cause of tooth decay and periodontal disease.

Overall, these results showed that water extracts from pecan shells have the potential for antimicrobial activity. Pecan shells have high levels of phenolics and flavonoids, but other chemicals could also contribute to antimicrobial activity, so more research is needed to determine if other chemicals also contribute. Further research on pecan shells is needed to determine their antimicrobial potential for other types of microorganisms. This study was for water extracts from pecan shells, and it is likely that other extraction methods may yield a stronger effect. Kaur et al. [33] showed that methanol, acetone, ethyl acetate, petroleum ether and chloroform extracts from the plant, *Parthenium hysterophorus* L., had much higher antimicrobial inhibition on *S. aureus*, *E. coli*, and *P. aeruginosa* than did water extracts. More research is needed to determine the best method of extracting the antimicrobial constituents of the pecan materials and how to use them to benefit society.

These results are only for a limited set of conditions for which the pecan shells were grown and handled prior to analysis. Samuelsson and Bohlin [34] reported that the levels of active constituents in plants could be affected by growth location, time of harvest, and storage conditions. Nahak et al. [35] reported that light intensity, season, climate, and temperature during growth and the extraction methods used could contribute to the wide variation in antioxidant activities and total phenols found in plant materials by various researchers. Chang et al. [36,37] reported that the time of day that plants are harvested might affect levels of some chemicals. Rezazadeh et al. [38] found that soil conditions could affect phenolic levels and antioxidant activity of plants. Additional research is required to determine factors that affect chemical levels in plant materials. Research should also be done on possible negative effects of pecan shell extracts, such as potential toxicity at certain levels.

5. Conclusions

The total phenolics, flavonoids, and phenolic acid contents were determined for native pecans from Central Texas and the *in vitro* antimicrobial activity of pecan shell water extracts was determined for four microbes (*Pseudomonas aeruginosa, Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus mutans*), plus general oral cavity bacteria. The total phenolic content of the shells was 60% higher and the total flavonoid content of the shells was five times higher than the kernels. Water extracts from pecan shells inhibited the growth of the bacteria studied, and inhibited the growth of oral cavity specimens. Overall, the pecan shell water extracts showed good potential for antimicrobial activity. The pecan shells contained gallic, vanillic and caffeic acid.

Acknowledgments

The authors wish to acknowledge the Ministry of Science and Technology of Taiwan for partially funding this research under Project Number 103-2918-I-197-004.

Conflict of interest

The authors declare no conflict of interest.

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