In vitro antibacterial activity of nut by-products against foodborne pathogens and their application in fresh-cut fruit model

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Revised: 24 July 2018 / Accepted: 31 July 2018 / Published online: 20 August 2018
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Abstract Aqueous extract of nut by-products (cashewnut shell, coconut shell, and peanut hull) were studied for their physicochemical properties, antibacterial activity and food preservation potential in an artificially inoculated fresh-cut fruit (papaya) model. Physicochemical characteristics revealed the colour, odor, nearly neutral pH (6.67–6.83), high water solubility (69.18–82.63%) and total phenolic content (1130.54–2403.41 mg GAE/100 g) of the extracts. The antibacterial property of the extracts evaluated by zone of inhibition assay revealed that cashew nut shell extract had a strong inhibition effect on *Escherichia coli* (18 mm), *Listeria monocytogenes* (18 mm), and *Salmonella enterica* (16 mm). Food preservative effect of extracts was examined in an artificially inoculated fresh-cut papaya model, and both cashewnut and coconut shell extracts significantly reduced the population of the above mentioned foodborne pathogens. However, when compared to coconut shell extract, the application of cashewnut shell extract was found to affect the sensory property of the fresh-cut fruit as darkening of the cut fruit was observed. So, the coconut shell extract could be considered as a natural source of antibacterial agent for food preservative applications.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13197-018-3373-x>) contains supplementary material, which is available to authorized users.

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Phytochemical investigation through LC–MS/MS technique revealed that luteolin as the major constituent of coconut shell extract.

Keywords Nut by-product · Extract · Polyphenols · Antibacterial · Foodborne pathogens

Introduction

Fresh-cut fruits and vegetables (FFV) is a fast growing sector due to their convenient usage and amounts to multi-billion dollar industry worldwide (Bhagwat 2006). The growing consumers' demand for safe food that can maintain their health has improved the consumption of FFV (Park et al. 2011). But, FFV is more prone to contamination with diverse pathogenic microorganisms, such as *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, *Shigella flexneri* and *Listeria monocytogenes* (Prakash et al. 2018). Several outbreaks associated with consumption of FFV have been reported in the past (Franz and Bruggen 2008). Various sanitizers such as chlorine dioxide (ClO₂), hydrogen peroxide (H₂O₂) and ozonized water have been used for enhancing microbiological safety of FFV. But the use of chlorine-based sanitizers is likely to produce toxic chemicals such as trihalomethanes with organic matter which are considered as carcinogens (Gomez-Lopez et al. 2013). Therefore, there is a continuous demand from consumers for safe and natural preservatives.

In recent years more attention has been shifted on plant extracts, which have been used for centuries to improve the shelf-life of foods (Fernandez-Gines et al. 2005). Agro-food by-products could be exploited as a natural source of phytochemicals for food preservation (Vadivel and Brindha

2015; Vadivel et al. 2017). In India, agro-food by-products such as shell of cashew nut (*Anacardium occidentale* L.), the shell of coconut (*Cocos nucifera* L.) and hull of peanut (*Arachis hypogaea* L.), are locally available in large scale. Even though the chemical constituents and some minor uses of these by-products have been reported earlier, their food preservation applications have seldom been investigated. Hence, in the present work we have attempted to reveal the protective effect of nut by-product extracts against the foodborne pathogens in fresh-cut fruit (papaya) model and also to study the phytochemical constituents of the active extract.

Materials and methods

Preparation of extracts

Nut by-products (cashew nut shell, coconut shell, and peanut hull) collected from the local food processing industries were shade dried and then powdered to 1 mm particle size using a lab mill. The extract was prepared by taking 100 g powdered samples (cashew nut shell, coconut shell, and peanut hull) with 1000 ml of distilled water and kept on an orbital shaker at 500 rpm for 3 h at 70 °C. The contents were then filtered, dried and re-dissolved in distilled water.

Characterization of extracts

The colour and odor of the dried extracts were assessed based on sensory evaluation carried out by a panel of untrained 15 volunteers from School of Chemical and Biotechnology, SASTRA Deemed University. The pH of the sample was measured using pH meter while the water solubility of the extracts was analyzed according to the method described by Joshi and Aeri (2009).

Total phenolic content of nut by-product extracts was analyzed using Folin–Ciocalteu reagent method with some modifications (Singleton et al. 1999). The extract (100 µl) was added to 250 µl of Folin–Ciocalteu reagent and vortexed for 1 min. Then, 1.0 ml of 5% sodium carbonate solution was added, and the mixture was vortexed again for 1 min. The samples were incubated at room temperature for 30 min in dark and the absorbance was read at 720 nm against the blank using Spectrophotometer (Make: Perkin-Elmer, Model: Lambda-365). The results were expressed as gallic acid equivalents (mg GAE/100 g).

Antibacterial activity

Antibacterial activity of the nut by-product extracts was evaluated against selected Gram-negative bacteria like

E. coli (ATCC 25922) and *S. enterica* (ATCC 13311) and the Gram-positive bacteria *L. monocytogenes* (ATCC 7644). *Escherichia coli* and *S. enterica* were maintained in Nutrient Agar medium while Brain–Heart–Infusion agar medium was used for *L. monocytogenes*. Antibacterial activity of the extracts were determined by agar well diffusion method as described by of Nithyanand and Pandian (2009) and the minimum inhibitory concentration (MIC) by broth microdilution method in a 96 well microtiter plate.

Food preservation studies

Escherichia coli, *S. enterica*, and *L. monocytogenes* were separately cultured over-night in the medium at 37 °C. Then all cultures were centrifuged at $4000 \times g$ for 10 min and the pellets were washed twice with 0.9% saline solution. Then, all these bacterial species were combined to construct a cocktail with approximately 10^5 – 10^6 CFU/ml. Fresh papaya fruits purchased from a local market were cut into small pieces ($3 \times 3 \times 3$ cm) under aseptic conditions. Later, 0.1 ml of the mixed strain cocktail with the initial inoculum level of 10^5 CFU/g was added to 25 g of fresh-cut fruit (Choi et al. 2015) and kept at room temperature in a laminar flow for 30 min to allow attachment.

Aqueous extract of cashewnut shell, coconut shell and peanut hull (1%, w/v) and 0.5% citric acid as positive control were used for the treatment. Inoculated fresh-cut papaya was randomly divided into five groups. Group-I was untreated control, group-II was treated with 0.5% citric acid, group-III in 1% cashewnut shell extract, group-IV in 1% coconut shell extract and group-V in 1% peanut hull extract. Each group of inoculated fruit sample was dipped in the respective solutions for 5 min and allowed to dry in laminar air flow for 30 min. Then the samples were aseptically packed in a sterile light-density poly-ethylene material (15×20 cm) and stored at 5 °C for 10 days and analyzed for the microbial count at regular time intervals. For counting of pathogens at different time intervals of the storage period, inoculated fruit samples (10 g) were aseptically homogenized with 50 ml sterile 0.9% (w/v) saline solution for 2 min using a tissue homogenizer (Make: Remi, Model: RQ127 A/D). Then the sample was serially diluted and plated onto Hicrome coliform agar medium to enumerate *E. coli*, *Salmonella* differential agar medium for *S. enterica* and *Listeria* selective agar medium for *L. monocytogenes*. All plates were incubated at 37 °C for 24 h, and then typical colonies were counted.

Sensory analysis was carried out for evaluating the overall quality of control and treated fresh-cut fruit samples with nut shell extracts as described in the previous section. The prepared samples were presented in random order to the panel of 15 members from School of Chemical and Biotechnology, SASTRA Deemed University at different

time intervals of storage period. Only the colour and appearance attribute was rated on a 9-point structured Hedonic scale (Wichchukit and O'Mahony 2015). Results were recorded and used to calculate the consumer acceptability.

LC–MS analysis

From the results of antibacterial assay, only the coconut shell extract was selected to investigate the phytochemical profile using LC–MS/MS. The extract of coconut shell was filtered using 0.45 µm syringe filter and analyzed using liquid chromatography coupled to mass spectrometer (LC–ESI–MS/MS, MicroTOF-Q II, Bruker, Germany) The solution (10 µl) was injected for liquid chromatography separations in a C18 reverse phase column (120 Å, 2.1 × 150 mm, 3.0 µm, Dionex, USA) at 330 nm. A discontinuous gradient elution at a flow rate of 0.2 ml/min was performed using mobile phase A containing methanol and mobile phase B containing MilliQ water with 1% acetic acid. The gradient started from 1% of A for 0.2 min and it was then brought to 75% A at 16th min and then reaching at 100% A at 19th min to 5% A at 21st min and was maintained at same condition till 40th min. Eluted compounds were then identified using MS and their respective MS/MS pattern. The results of molecular mass were compared with mass bank data and the phytochemicals were identified. Further, luteolin content was quantified HPLC (Make: Agilent, Model: Infinity 1200) through calibration curve using isocratic mobile phase containing methanol, water and acetic acid (20:78:2, v/v/v) in C-18 column at 280 nm.

Statistical analysis

All experiments were carried out with three replicates and reported data were represented as the mean ± standard deviation. CFU data were transformed to log CFU/g. Analysis of variance (ANOVA) of data was determined using Minitab software (version 17, developed by Minitab Inc, Pennsylvania) and the difference between the treatment groups ($p < 0.05$) was considered as significant using Turkey analysis.

Results and discussion

Physicochemical properties

In the present study, agro-food by-products such as cashew nut shell, coconut shell and peanut hull were used as a natural source of phytochemical compounds, which are available enormously in India. Distilled water was used to

extract the polyphenols at 70 °C, because of its safety and suitability for food preservation studies. The physical properties like colour was orange–yellow in the case of peanut hull, whereas the cashew nut shell extract was dark brown in colour and coconut shell extracts had light brown colour (Table 1). Both peanut hull and coconut shell extracts showed acceptable odor while cashewnut shell extract had no characteristic odor. Measurements of pH indicated that all the extracts were nearly neutral (6.67–6.83). Water solubility is an important parameter, which can determine the suitability of the extracts in food applications. All the three by-product extracts exhibited significant water solubility with highest level recorded by peanut hull extract (82.63%), and least by cashew nut shell extract (69.18%). Among the presently studied samples, cashewnut shell extract showed the significantly higher level of total phenolic content (2403.41 ± 252.15 mg GAE/100 g), which was followed by coconut shell extract (1845.73 ± 109.63 mg GAE/100 g) and peanut hull extract (1130.54 ± 331.08 mg GAE/100 g).

Antibacterial activity

Antibacterial activity of nut by-product extracts against selected foodborne pathogens are shown in Table 1. Measurement of the zone of inhibition indicated that cashewnut shell extract was more effective in preventing the growth of *E. coli* (18 mm), *L. monocytogenes* (18 mm), and *S. enterica* (16 mm) (Supplementary Fig. 1). Among the investigated extracts, peanut hull exhibited weaker activity against all the studied pathogens. MIC values also revealed the same trend, and cashewnut shell extract at 0.63 and 1.25 mg/ml concentration inhibited the growth of *E. coli* and *L. monocytogenes*, respectively (Table 1). Coconut extract was found to be effective at 1.25 mg/ml towards inhibiting the growth of *S. enterica*. In another related study, Martin et al. (2012) evaluated the antimicrobial potential of seven different agro-industrial wastes and reported the MIC range from 0.78 to 25 mg/ml. Both cashewnut and coconut shell extracts could be explored as a natural source of antimicrobial compounds to inhibit the growth of presently studied foodborne pathogens.

Food preservation studies

Nut by-product extracts (cashewnut shell, coconut shell and peanut hull) were applied to inhibit the selected bacterial pathogens in papaya fruit model (Supplementary Fig. 2). The effect of nut by-product extracts on the *E. coli*, *S. enterica* and *L. monocytogenes* of artificially inoculated on fresh-cut papaya fruit are shown in Fig. 1. The control group showed high growth of bacteria, whereas when the fruit samples were treated with a commercial organic

Table 1 Physico-chemical properties and antibacterial activity of aqueous extract of nut by-products¹

S. No.	Physico-chemical properties	Nut byproduct extracts		
		Cashewnut shell	Coconut shell	Peanut hull
1	Colour	Dark brown	Light brown	Orange yellow
2	Odour	Odourless	Pleasant odour	Nut odour
3	pH	6.67 ^a ± 0.25	6.74 ^a ± 0.22	6.83 ^a ± 0.16
4	Water solubility (%)	69.18 ^c ± 1.42	76.29 ^b ± 1.34	82.63 ^a ± 2.07
5	Total phenolic content (mg GAE/100 g)	2403.41 ^a ± 252.15	1845.73 ^b ± 109.63	1130.54 ^c ± 331.08
6	Zone of inhibition (mm)			
	<i>Escherichia coli</i>	18 ^a ± 0.22	17 ^b ± 0.28	5.0 ^c ± 0.14
	<i>Salmonella enterica</i>	16 ^a ± 0.65	16 ^a ± 0.42	3.0 ^b ± 0.12
	<i>Listeria monocytogenes</i>	18 ^a ± 0.42	17 ^a ± 0.26	2.0 ^b ± 0.06
7	Minimum inhibitory concentration (mg/ml)			
	<i>Escherichia coli</i>	0.63	2.50	5.00
	<i>Salmonella enterica</i>	1.25	1.25	10.0
	<i>Listeria monocytogenes</i>	1.25	5.00	5.00

Values in the same row with different letter superscripts are significantly different ($p < 0.05$)

¹Values are mean of three separate analyses

preservative (e.g. citric acid) and nut by-product extracts, the bacterial count was reduced significantly ($p < 0.05$).

Escherichia coli population was reduced to 0.89, 1.51, and 2.49 log CFU/g in the samples treated with cashewnut shell, coconut shell, and peanut hull extracts, respectively (Fig. 1a). Among the by-products, cashewnut shell extract was found to be very effective in inhibiting the growth of *E. coli* when compared to other by-products as well as the synthetic preservative (citric acid). Pathogenic *E. coli* has an infectious dose of less than 50–100 cells and has caused several product-associated outbreaks in the past (Franz and Bruggen 2008). Therefore, identification of natural antimicrobials, which can control the growth of *E. coli* can be very beneficial to food industries.

The population of *S. enterica* was significantly reduced in fruits treated with nut by-products when compared with control (Fig. 1b). The initial count of *S. enterica* was 5.42–5.87 log CFU/g among different groups and treatment with cashewnut shell, coconut shell and peanut hull extracts reduced the population to 1.14, 1.47 and 1.82 log CFU/g, respectively. Cashewnut shell extract was very effective in controlling the growth of *S. enterica* on the fresh-cut papaya fruit when compared to other nut by-products. As *Salmonella* spp. is a serious foodborne pathogen which causes large number of deaths (Andino and Hanning 2015) the presently investigated nut by-product extracts might offer an alternative strategy to control their growth on fresh-cut fruits.

Listeria monocytogenes can cause listeriosis and often results in high hospitalization and mortality rates and has

been recently identified as one of the emerging infections associated with foodborne diseases (Sivaranjani et al. 2016). Effects of nut by-product extract on *L. monocytogenes* on artificially inoculated fruit sample during storage up to 10 days is shown in Fig. 1c. *Listeria monocytogenes* count increased from 4.68 to 5.62 log CFU/g in control group during the storage period of 10 days at 5 °C. But, treatment with synthetic preservative (citric acid) as well as nut by-product extracts decreased the *L. monocytogenes* count significantly ($p < 0.05$) when compared to control group. Among the three different extracts investigated, coconut shell extract was found to be more effective in reducing the *L. monocytogenes* count in fresh-cut papaya.

A significant reduction ($p < 0.05$) was observed in *E. coli*, *S. enterica* and *L. monocytogenes* populations in cashewnut and coconut shell extracts treated group at the end of storage period. Peanut hull extract showed weak inhibition effect on the presently studied pathogens (Fig. 1). Hence, it could be concluded that the treatment with cashewnut and coconut extracts are more effective to reduce the foodborne pathogens on fresh-cut papaya. Studies in the past have also reported the antibacterial effectiveness of natural substances against foodborne pathogens (Park et al. 2011; Kim et al. 2016).

All the samples were visually acceptable on the initial day and samples did not show any significant difference among the treatments (Fig. 2). On the 5th day of storage, the colour change was observed in the samples treated with nut by-product extracts and the severity of colour change noticed was in the following order: cashewnut

Fig. 1 Effect of nut by-product extracts on *E. coli* (a), *S. enterica* (b), *L. monocytogenes* (c) in fresh-cut papaya fruit model

Fig. 2 Sensory (colour and appearance) value of raw and treated fresh-cut fruit samples stored at refrigerated condition

shell > coconut shell > peanut > control > citric acid. Citric acid treated samples were best among them all, whereas control samples were also acceptable. Even though the cashewnut shell extract was very effective in controlling the growth of pathogens, fruit samples turned to dark brown in colour during refrigerated storage. Gibis and Weiss (2012) similarly observed a discoloration of fried beef patties upon the addition of higher concentration of rosemary extract. Although the colour changes due to lipid oxidation are widely reported, the differences found in the present study may not be due to lipid or protein oxidation and probably because of the dark colour of the cashewnut shell extract.

Phytochemical profile

Since the coconut shell extract exhibited maximal antibacterial property without affecting the sensory aspect of fresh-cut fruit, only this extract was analyzed in LC–MS to reveal its phytochemical composition (Supplementary Fig. 3). Based on mass/ion ratio (M–H) of 285.1 and fragmentation pattern of the daughter ions, the major compound was identified as luteolin, which is known to possess various therapeutic effects. Based on the calibration curve, the luteolin content of coconut shell extract was found to be 78.24 mg/100 g on dry weight basis, which might have contributed to its high antibacterial activity. Antibacterial activity of luteolin has been reported against *S. aureus* (Joung et al. 2016). The phytochemical constituents like anacardic acid and cardanol in cashew nut shell (Kubo et al. 2003; Boonsai et al. 2014), catechin and luteolin in the coconut shell (Esquenazi et al. 2002; Kajiya et al. 2004; Qin et al. 2013; Joung et al. 2016) and resveratrol in peanut hull (Dean et al. 2008; Hwang and Lim 2015) might have been responsible for their antimicrobial activity.

Conclusion

In the present study, physicochemical properties and antibacterial activity of nut by-product extracts were evaluated and their effect on artificially inoculated food-pathogens in fresh-cut papaya during refrigerated storage was assessed. Antibacterial studies revealed that cashewnut shell and coconut hull extracts have potent inhibition against food pathogens like *E. coli*, *S. enterica*, and *L. monocytogenes*. Even though the cashewnut shell extract showed significant reduction of foodborne pathogens in fresh-cut papaya fruit model, it also affects the colour of fruit samples. Hence, coconut shell extract with appreciable luteolin content could be considered as a natural source of antibacterial compounds for food preservation applications.

Acknowledgements Author (VV) is thankful to SERB for having provided the financial support (Start-up Grant No. YSS/2014/000332), and authors are grateful to the honorable Vice-Chancellor of SASTRA Deemed University, Thanjavur, Tamilnadu for their encouragement to conduct this research project.

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