

# Antioxidant, Antibacterial, and Cytotoxicity Activities of Cashew (*Anacardium occidentale*) Nut Shell Waste

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## Abstract

**Context:** Antioxidants have been used in the food industry particularly as dietary supplements and additives to preserve food quality during transportation and storage. In the Southern of Thailand, especially in Ranong Province, cashew nut is one major of agriculture product and the volume of cashew nut shell is becoming increase as by-product. **Aims:** The aim of this study is to evaluate antioxidant activity, antibacterial activity against to common food borne pathogens, and cytotoxicity to mammal cells of cashew nut shell waste. **Materials and Methods:** Antioxidant activity of cashew nut shell waste was 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) cation radical scavenging assays. Evaluation of antibacterial activity of water extract (CW) and ethanol extract (CE) of cashew nut shell waste was done against *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* by resazurin microplate assay (REMA). Cytotoxicity of CW and CE was determined by REMA and used human dermal fibroblasts, a neonatal (HDFn)-neonatal dermal fibroblast and Vero cells as mammal cells. **Results and Discussion:** Cashew nut shell waste extract (150 µg/ml) was inhibited DPPH and ABTS radical by  $75.5 \pm 1.4$  and  $97.1 \pm 1.4\%$ , which calculated to  $57.1 \pm 1.0$  and  $56.2 \pm 0.6$  µmol trolox equivalent antioxidant capacity, respectively. Water (CW) and ethanol (CE) extracts of cashew nut shell waste were inhibited *S. aureus*, *B. cereus*, and *E. faecium* with minimal inhibitory concentration = 3.13, 3.13, and 6.25 µg/ml, respectively. CW and CE (3.13-100 µg/ml) were insufficient cytotoxic against Vero cells and HDFn-neonatal dermal fibroblast. **Conclusions:** Cashew nut shell waste may apply for food additives used, as well as, to reduce or diminish agricultural waste production.

**Key words:** *Anacardium occidentale*, antibacterial activity, antioxidant, cashew nut shell, cytotoxicity, food additives

## INTRODUCTION

The focus on natural antioxidant from plant origin is currently reported and be able to prevent chronic diseases. Fruits and vegetables are common antioxidant-rich diet, and based on epidemiological data, high consumption can reduce risk of chronic diseases.<sup>[1]</sup> Natural antioxidants are widely distributed in food and medicinal plants and exhibit a wide range of biological effects, including anti-inflammatory, antiaging, antiatherosclerosis, and anticancer. Extraction and assessment of antioxidants from food and medicinal plants are crucial to explore the potential antioxidant sources and promote the application in functional foods, pharmaceuticals, and food additives.<sup>[2]</sup> In addition, the long-standing empirical use of herbal medicine against infectious

ailments is consistent with the previous observation.<sup>[3]</sup> At this point, natural products have garnered substantial interest as lead sources for identification of new pharmaceutical agents.<sup>[4]</sup>

Cashew (*Anacardium occidentale* L.) is well-known species belongs to the *Anacardiaceae* family, which is a

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perennial-flowering plant and originates from Brazil. These plants are worldwide cultivated in various tropical areas for fruit and nut production. Plants are 10–12 m of height with short and disorganized branches. Leaves are considerably glazed with a curve-to-oval shape and arranged in spiral pattern. Their leaves are 4–22 cm long and 2–15 cm in width. Flower consists of five petals 7–15 cm long with sharp tip and have a pale-green color and turn to red color after blooming. Kidney shaped of true fruit with a single seed is located on the tip of pseudo carp. Currently, Brazil, India, Vietnam, Tanzania, and also Thailand are the main producers of cashew nuts. Southern Thailand is located in tropical zone and characterized by high humidity and rain throughout the year, which is the major region of cashew nut production.<sup>[5,6]</sup> Different parts of the cashew tree have traditionally been used across the world to treat various diseases. Leaves or nut shell extracts from cashew, commonly known as the cashew tree, have long been used to treat inflammation and other conditions, including asthma, ulcers, and cancer.<sup>[7,8]</sup> Although the efficacy of these compounds for treating such disorders has not been established in controlled trials, the major component of cashew nut shell extract, anacardic acid, has been shown to exert a variety of effects on both prokaryotic and eukaryotic cells.<sup>[9,10]</sup> Anacardic acid and related compounds are chemical closely related organic compounds, each consisting of salicylic acid substituted with an alkyl chain. The enduring research and emerging evidence suggest that anacardic acid could be a potent target molecule with bactericide, fungicide, insecticide, anti-termite, and molluscicide properties and as a therapeutic agent in the treatment of the most serious pathophysiological disorders such as cancer, oxidative damage, inflammation, and obesity. Furthermore, anacardic acid was found to be a common inhibitor of several clinically targeted enzymes such as nuclear factor- $\kappa$ B kinase, histone acetyltransferase, lipoxygenase, xanthine oxidase, tyrosinase, and ureases.<sup>[8]</sup>

Antioxidants control and reduce the oxidative damage in foods by delaying or inhibiting oxidation caused by reactive oxygen species, ultimately increasing the shelf life and quality of these foods.<sup>[11-13]</sup> Antioxidants have recently been used in many sectors in the food industry particularly as dietary supplements and additives to preserve food quality during transportation and storage. This is due to their ability to influence some enzyme systems involved in the pathogenesis of some diseases.<sup>[13]</sup> Lipid oxidation and enzymatic activities are some of the most significant problems in the food industry. These reactions lead to changes in chemical composition which in turn reduce the quality and shelf life of food products.<sup>[13,14]</sup>

Cashew nut is one of most agricultural products from Southern of Thailand, especially Ranong province, and after nut processing, the volume of cashew nut shell is becoming increasing as by-product. Thus, the aims of this study were to evaluate antioxidant and antibacterial activities of cashew nut shell waste extracts. Cytotoxicity of cashew nut shell waste to mammal cells was also evaluated. This finding may provide useful data for antioxidant and antibacterial

activities of cashew nut shell as low cost of food additive, and cytotoxicity of cashew nut shell was also evaluated.

## MATERIALS AND METHODS

### Sample Collection and Preparation

Cashew nut shell waste was collected on May 2017 (harvest season during February to May annually) and obtained from local agriculture group, which cultivated cashew plants and produced cashew nuts, stayed nearby Suan Sunandha Rajabhat University, Ranong academic campus, Thailand. Botanical identification for cashew tree had done by expert agriculturalist and identified as Koh Phayam or Phayam island variety, which is most favorite cashew in Ranong Province. Fresh cashew nut shell (1 kg) was cleaned, cut into small pieces, and then airdried. The airdried nut shell (780 g) was ground in powder form. The extractions were separately prepared for antioxidant and for antibacterial activity and cytotoxicity tests. First, 5 g of cashew nut shell powder was extracted with 50 ml of distilled ethanol for 24 h after sonication in 1<sup>st</sup> h. Extracted solutions were filtered through vacuum and collected solution layer for antioxidant testing. Second, 1 g of shell powder was macerated with 1 L of water and with 1 L of 95% ethanol (W/V) during 3 days. Each extract solution was evaporated through rotary evaporation apparatus under vacuum in constant weight for determination of antibacterial activities and cytotoxicity from extracts of cashew nut shell waste.

### Assays for Antioxidant Activity

#### DPPH radical scavenging assay

Briefly, 100  $\mu$ l of sample solution (300  $\mu$ g/ml in ethanol) was added to 100  $\mu$ l of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical reagents ( $6 \times 10^{-5}$  M) into 96-well plate for triplicate samples. The final concentration of sample in reaction mixture was 150  $\mu$ g/ml. The concentration of 6-hydroxy-2, 5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) standard solution was 50, 25, 12.5, and 6.25  $\mu$ M, and 100  $\mu$ l of each concentration was added to DPPH reagent/each well. Reagent and control blank were done along with running of samples and standards. The 96-well plate was gently shaken for 5 s and incubated in the dark for 30 min. The decrement of light absorbance of DPPH radical was promptly measured at 517 nm. Percentage inhibition of DPPH radical for extracted samples and trolox standard solutions was calculated, and antioxidant activity of samples was reported as  $\mu$ M of trolox equivalent antioxidant capacity (TEAC).<sup>[15,16]</sup> The assay was repeated in 5 times for averaging of data (mean  $\pm$  standard deviation).

#### ABTS cation radical scavenging assay

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) cation radical was prepared by mixing 7 mM

ABTS stock solution with 2.45 mM of potassium persulfate (1/1, V/V) and leaving for 16 h. ABTS cation radical solution was diluted with ethanol to an absorbance of  $0.700 \pm 0.05$  at 734 nm before assay starting. 20  $\mu$ l of sample solution ( $\mu$ g/ml) was added to 180  $\mu$ l of 2, and ABTS reagent into 96-wells plate for triplicate samples. The concentration of trolox standard solution was ranged at 50, 25, 12.5, and 6.25  $\mu$ M, and 20  $\mu$ l of each concentration was added to DPPH reagent/each well. The mixture reaction was processed and calculated the same as DPPH radical scavenging assay; however, the absorbance measurement was at 734 nm.<sup>[15,17]</sup>

### Antibacterial activity test

Seven bacteria including three Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), and *Enterococcus faecium* (ATCC 51559) and four Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 70063), *Pseudomonas aeruginosa* strain PAO1, and *Acinetobacter baumannii* (ATCC 19606) were used as tested organisms, and the evaluation of antibacterial activity of water and ethanol extracts of cashew nut shell waste was done by resazurin microplate assay (REMA).<sup>[18,19]</sup> Each bacterium was cultured by streaking on to medium called Tryptic Soy Agar. The culturing lasted 1-day incubation at 37°C. Next, the single colony was cultured in Tryptic soy broth (TSB) for 5 mL and incubated at 37°C in 200 rpm shaker incubator for 30 min ( $OD_{600} \sim 0.1$ ). Activated cultures were diluted for 200 times. The 20 mL TSB medium was incubated at 37°C in 200 rpm shaker incubator for another 3 h ( $OD_{600} \sim 0.4-0.5$ ). After that, the test to find out antimicrobial activity continued by adding 7.5  $\mu$ L extract, 25  $\mu$ L of resazurin 0.25 mM, and bacterial cell suspension until the final volume. Furthermore, 75  $\mu$ L ( $\sim 15,000$  cells) in Mueller Hinton broth was put onto 384-well plate. The sample was taken to incubate at 37°C for 2 h, following by measuring signal by SpectraMax M5 multidetection microplate reader (Molecular Devices, USA) at excitation and emission wavelength of 530 and 590 nm.<sup>[18,19]</sup> Amikacin and ofloxacin were used as positive control for Gram-negative bacteria. Vancomycin was used as positive control for *B. cereus*. Tetracyclin and rifampicin were used as positive control for *E. faecium*. 0.5% dimethyl sulfoxide (DMSO) was used as negative control.

### Cytotoxicity test

Vero cells (African green monkey) and human dermal fibroblasts, a neonatal (HDFn) C-004-5C, were used for evaluated cytotoxicity of cashew shell extracts. The REMA developed by O'Brien *et al.*<sup>[20]</sup> was performed for anticancer test. In brief, the cells were cultured in proper condition and diluted by culture medium at  $2.2-3.3 \times 10^4$  cells/ml. The next step was to add the 5% DMSO 50  $\mu$ l into cell suspension 45  $\mu$ l in the 384-well plates. Then, the extract was incubated at 37°C in the incubator which contained 5% of CO<sub>2</sub>. After incubation (3–5 days), 12.5  $\mu$ l of resazurin (62.5  $\mu$ g/ml) was added. The incubation was continued for 4

h and then measured fluorescence signal by SpectraMax M5 multidetection microplate reader (Molecular Devices, USA) at excitation and emission wavelength of 530 and 590 nm, respectively. Dose-response curve could be done in the sixth test. 3-fold serial intensity dilution and the intensity of the cell-restraint extract 50% of inhibitory concentration (IC<sub>50</sub>) could be calculated by SOFTMax Pro software (Molecular Devices, USA). Ellipticine was used as positive control. 0.5% DMSO and water were used as negative control.

## RESULTS AND DISCUSSION

Cashew nut shell waste extract (150  $\mu$ g/ml) was inhibited DPPH and ABTS radical by  $75.5 \pm 1.4$  and  $97.1 \pm 1.4\%$ , which calculated to  $57.1 \pm 1.0$  and  $56.2 \pm 0.6$   $\mu$ mol TEAC, respectively [Table 1]. Water (CW) and ethanol (CE) extracts of cashew nut shell waste were inhibited all of Gram-positive bacteria including *S. aureus*, *B. cereus*, and *E. faecium* at minimal inhibitory concentration (MIC) = 3.13, 3.13, and 6.25  $\mu$ g/ml, respectively [Table 2]. CW and CE (3.13-100  $\mu$ g/ml) were insufficient cytotoxic against Vero cells and HDFn-neonatal dermal fibroblast [Table 3].

Cashew nut shell liquid (CNSL), the reddish brown viscous liquid extracted from the pericarp of the cashew nut, is the major by-product of the cashew nut industry and has numerous medicinal and industrial applications.<sup>[21]</sup> Its major constituents include anacardic acid, cardol, cardanol, and methyl cardol,<sup>[22]</sup> and the ratio of these components varies based on the method of extraction.<sup>[23]</sup> Thus, cashew nut shell waste in our study was focused on biological activities of CNSL and its composition. We found that cashew nut shell waste extract was possessed preferable antioxidant activity when compared with trolox. Moreover, industrial CNSL was reported as DPPH and hydroxyl radical scavengers, and inhibited xanthine oxidase activity.<sup>[23]</sup> CNSL and some derivatives (cardanol, hydrogenated cardanol, and alkylated hydrogenated cardanol) were also had antioxidant activities using Fourier transform infrared measurements.<sup>[24]</sup> In addition, CNSL exerts an important protective effect against oxidative stress in yeast when exposed to paraquat or H<sub>2</sub>O<sub>2</sub>, indicating an *in vivo* antioxidant effect.<sup>[23]</sup>

In case of antibacterial activity, CW and CE were inhibited *S. aureus*, *B. cereus*, and *E. faecium* (MIC = 3.13, 3.13 and 6.25  $\mu$ g/ml). Compared to previous studies, CNSL was inhibited the growth of *Bacillus subtilis* with an IC<sub>50</sub> of 0.35% (v/v) and the treated cells exhibited elongated morphology, indicating that suppression of cell division is one of the possible mechanisms of its action.<sup>[25]</sup> Moreover, anacardic acid, the one of major components of CNSL, has been shown to exhibit direct antimicrobial activity against a number of bacterial species, including *Propionibacterium acnes*, *S. aureus*, and *Helicobacter pylori*.<sup>[9,26,27]</sup> Hence, there was concluded that antibacterial activity of CNSL or cashew nut shell waste was affected to Gram-positive bacteria.

**Table 1:** Antioxidant activities of cashew nut shell waste extract

Standard/sample	% Inhibition of DPPH-	% Inhibition of ABTS <sup>+</sup>
50 µM of Trolox	66.3	87.1
25 µM of Trolox	32.2	41.7
12.5 µM of Trolox	15.4	17.7
6.25 µM of Trolox	7.3	9.3
Cashew nut shell waste extract (150 µg/ml)	75.5±1.4	97.1±1.4
	57.1±1.0 µmol TEAC	56.2±0.6 µmol TEAC

TEAC: Trolox equivalent antioxidant capacity

**Table 2:** The antibacterial activity of cashew nut shell water (CW) and 95% ethanol (CE) extracts<sup>a</sup>

Specie/MIC <sub>90</sub> <sup>b</sup> (µg/ml)	CW	CE	V <sup>c</sup>	T <sup>d</sup>	R <sup>e</sup>	A <sup>f</sup>	O <sup>g</sup>
<i>S. aureus</i>	3.13	3.13	0.75	-	-	-	-
<i>B. cereus</i>	3.13	3.13	2.00	-	-	-	-
<i>E. faecium</i>	6.25	6.25	-	0.195	1.56	-	-
<i>E. coli</i>	-	-	-	-	-	4.00	0.031
<i>K. pneumoniae</i>	-	-	-	-	-	0.50	1.00
<i>P. aeruginosa</i>	-	-	-	-	-	0.391	0.391
<i>A. baumannii</i>	-	-	-	-	-	3.13	0.391

<sup>a</sup>Negative control: 0.5% DMSO, <sup>b</sup>MIC: 90% of minimal inhibitory concentration, <sup>c</sup>V: Vancomycin, <sup>d</sup>T: Tetracycline,<sup>e</sup>R: Rifampicin, <sup>f</sup>A: Amikacin, <sup>g</sup>O: Ofloxacin, DMSO: Dimethyl sulfoxide, *S. aureus*: *Staphylococcus aureus*, *B. cereus*: *Bacillus cereus*, *E. faecium*: *Enterococcus faecium*, *E. coli*: *Escherichia coli*, *K. pneumoniae*: *Klebsiella pneumoniae*, *P. aeruginosa*: *Pseudomonas aeruginosa*, *A. baumannii*: *Acinetobacter baumannii*

The CW and CE (up to 100 µg/ml) were lack of cytotoxicity on against Vero cells and HDFn–neonatal dermal fibroblast. Leaves or nut shell extracts from cashew have long been folk medicinal used to treat inflammation and other conditions, including asthma, ulcers, and cancer.<sup>[8]</sup> In the previous study, toxicity testing of anacardic acids in the CNSL had measured the acute, subacute, and mutagenic effects of anacardic acid administration in BALB/c mice, and doses <300 mg/kg did not produce biochemical and hematological alterations in BALB/c mice.<sup>[28]</sup> In addition, CNSL had been reported sustainable and environmental safe plant-based larvicidal on larvae of *Aedes aegypti*.<sup>[29-31]</sup> The study on Thai medicine herbal infusions for biochemical constituents and antibacterial properties, which were included chrysanthemum, lotus stamen, bullet wood, cananga, safflower, roselle, and licorice as well as cashew flower, leaf buds, and leaves, found antioxidant and antibacterial activities of the dried cashew flower, leaf buds, and leave extracts contained highest amount of total phenolics and antioxidant activity when compared to the other herbs.<sup>[32]</sup> Compared with previous studies, we suggested that the chemical composition of cashew in different parts are similar type such as phenolic compounds; however, these are varied on derivatives and amounts, which may implied for different traditional medicinal used in different tropical countries. Furthermore, the part of plant, dose of usage and preparation of cashew nut shell for food additive use are need to consider.

The abundant and cheap source of natural waste products is trend to apply in accordance with the concept of green

chemistry and environmental friendly safe. Due to biological activity and toxicity from our results and previous studies, cashew nut shell waste may apply for food additives used, as well as, to reduce or diminish agricultural waste production. However, additional studies should be conducted to evaluate pharmacological characteristics of its constituents in greater detail for ensuring safety use.

## CONCLUSION

Cashew nut shell waste (150 µg/ml) activity was inhibited DPPH and ABTS radical (57.1 ± 1.0 and 56.2 ± 0.6 µmol TEAC). CW and CE were inhibited *S. aureus*, *B. cereus*, and *E. faecium* (MIC = 3.13, 3.13, and 6.25 µg/ml) with no cytotoxicity against to Vero cells and HDFn–neonatal dermal fibroblast. Cashew nut shell waste may apply for food additives used, as well as, to reduce or diminish agricultural waste production.

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**Table 3:** Cytotoxicity of cashew nut shell water (CW) and 95% ethanol (CE) extracts

Extract/cytotoxicity <sup>a</sup>	HDFn (%)	Vero (%)
CW		
100 µg/ml	31.26	9.25
50 µg/ml	13.19	-24.13
25 µg/ml	8.73	-34.27
12.5 µg/ml	-6.86	-36.56
6.25 µg/ml	-5.57	-33.55
3.13 µg/ml	-15.95	-33.92
CE		
100 µg/ml	14.25	-7.74
50 µg/ml	15.90	-31.19
25 µg/ml	15.21	-30.45
12.5 µg/ml	18.13	-26.88
6.25 µg/ml	15.63	-23.58
3.13 µg/ml	16.49	-31.28
Control	IC <sub>50</sub> <sup>ob</sup> (µg/ml)	IC <sub>50</sub> <sup>b</sup> (µg/ml)
Positive control: Ellipticine	4.59	1.28
Negative control: 0.5% DMSO	-	-

<sup>a</sup>Interpretation: % Cytotoxicity < 50% = non-cytotoxic, % Cytotoxicity ≥ 50% = cytotoxic, <sup>b</sup>IC<sub>50</sub>: 50% of inhibitory concentration. DMSO: Dimethyl sulfoxide, HDFn: Human dermal fibroblasts, a neonatal

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