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Influence of gamma radiation on the antimicrobial activity of crude extracts of *Anacardium occidentale* rich in tannins

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Abstract: *Anacardium occidentale* L. Anacardiaceae, known as cashew, commonly found in northeastern of Brazil, has high levels of secondary metabolites, particularly tannins, used as raw material for herbal medicines. An efficient alternative to decontaminate plant products is the total sterilization or reduction of the initial microbial count, the process of gamma irradiation with ^{60}Co. The objective of this study was to analyze the antimicrobial activity of crude extracts of bark and leaves of A. occidentale, based on the quantification of total phenols and tannins, before and after exposure to gamma radiation from ^{60}Co. The extracts were obtained in the laboratory by cold maceration in ethanol, filtered and dryness. They were divided into non-irradiated control group (0 kGy) and irradiated: groups exposed to gamma radiation at doses of 5, 7.5 and 10 kGy. The total phenols was obtained by the Folin-Ciocalteau method and tannins, by the precipitation of casein. The antimicrobial potential activities of these extracts were also evaluated. The results showed that gamma radiation doses employed in this study did not influence statistically the percentage of total phenols and tannins in the bark extracts, at levels ranging from 5.73±0.14 and 5.20±0.14, respectively. The levels of metabolites in the leaves were statistically (p<0.05) influenced by radiation, observed average total phenols between 3.13±0.04 (0 kGy) and 3.50±0.08 (10 kGy), and tannin between 2.47±0.06 (0 kGy) and 2.93±0.04 (10 kGy). The extracts of bark and leaves were active against *Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis, Enterococcus faecalis, Mycobacterium smegmatis, Candida albicans*. Gamma radiation caused an increase in antimicrobial activity of extracts against *Staphylococcus aureus* (Gram positive), with average inhibition zones for shells: 14.33±058 (0 kGy) and 22.33±0.58 (10 kGy), and leaves: 11.33±0.58 (0 kGy) and 19.00±1.00 (10 kGy). Exposure to radiation caused changes in physical and chemical constituents of phenolic extracts of leaves of cashew, increasing levels of tannins.

Introduction

Anacardium occidentale L., plant species tree popularly known as the cashew, the family Anacardiaceae, produce tasty fruit, excellent wood, compounds used in industry and medicine. Originally from South America, widely spread in the coastal region of tropical and subtropical regions of Brazil that is endemic in the Northeast, with temperatures above 22 ºC and relative rainfall, requiring a period of drought to produce (Panizza, 1997). Chemical constituents in *A. occidentale* can allow its use in medicine or cosmetics and food industries (Calixto, 2000, Newman, et al. 2003, Burnes et al. 2001; Sluis et al., 2001, Novais, et al., 2003). There is also used to get soft leather with higher durability, cleanse impurities from gasoline, food & beverage manufacturing, etc (Bernays et al., 1989). Harbone et al., 1991; Schreiber et al., 1993).

The usual procedure to total sterilization or just reducing the initial microbial contamination through chemical processes is the gamma irradiation (Owczarczyk et al. 2000; Ministério da Saúde 2004; Satomi, et al., 2005), using a no toxic dose level.

Whereas there are studies that relate to antimicrobial extracts of *A. occidentale* by tannin contents, this study aimed to analyze the influence of gamma radiation from ^{60}Co on the crude extracts showing its potential as an antimicrobial agent in order

to reveal the effectiveness of the extracts after the process of gamma radiation.

Methodology

Plant material

Bark and leaves of *Anacardium occidentale* L. Anacardiaceae, was collected in the Caatinga (semi arid region) of about 20 hectares in the Experimental Station in the city, a body belonging to the Agronomic Institute of Pernambuco, located in the micro Ipojuca Valley, one of six geography of the Pernambuco (08º 14'18.2 "S and 35º 54'57.1" W) (Alcoforado Filho et al., 2003). The study area was chosen due to the large number of species and provides good storage conditions. The voucher specimen is deposited at the herbarium of Department of Botany of Federal Rural University of Pernambuco under the number 46236.

Preparation of plant extracts

Extracts of *A. occidentale* were obtained from bark and leaves, which were macerated for 72 h for four consecutive extractions in ethanol/water (70%). Subsequently, the extracts obtained were filtered and evaporated until dryness.

Irradiation of extracts

Crude extracts of bark and leaves of *A. occidentale* were divided into aliquots of 20 g, placed in glass and through the process of gamma irradiation.

The experiment consisted of the format: 9 x 2 x 3, nine aliquots of homogeneous material for bark and leaves, three doses of radiation used (5, 7.5 and 10 kGy). In addition, the controls were regarded bark and leaves, totaling 56 samples and two samples irradiated controls. Irradiation was performed in Gamma cell source irradiator with 60-Co (model 220 Excel-MDS Nordion, dose rate of 10.040 kGy/h) from the Department of Energy Nuclear, UFPE.

Determination of phenols and tannins contents

From the crude extracts of bark and leaves of *A. occidentale*, 500 mg were weighed and transferred to flasks of 50 mL, and dissolved with 80% methanol, checking the final volume of the balloon with the above solvent. All treatments, control and irradiated were performed in triplicate.

The determination of total phenols was based by the Folin-Ciocalteau methods and to total tannins by chemical precipitation of casein (Folin & Ciocalteau, 1927; Seigler et al. 1986; Mueller-Harvey, 2001; Queiroz et al., 2002). Some Changes in this method have been inserted to adequate of phenolic and tannin levels in the plant.

The adaptation of the Folin-Ciocalteau method was to add 0.20 mL of extract diluted in volumetric flask containing 100 mL before 50 mL of water, adding 5 mL of Folin-Ciocalteau (10% aqueous solution) 10 mL solution of sodium carbonate 7.5% and volume completed with distilled water. The solution was stirred and allowed to rest for 30 min at room temperature and ambient lighting. After this period, the reading of absorbance was performed at 760 nm. The same procedure was adopted for the standard solutions of tannic acid at concentrations of 0.5; 1.0; 1.5; 2.0; 2.5; 3.0 and 3.5 µg/mL. The relationship between concentrations and absorbance was used to get the calibration curve, using a spectrophotometer UV-visible Hewlett Packard - HP-8453E.

The tannins were determined by chemical precipitation of casein, which consisted in adding a 50 mL flask, 1 g of casein powder and aliquots of 5 mL of extract diluted in 12 mL of water, which were kept under constant agitation for 3 h at room temperature and ambient lighting. Then, the samples were filtered on 9 cm filter paper and the completed to 25 mL of final volume. Aliquots of 5 mL were drawn from this solution and the phenol plant, as determined by Folin-Ciocalteau. The tannin quantity corresponded to the difference between the founded value and the obtained in theoretical determination of total phenols. The total phenols and tannins were expressed as percentage of mg dry matter.

The Kolmogorov-Smirnov test was used to analyze the normality of the data. The results were evaluated by analysis of variance and was performed using the BioEstat 5.0, with significance level of 95% ($p<0.05$).

Evaluation of antimicrobial activity

The antimicrobial qualitative essay was performed by diffusion test (Bauer et al., 1966) and the quantitative test was the dilution technique for the MIC (Minimal Inhibitory Concentration) determination (Carvalho et al., 2002). The extracts were tested against the following microorganisms from the Culture Collection of Department of Antibiotics, at the Federal University of Pernambuco. The Gram-positive bacteria were *Staphylococcus aureus, Bacillus subtilis, Enterococcus faecalis, Micrococcus luteus.* The Gram-negative bacteria were *Escherichia coli, Pseudomonas aeruginosa, Serratia marcescens*. Alcohol-Acid Bacteria: *Mycobacterium smegmatis*, and it was used *Candida albicans* as a representant of yeasts. The antimicrobial assays were standardized by

turbidity equivalent to 0.5 McFarland scale (Koneman, 1997).

The culture media used were Mueller Hinton Agar (*S. aureus, B. subtilis, M. luteus, E. coli, P. aeruginosa* and *S. marcescens*), glucose yeast extract (*E. faecalis* and *M. smegmatis*) and Sabouraud (*C. albicans*). It was placed on paper discs syrup sterile, 6 mm in diameter, soaked with 10 mL of the extract solution to 200,000 g/mL. After placement of the disks, the plates were incubated for 24 and 48 h at temperatures of 30 and 37ºC. The extracts were tested six times and the diameters of the halos were expressed as mean standard deviation of the results obtained in six repetitions. Halos equal and greater than 10 mm were considered significant antimicrobial activity (Awadh Ali et al. 2001; Bakshu et al., 2001, Khan et al., 2001, Chowdhury et al. 2002, Ferronatto et al., 2007, Silva et al., 2007, Nascimento et al., 2007, Aguiar et al., 2008).

The MIC was determined for the extracts that exhibited better antimicrobial activity, the method of serial dilutions on solid medium (Carvalho et al., 2002). Aliquots of different volumes (1.0; 0.5; 0.25; 0.125; 0.06 and 0.03 mL) of a solution of 20,000 mg/mL were placed in Petri dishes and mixed with 10 mL of medium for the growth of each organism. The microorganism tests were grown in grooves on the surface of the medium and the plates were incubated between 30-37°C for 24-48 h. MIC values equal to or greater than 1000 mg/mL were not considered (Holetz et al., 2002).

Results and Discussion

The average values of total phenols and tannins in the bark and leaves of *A. occidentale* are presented in Table 1. The variation of percentage of shells was between: 5.45±0.18 to 5.96±0.04 (phenol), 4.92±0.20 to 5.34±0.07 (tannins), higher found in the leaves: 3.13±0.04 to 3.50±0.08 (phenol), 2.47±0.06 to 2.93±0.04 (tannins). It was observed that tannins are the main constituents, showing 89 and 92% to barks and 79 to 84% to leaves. These findings confirmed the suggested by Paes et al. (2006).

The statistical analysis of extracts from *A. occidentale* to 0, 5, 7.5 and 10 kGy, shows that gamma radiation change the contents of total phenols and tannins from the barks and have an influence, increasing the total phenols and tannins from the leaves, showing higher concentrations in a dose of 10 kGy (Table 1).

It were shown that gamma radiation can increases (Moussaid et al., 2000, Mechi et al. 2005, Miranda et al. 2006, Bhat et al. 2007, Stajner et al. 2007), decreases (Villavicencio et al. 2000; Brigide & Canniatti-Brazaca, 2006, Toledo et al., 2007) or unchanged levels of total phenols and/or tannins (Koseki et al., 2002) in plant extracts from some plants, and raw or cooked, which had considerable percentage of moisture, favoring the indirect effects of radiation (Mechi et al., 2005, Brigid & Canniatti-Brazaca, 2006). These studies are unprecedented as the characterization of dry vegetable matter, which dominates the interaction of gamma radiation from the direct effect.

The experiments here showed that gamma radiation caused changes in their physical-chemical properties of leaves extracts of *A. occidentale*, increasing levels of total phenols and tannins.

Antimicrobial activities

Tables 2 and 3 shows that *A. occidentale* extracts of bark and leaves can inhibit the growth of Gram-positive and alcohol-acid-resistant bacteria and yeast. The extracts were inactive against Gram-negative bacteria. Laurens et al, 1992 indicate the inactivation of plant extracts against Gram-negative bacteria, due to their action on the outer cell wall.

The antimicrobial properties can be attributed to the phenolic compounds, mainly tannins (Oliveira, 2008). These actions can be observed because the tannins can inhibit bacterial enzymes and/or complexing with the enzyme substrates, or its action on bacteria

Table 1: Total phenols and tannin contents in barks and leaves of *Anacardium occidentale* L. Anacardiaceae. Control and irradiated samples (5, 7.5 and 10 kGy).

Dose (kGy)	Percentual Values $(x \pm \sigma)$ *			
	Barks		Leaves	
	Total Phenols	Percentual** of tannins	Total phenols	Percentual** of tannins
0	5.79±0.07 ab	5.34±0.07 a	3.13±0.04 a	2.47±0.06 a
5	5.45±0.18 a	4.92±0.20 a	3.33±0.15 ab	2.77±0.19 b
7,5	5.74±0.28 ab	5.21±0.28 a	3.45±0.07 b	2.83±0.07 b
10	5.96±0.04 b	5.34±0.04 a	3.50±0.08 b	2.93±0.04 b

* Mean of six repetitions and standard deviation.
** From total phenol content.

Table 2. Antimicrobial activity of bark irradiated extracts of *Anacardium occidentale* L. (2000 µg/disco).

Microorganisms	Irradiated extracts			
	0 kGy (X±δ)	5,0 kGy (X±δ)	7,5 kGy (X±δ)	10 kGy (X±δ)
Staphylococcus aureus	14,33±0,58	15,33±0,58	16,33±1,15	22,33±0,58
Micrococcus luteus	20,67±0,58	21,00±1,00	23,67±3,21	21,00±1,00
Bacillus subtilis	12,00±1,00	13,00±0	13,67±0,58	14,00±0
Enterococcus faecalis	11,67±0,58	11,67±0,58	12,67±0,58	12,33±0,58
Pseudomomas aeruginosa	9,00±0	9,00±0	9,00±0	9,00±0
Escherichia coli	0	0	0	0
Serratia marcescens	0	0	0	0
Mycobacterium smegmatis	10,33±0,58	13,00±1,00	13,00±0	12,00±0
Candida albicans	14,00±1,00	12,50±0,58	0	13,00±0

X → Arithmetic Mean; δ → Standard Deviation. Averages of six trials.

Table 3. Antimicrobial activity of leaf irradiated extracts of *Anacardium occidentale* L. (2000 µg/disco).

Microorganisms	Irradiated extracts			
	0 kGy (X±δ)	5,0 kGy (X±δ)	7,5 kGy (X±δ)	10 kGy (X±δ)
Staphylococcus aureus	11,33±0,58	13,33±0,58	12,33±0,58	19,00±1,00
Micrococcus luteus	20,00±0	18,33±2,08	18,67±1,15	17,33±1,53
Bacillus subtilis	10,00±0	10,33±0,58	11,00±1,00	11,00±1,00
Enterococcus faecalis	9,67±1,53	8,50±0,58	9,00±0	10,00±0
Pseudomomas aeruginosa	0	0	0	0
Escherichia coli	0	0	0	0
Serratia marcescens	0	0	0	0
Mycobacterium smegmatis	11,00±0	11,00±1,00	11,00±0	13,67±1,15
Candida albicans	10,50±0,58	10,00±0	0	11,00±0

X → Arithmetic Mean; δ → Standard Deviation. Averages of six trials.

Table 4. Minimum Inhibitory Concentrations in µg/mL of crude extracts of leaves of *Anacardium occidentale* L. against the tested microorganisms.

Microorganisms	Bark extracts (µg/mL)			
	0 kGy	5,0 kGy	7,5 kGy	10 kGy
Staphylococcus aureus	25	25	25	25
Micrococcus luteus	6,25	12,5	6,25	6,25

Table 4. Minimum Inhibitory Concentrations in µg/mL of crude extracts of barks of *Anacardium occidentale* L. against the tested microorganisms.

Microorganisms	Leaf extracts (µg/mL)			
	0 kGy	5,0 kGy	7,5 kGy	10 kGy
Staphylococcus aureus	>100	>100	100	50
Micrococcus luteus	6,25	25	25	25

end yeasts cell membranes, or maybe the tannins can acts as a chelate, reducing availability of essential ions for microbial metabolism (Haslam, 1995, Silva et al., 2007).

Doses of 5, 7.5 and 10 kGy promoted some changes in the antimicrobial activity of *A. occidentale*, facing the Gram-positive and alcohol-acid-resistant bacteria as showed in Tables 2 and 3.

Gamma radiation is dose-dependent to antimicrobial activity, mainly against *S. aureus* for the

bark (0 kGy 14.33±0.58, 5 kGy 13.33±0.58, 7.5 kGy 12.33±0.58; 10 kGy 22.33±0.58) and leaves (0 kGy 11.33±0.58, 5 kGy 15.33±0.58, 7.5 kGy 16.33±1.15; 10 kGy 19.00±1.00) (Tables 2 and 3). The gamma radiation caused chemical modifications in compounds, enhancing its action against the *S. aureus* and also the phenolic contents. These data suggest that leaf extracts of *A. occidentale* can be used as substitute of the bark extract (Tables 4 and 5), usually used as therapeutically sources (Mulligan et al., 1993, Blatt & Piazza, 2004).

Conclusions

Crude extracts of barks and leaves of *A. occidentale* submitted or not to gamma irradiation, showed a pronounced activity against the Gram-positive, alcohol-and acid-resistant bacteria, which is associated to tannin contents. The extracts showed a dose dependent activity against *S. aureus*.

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