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Antimicrobial activity of extracts obtained from *Anacardium excelsum* againts some pathogenic microorganisms

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Abstract: In this study,we evaluated the antimicrobial activity of secondary metabolites presents in extracts and fractions of *Anacardium excelsum* (Anacardeaceae), using agar well diffusion assay and bioautography. As a general result was determined that *Anacardium excelsum* has good antimicrobial activity against gram positive microorganisms. Regarding the fractions in study, these were tested against *Bacillus subtilis* where the integument, seed and seed coat showed the best antimicrobial activity. Analysis by gas chromatography (GC) with mass selective detector (MSD) established the presence of some phenolic compounds like 2-(1,1-dimethylethyl)-4-(1,1,3,3-tetramethylbutyl)phenol, which is responsible of the activity found.

Key words: *Anacaurdium excelsum*, microbial activity, phenolic compounds

نشاط مضادات الميكروبات لمستخلص نبات ***Anacardium excelsum*** ضد بعض الكائنات الحية الدقيقة المرضة

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الملخص: في هذه الدراسة تم تقييم نشاط مضادات الميكروبات فى المركبات الثانوية الموجودة في مستخلصات ومقاطع نبات (Anacardeaceae) *Anacardium excelsum*، باستخدام طريقة فحص في مادة الاجار bioautography . كنتيجة عامة تحددت ان نبات *Anacardium excelsum* يمتلك نشاط جيد ضد الميكروبات الموجبة ضد الكائنات الحية الدقيقة الموجبة لصبغة جرام. وفيما يتعلق بالمقاطع فقد تم اختبارها ضد ميكروب *Bacillus subtilis* حيث اظهرت افضل اداء ونشاط مضاد للميكروبات في مستخلصات البذور والغطاء الخارجي للبذور. وبالتحليل بواسطة gas chromatography ومع جهاز mass selective detector (MSD) تم العثور على بعض المركبات الفينولية مثل 2-(1,1-dimethylethyl)-4-(1,1,3,3-tetramethylbutyl) وهي المسؤولة عن هذا النشاط.

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Introduction

The wild cashew (*Anacardium excelsum*; *Anacardium rhinocarpus, Rhinocarpus excelsa*), Ber. et Balb (Bartholomaus, 1995) is a tree in the flowering plant Anacardiacea. The tree is common in the Pacific and Atlantic watersheds, growing from the sea level to 1000 m, found as far north as Guatemala and extending south into Ecuador. It received different names like cajuhy or cashu (Brasil), espavel (Costa Rica), nariz (Cuba), caschou (Guyana), caracoli (Panamá, Ecuador, Colombia), merey (Venezuela) (Cáceres, 1980), (Programa Colombia forestal, 2005). In Colombia is found in the lower valley of Atrato river, in the foothills of Andes mountains and in the Colombian Amazon region (Cáceres, Meneses and Quintero, 1980).

It is a large evergreen tree growing to 45 m tall, with a straight, rose-hued trunk reaching 3m in diameter, brown color with medium size lenticels equi-dimentionals, and few inconspicuous (Maderas Colombianas, 1970). The leaves are simple, alternate, oval-shaped, 15–30 cm long and 5–12cm broad (Cáceres, Meneses and Quintero, 1980). The flowers are produced in a pinnacle up to 35cm long, each flower small, pale green to white (Smith et al., 2004, Espinal, 1963). The true fruit is a 2–3cm long drupe shaped like a kidney supported by a green stalk (Cáceres, 1980). The nuts of wild cashew are a treat for monkeys but raw nuts contain oil poisonous for man. Roasted nuts are free from the poison and as tasty as their relatives, cashew nuts. In Valledupar region in Colombia is used cooked and ground to make a kind of edible bread called Caracoli bread (Araujo, 2008; Smithsonian, 2008).

Maturation occurs in from March to May (Nichols, 1991). Anacardium is one of the most studied genera, determining multiple chemical and biological activities; these are summarized in Table 1. The aims of this study were to determine the antimicrobial activity of total extracts and fractions of *Anacardium excelsum* against some microorganisms pathogens such as *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Salmonella*, and determinate the kind of chemical compounds related to such activity.

Material and Methods

Samples of plant material were collected during the flowering season in the metropolitan area of Bucaramanga, Santander-Colombia, under the following coordinates 7°06'32 .51"N and 73°06'15.19"W, at elevation of 986 m. Specimens of the plats were identified by the National Herbarium of Colombia as *Anacardium excelsum* under classification number 520397 (Figure 1). The material was thoroughly washed in water; leaves, fruits, flowers, bark, seed, seed coat and integument were separated and dried to the environment for 15 days until humidity between 7 and 15% (Figure 1). The dried material was powdered to size of 5 mm and stored in sterile conditions at 3°C until use. From 400 to 600g of dried material were brought under solid-liquid extraction by soxhlet technique for 15 days to obtain the total extract in ethanol. Partial liquid-liquid extraction was made in continuously using solvents in different polarities. Total extracts and fractions were concentrated using vacuum at 40°C (Santos et al., 2010). The antimicrobial activity was tested against *Staphylococcus aureus* and *Bacillus subtilis* (Gram positive) and *Escherichia coli* and *Salmonella* (Gram negative) (Dobner et al., 2003). These microorganisms were obtained from the strain collections of Microbiology Department of Pontificia Universidad Javeriana-Bogota (Colombia). It was tested by agar well diffusion on Mueller Hinton medium. Four holes of 5mm diameter were made by each petri dish and 50µl of extracts and fractions at different concentrations (10, 20 and 40 mg/mL) dissolved in dimethyl sulfoxide (DMSO). The remained hole was use like positive control (Gentemicin) or negative control (DMSO). Then assay samples were incubated for two days at 35°C and readings were made at 24 and 48 hours.

Table 1. *Anacardium* genus plants with chemical and biological activity

Plant	Part Used	Method / Assay / Determination	Activity / Effect	Metabolite	Reference
Anacardium occidentale	Fruit: Anacardic acids extracted and HPLC purified.	RP-HPLC. Anion superoxide. Auto oxidation. Lipoxigenasa. DPPH. Uric Acid generate by Xantina Oxidized	Antioxidant.	Anacardic acids: acid 6-pentadec(en) salicylic.	Kubo et al., 2006
	Fruit: Anacardic acids extracted and HPLC purified.	HPLC. Nano-ESI-MS-MS, GC-MS and NMR. Cinamic acid. Anion superoxide. Uric acid. 2-deoxi guanosine.	Antioxidant.	Alkyl phenols: Anacardic acids, cardanols and cardols.	Trevisan et al., 2006
	Extract from stem bark.	Induced septic shock. Plasma leakage in mouse skin.	Inflammatory.	No reported.	Olajide et al., 2004
	Extract from bark.	Agar Diffusion.	Antimicrobial.	Tannins, alkaloids.	Akimpelu, 2001
	Fruit: Anacardic acids extracted and HPLC purified.	Macrodilution.	Antibacterial.	Acid 6-alqu(en)ilsalicylic.	Kubo et al., 2003
	Fruit Extracts.	RP-HPLC. Agar Dilution.	Antibacterial.	Anacardic acids and cardanols.	Kubo et al., 1995
	Fruit Extracts.	RP-HPLC. UV-VIS. IR. RMN.	Antimollusk.	Anacardic acids, cardanols, cardol and 2-methyl-cardol.	Kubo et al., 1986
	Fruit Extracts.	Mutagenicity.Promotion-initiation in mouse skin tumors. Cocarcinogenic.	Mutagenic, carcinogenic, cocarcinogenic.	No reported.	George and Kuttan, 1997
Anacardium excelsum	All tree (*in situ*).	Estimation of carbon in trees.	Carbon Interchange.	No reported.	Losi et al., 2003
Anacardium humile	Total Extract.	Maceration. Qualitative assay of functional groups.	Anti ulcer.	Bi flavonoids.	Ferreira et al., 2004
Anacardium giganteum	Fruit Extracts.	HPLC. HRGC-MSD. RMN. Citotoxicity of human cell lines.	Antitumor.	Alkyl resorcinols and acid 6-alkilsalic.	Ramos et al., 2004
Anacardium semecarpus	Nuts Extracts.	Necrosis tumor factor. Analysis hispatologic and radiologic.	Anti-arthritic (rheumatoid arthritis). Metabolism of bones.	No reported.	Ramprasath et al., 2006
	Fruits Extracts in formulation ingredients.	Monitoring of lipids, enzymes of lipid and lipoprotein metabolism.	Anti-carcinogenic.	No reported.	Veena et al., 2006
	No reported.	Superoxide dismutase. Catalase and glutathione peroxidase. Lipid peroxides in tissues. Glutathione reductase.	Antioxidant.	No reported.	Ramprasath et al., 2005.

Figure 1. (a) *Anacardium exelsum* plant with flowers; (b) fruit; (c) powdered integument and (d) powdered fruit.

The antibacterial effect was classified based upon inhibition zone diameters, halo greater than 5mm present activity and halo iqual or lower than 5mm without activity (Jasir et al., 2004). Thin layer chromatography (TLC) and bioautography was realized to fractions with microbial activity using Silica gel G60 F_{254} alumina backed plates (3cm × 8cm). Two TLC plate were run, in the first, the sample was applied directly and the chromatograph was run. The layers were developed with Vanillin to determine the region with active compounds. The second chromatographic plate was run and placed in direct contact in the petri dishes with *Bacillus subtilis* culture for 17h at 4°C. Subsequently, plates were removed from each of the petri dishes and incubated at 35°C for 24h (Schmourlo et al., 2005). The active compounds were established in the region where was generated growth inhibition of pathogenic microorganism. To identify the kind of compounds with antimicrobial activity was made a column chromatography according to the results of TLC and bioautography and the fractions obtained were analyzed by GC-MSD.

Results and Discussions

The results of extractions performed with different solvents polarities are shown in Table 2. The efficiency for ethanol extractions in dry material reached the higher yield for seed coat, fruit and seed with 44.6, 36.0 and 29.4% respectively, while the higher extraction efficiencies for partial fractions were: low polarity in fruits (61.7%), medium polarity in leaves (71.7%) and high polarity in bark (63.2%).

Table 2. Weights of total extracts and partial extracts in differents solvents obtained from *Anacardium excelsum.*

				Partial Extracts [g], in:		
Part Plant	Dry Sample Weight [g]	Total Extract in EtOH [g]	Initial Weight [g]	Petrol	DCM	BuOH
Integument	53,4	23,8	23,8	5,1	2,4	12,6
Seed	614,8	140,1	98,1	6,4	4,1	42,6
Fruit	39,2	14,1	14,1	8,7	0,8	1,8
Flower	263,1	53,3	22,2	7,2	5,4	2,7
Seed Coad	476,7	61,3	34,8	10,6	11,1	---
Bark	273,3	73,1	30,1	2,2	2,0	19,0
Leave	325,7	73,2	14,6	3,9	10,4	---

The results of antimicrobial activity (Figure 2) with total ethanolic extract not showed inhibition against *Escherichia coli* and *Salmonella,* possibly these gram negative microorganisms did not generate sensitivity to the total extracts, because of the complexity of its cell wall. Gram negative bacteria contain a very thick layer of peptideglycan composed of polysaccharides which develop links among themselves and also with amino acids preventing the entry of some kind of chemical compounds. In the other hand, Gran positive bacteria, *Staphylococcus aureus* and *Bacillus subtillis,* were inhibited. The table 3 summaries the results of this inhibition.

The ethanolic extracts were fractioned with petroleum ether (petrol), dichloromethane (DCM) and butanol (BuOH) and used them in the microbial assay inhibition with *Bacillus subtillis*. As result we found that only the fraction of integument, seed and seed coat presented microbial inhibition (Table 4). The concentration used in this assay was 40 mg/ml and dissolved in DMSO. The positive control was gentamicin and negative control DMSO.

Table 3. Antimicrobial activity of total extracts from *Anacardium excelsum* in differents concentration against *Staphylococus aureus* and *Bacillus subtillis.* The microbial activity was measured according to the inhibition grown in milimeters (mm).

	Staphilococcus aureus (mm)			*Bacillus subtillis* (mm)		
Part Plant	10 ug/mL	20 ug/mL	40 ug/mL	10 ug/mL	20 ug/mL	40 ug/mL
Integument	12	15	19	14	16	20
Seed	0	11	12	13	15	17
Fruit	12	14	18	10	13	13
Flower	11	12	14	14	15	17
Seed Coat	13	14	16	10	13	16
Bark	9	10	12	7	10	12
Leave	12	14	14	15	15	18

Table 4. Antimicrobial activity of partial extracts from Integument, seed and seed coad against *Bacillus subtillis*. The microbial activity was measured according to the inhibition grown in mm.

	Partial Fraction (mm)		
Part Plant	Petrol	DCM	BuOH
Integumento	15	16	19
Seed	13	13	16
Seed Coad	17	11	13

The bioautography assay was carried out only for the extracts that showed antimicrobial activity against *Bacillus subtillis*, using a silica gel TLC plates. Them were revealed with long-wave U.V. light and fluorescence was detected in the spots where are presents the antimicrobial compounds. Clear inhibition zones in the Petri dishes incubated with TLC were observed to integument fraction. These bands are in different polarities but only the high polarity zone showed inhibition against *Bacillus subtillis* (Figure 3).

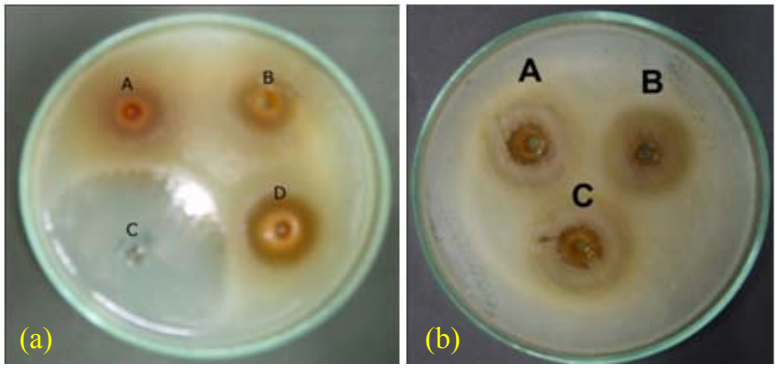


Figure 2. (a) Antimicrobial activity of total seed extract *A. excelsum* against Bacillus subtilis, A: 20 mg / ml, B: 10 mg / ml, C: 40 mg / ml; (b) Antimicrobial activity of fractions obtained from ethanolic extracts of integument; A: petrol, B: Dichloromethane; C: Positive control (gentamicin); D: Ethanol.

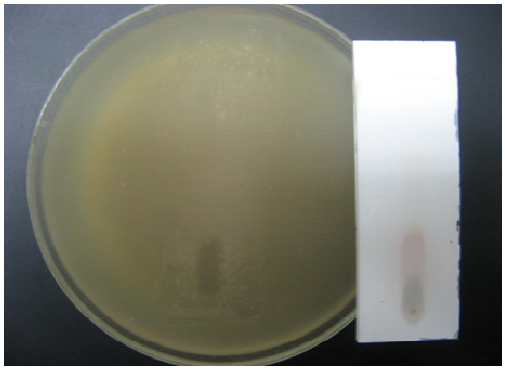
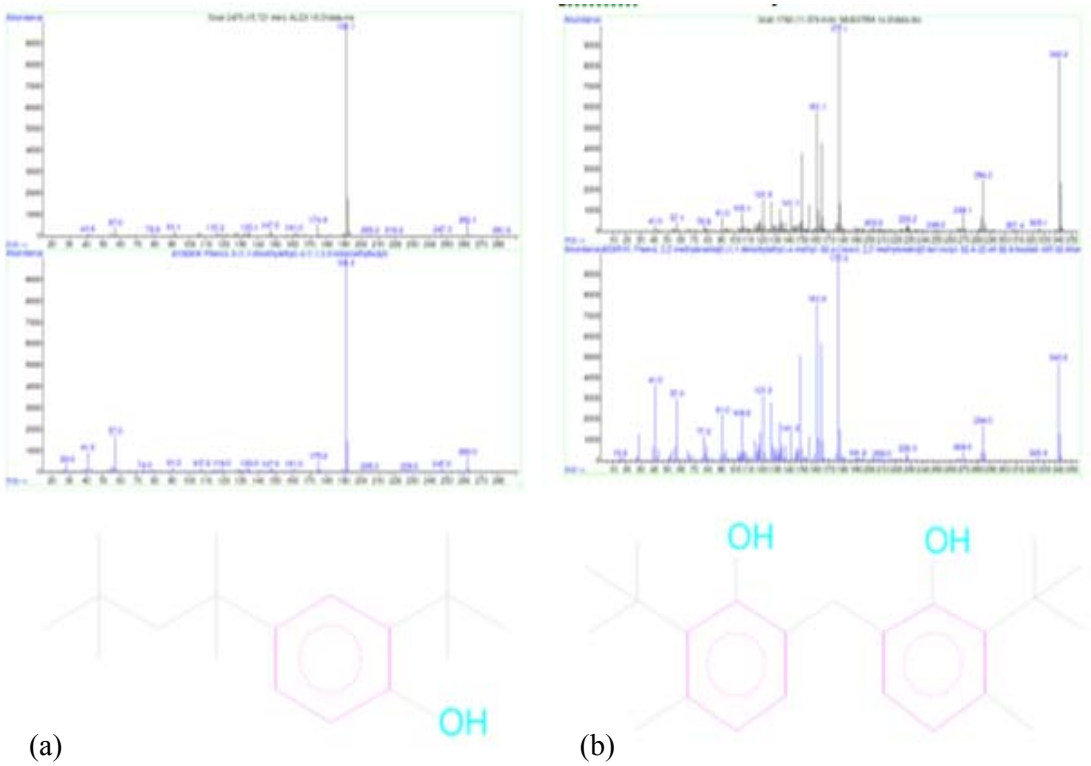


Figure 3. Bioatography in TLC using DCM:MeOH (1:1) as movil phase to the fraction methanol/butanol obtained from integument of *Anacardium excelsum*.

To determine the type of compound microbiologically active was made a percolation column with different solvents using the polar fraction of integument. These fractions were analyzed in a gas chromatograph coupled a mass selective detector. Sixteen fraction from the percolation process were obtained and their analyses by GC/MSD are summarized in the table 5 and figure 4.

Table 5. Compounds identified by GC/MS, according to mass spectra library Wiley.

Percolation Sample	Retention Time (min)	Molecule
2	9.36	2-methyl-2-propenoic acid
4	6.30	2,2',6,6'-Tetramethyl-4,4'-methylenediphenol
	8.90	Hexadecane
5	15.72	2-(1,1-dimethylethyl)-4-(1,1,3,3-tetramethylbutyl)phenol
6	7.36	1,2,3-Benzenetriol
	16.75	N-(Benzyl) benzenesulfonamide
7	11.26	9-Octadecenoic acid,12-hydroxy-, methyl ester
10	15.75	2-(1,1-dimethylethyl)-4-(1,1,3,3-tetramethylbutyl)phenol
14	4.6	2,4-Dioctylphenol
	9.9	2,6-Di[p-cyanophenyl]-4-picoline
	11.57	2,2'-methylenebis(6-(1,1-dimethylethyl)-4-ethyl-pheno
	13.56	methyl 2-[2-(2-ethyl-1,3-dioxolan-2-ylmethyl)-1-hydroxipent-4-enyl]-4-methyl)furan-3-carboxylate



(a) (b)

Figure 4. (a) 2-(1,1-dimethylethyl)-4-(1,1,3,3-tetramethylbutyl) phenol identified by GC/MS; (b) *2,2'-methylenebis*(*6-*(*1,1-dimethylethyl*)-4-ethyl-phenol identified by GC/MS.

According to the results obtained by GC/MSD, it was achieved data only from the percolate samples number 2,4,5,6,7,10 and 14 which evidenced the presence of certain chemical groups derived from terpene, benzene and phenols. These results suggest that the possible compound with antimicrobial activity are the 2-(1,1-dimethylethyl)-4-(1,1,3,3-tetramethylbutyl)phenol present in the fraction 5 and 10 with retention time of 15.7 minutes and the 2,2'-methylenebis(6-(1,1-dimethylethyl)-4-ethyl-phenol found in the fraction 14 with retention time of 11.57 minutes. This kind of compounds has powerful antimicrobial and antiseptic properties resulting in an active germicidal action, due to its toxic potential (Routh, 1990). It is also possible to correlate the presence of this type of phenols with the results of the autobiography analysis where the band of very high polarity, was the one that showed higher antimicrobial activity against *bacillus subtillis*.

Conclusions

The data collected during chemical analysis and bioactivity test of *Anacardium excelsum* allows to draw following conclusions.

The extracts from leaves, fruits, coat, seed coat, seed, flower and bark of *Anacardium excelsum* have antimicrobial activity against gram positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* and they are no active against gram negative bacteria *Escherichia coli* and *Salmonella*.

The ethanol fraction of seed coat and integument, and petrol fraction from seed coat have the higher antimicrobial activity against *Bacillus subtilis*, the activity was evidenced through autobiographical analysis.

It was evidenced by GC/MS the presence of phenolic compounds characteristic of *Anacardiacea* family which are associated with the high antimicrobial activity determined in the study.

Some of these phenolic compounds are toxic to the man but those present in the fruit of *Anacardium excelsum* specie are easily broken by temperature when cooked and used as food.

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