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Anacardic acid derivatives from brazilian propolis and their antibacterial activity

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Abstract: Propolis is a sticky, gummy, resinous substance collected by honeybees (*Apis mellifera* L.) from various plant sources, which has excellent medicinal properties. This paper describes the isolation and identification of triterpenoids and anacardic acid derivatives from Brazilian propolis and their antibacterial activity. Their structures were elucidated by ^{1}H and ^{13}C NMR, including uni- and bidimensional techniques; in addition, comparisons were made with data from academic literature. These compounds were identified as: cardanols (1a + 1b), cardols (2a + 2b), monoene anacardic acid (3), α-amirine (4), β-amirine (5), cycloartenol (6), 24-methylene-cycloartenol (7) and lupeol (8). The determination of the position of the double bond after a reaction with Dimethyl disulfide (DMDS) is described for the phenol derivatives. The ethanolic extract was tested *in vitro* for antimicrobial activity by using the disc diffusion method and it showed significant results against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Shigella* spp.

Keywords: Anacardic acid; Antibacterial activity; Triterpenoids; Brazilian propolis; Alkylthiolation.

Introduction

Propolis is a natural resinous product collected by bees from the buds and exudates of plants. It is used to seal the hives and, more importantly, to prevent the decomposition of creatures that have been killed by bees after invading the hive [1-2]. The use of propolis for various purposes has a long history dating back to at least 300 BC [1] and even now, in the 21st century, it is used in home remedies and personal products [3]. Propolis has also gained popularity in various parts of the world, including the United States, Japan, and the European Union, where it is claimed that it improves human health and prevents conditions such as inflammation, heart disease, diabetes and even cancer. Propolis and its constituents [4-6] are reported to have biological effects, such as being antibacterial, antifungal, cytotoxic, anti-protozoan, antioxidant, anti-inflammatory, antiseptic, spasmolytic and having anesthetic properties.

Although research has concentrated on the *Apis mellifera* L. bee - because of its commercial appeal - about 20,000 bee species are known worldwide, 1,000 of which collect propolis. Brazil has an estimated 350–600 species, where flora is abundant. Propolis has a very complex chemical composition that varies according to which vegetable source the bees use [7]. The composition of South American propolis varies considerably, as has been shown with Brazilian samples [4.8]. Recently, there has been increasing interest in

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Brazilian propolis [8-9] and many new compounds have been identified [10]. Because most of these studies used propolis from Brazil's south and southeast, we decided to carry out a study on northeastern propolis from the state of Piauí.

This paper describes the isolation and identification of the triterpenoids α-amyrin, β-amyrin, lupeol, cycloartenol and 24-methylenecycloartanol and the phenolic derivatives cardanol, cardol and anacardic acid from propolis produced by *Apis mellifera*. These compounds are characteristic of *Anacardium occidentale* L., a family of trees that produces cashew nuts and is commonly called "cajueiro". This paper also describes *in vitro* evaluation of the antibacterial activity of the ethanolic propolis extract against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Shigella* spp.

Experimental

Propolis origins

The sample of propolis, produced by bees of the species *Apis mellifera*, was collected in the apiary of the Center for Agrarian Sciences of the Federal University of Piauí, in the city of Teresina - PI (Northeastern Brazil).

Chemical analyses

Chromatography was carried out on Vetec silica gel 60 (0.063-0.200 mm); Merck silica gel 60 (0.2-0.5 mm) and Sigma Sephadex LH-20 columns. Hexane, ethyl acetate, methanol, dichloromethane and acetone were obtained from Merck (analytical grade), Vetec (analytical grade) and Synth (analytical grade).

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX 500 spectrometer in CDCl$_3$ using TMS as the internal standard (δ=0).

Chromatographic analysis

Analysis of the fractions was performed on Shimadzu GC-17A / MS QP5050A (GC/MS system): DB-5HT capillary column (30 m x 0.251 mm, 0.1 μm film thickness); carrier gas: helium 1.7 mL/min; column inlet pressure 107.8 kPa; column flow = 1.7 mL/min; linear velocity = 47.3 cm/sec; total flow 24 mL/min; carrier flow 24 mL/min; injector temperature 300 °C; detector temperature 310 °C; column temperature 100 (1 min) - 310 °C at 10 °C/min (15 min). Mass spectrometer operating conditions 70 eV of ionization energy.

Extraction and isolation of the compounds

Constituents from a 5.0g sample collected in January were isolated by extraction using 50 mL of methanol in a Soxhlet apparatus for two hours. The extract was filtered while hot, diluted with 25 mL of water and extracted successively with petroleum ether (3×75 mL) and diethyl ether (3×75 mL). The diethyl ether extract (3.29 g) was concentrated and subsequently chromatographed on a silica gel column with mixtures of hexane and diethyl ether of increasing degrees of polarity. Sixty fractions (100 mL each) were collected. The fractions F-4, F-5 and F-13 eluted with hexane-diethyl ether (3:1) and F-51 with hexane-diethyl ether (1:1) were analyzed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Another propolis sample was collected in the same apiary in June and used in an ethanolic extraction carried out by maceration, at room temperature. It was then divided into two parts: one was analyzed to confirm the presence of anacardic acid derivatives and the other part was submitted to antibacterial activity tests.

Methylation

The fraction containing anacardic acid was subjected to methylation with diazomethane [11]. Methyl esters were prepared by treatment with an excess of previously prepared ethereal diazomethane and maintained at –20 °C. The reaction, after 3 hours, yielded methyl esters quantitatively, and took place at 5 °C. The solvent was then evaporated in a vacuum. A small portion of the crude reaction was analyzed in GC-MS.

Alkylthiolation reaction

The position of the double bonds of unsaturated hydrocarbons was determined by iodine-catalyzed methylthiolation using dimethyl disulphide – DMDS [12-14, 22-23]. Approximately 50 mg of the phenolic fraction was kept dehydrated using anhydrous calcium chloride for 24 hours and then incubated with 0.3 mL of DMDS containing 10 mg/mL in ethyl ether of I$_2$ at 25 °C, for 4 hours. After reduction of the excess I$_2$ with a saturated NaHSO$_3$ aqueous solution, 3 mL of hexane was added into the mixture. 1 μL of the hexanic phase was injected into the GC-MS.

Antibacterial activity

The ethanolic extract of the propolis studied was tested against the following micro-organ-
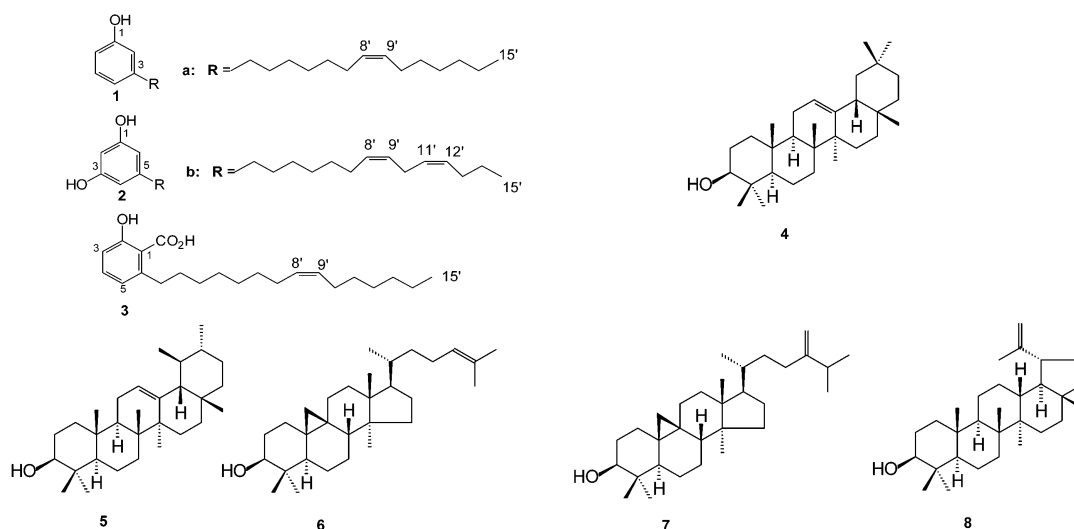
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Figure 1. Compounds isolated and/or identified in the methanolic extract of Brazilian propolis (*Apis mellifera*).

isms: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 26753), *Staphylococcus aureus* (ATCC 25923) and *Shigella sp.* The microbiological experiments were carried out using the disc diffusion technique [15]. The inoculations were standardized to 10^6 cfu (colony former unit), using the MacFarland scale. In a Petri dish, a volume of 1 mL of each suspension was added to 20 mL of the medium BHI (Brain Heart Infusion), which had been previously melted and subsequently cooled, and homogenized until it solidified. After the medium solidified, filter paper discs (cecon) impregnated with 0.01 mL of the sample was placed on top of the medium. The plates containing the culture medium were incubated for 24 hours. The results measured the inhibition halos using calipers or an ordinary ruler.

Results and discussion

The structures were elucidated by ^{1}H and ^{13}C NMR, including uni- and bidimensional techniques, and also compared with data from scientific literature. It was concluded that fraction F5 is a mixture of triterpenoids: α-amyrin (4); β-amyrin (5); cycloartenol (6); 24-methylenecycloartanol (7) and lupeol (8) [9,16-19]. The fractions F4, F-13 and F-51 are composed of a mixture of cardanols (1a+1b), cardols (2a+2b) and anacardic acid (3), respectively (Figure 1).

The ^{1}H NMR spectrum of fraction F-5 showed signals of between δ 5.15 and 5.06 of olefinic hydrogens, two signals of δ 3.20 and 3.18 of hydroxymethine groups, singlets of methyl hydrogens and allylic methyl group (δ 0.6-2.0) of triterpenoids and characteristic signals of cyclopropane methylene hydrogens (δ 0.28 and 0.52, d, J = 4.1 Hz). The structures were also suggested based on some C-13 data, such as 17.5 (C-29), 21.4 (C-30), 124.4 (C-12) and 139.6 (C-13) to α-amyrin (4); 121.7 (C-12) and 145.2 (C-13), 33.3 (C-29), 23.7 (C-30) to β-amyrin (5); 20.4 (C-9), 26.0 (C-10), 125.3 (C-24), 130.9 (C-25) to cycloartenol (6); 20.4 (C-9), 26.0 (C-10), 156.9 (C-24) and 105.9 (C-31) to 24-methylenecycloartanol (7); 16.1 (C-25), 150.9 (C-20), 109.3 (C-29) and 19.7 (C-30) to lupeol (8). The NMR ^{1}H and ^{13}C data coincides with that found in other studies [16, 18].

The ^{1}H NMR spectrum of F-4 fraction presented four signals in the aromatic region with the pattern of a 1.3-disubstituted ring, δ 6.75 (d, H-4) 6.64 (d, H-6), δ 6.68 (s, H-2) and δ 7.12 (t, H-5). Signals at δ 5.53-5.42, δ 2.54 – 2.59 and δ 2.79 were assigned to olefinic, benzylic and double allylic hydrogens, respectively.

The ^{1}H NMR spectrum of F-13 showed two singlets (δ 6.22 and 5.15) with a relative integration of 2:1, respectively. These NMR signals are characteristic of hydrogen nuclei in a 1, 3, 5-trisubstituted aromatic ring with two of them being equivalent. Signals due to olefinic (δ 5.33), double allylic (δ 2.77-2.74), benzylic (δ 2.54) and allylic

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hydrogens (δ 2.04-1.98) were also observed. Comparison with data found in other studies [20], showed that fractions F-4 and F-13 are composed of mixtures of cardanols and cardols, respectively. From the relative integration between aromatic hydrogen (H-2) and allylic hydrogen, it was concluded that the mixtures only contained the mono-ene and the diene. Because of the double allylic hydrogen signal integration, it was concluded that the mixture is composed of 27% of diene and 73% of monoene for the cardols and 60% of monoene and 40% of diene for cardanols. This analysis was confirmed by ^{13}C NMR spectroscopy (Table 1) which showed a good correlation between observed and calculated ^{13}C chemical shifts, using the principle of substituent additivity [21].

The ^{1}H NMR spectrum of the F-51 fraction showed it is composed of the anacardic acid monoene. The spectrum consists of three signals for a 1,2,3-trisubstituted aromatic ring at δ 7.30 (t, J=7.9 Hz, H-4) δ 6.83 (d, J=8.3, H-5) and δ 6.72 (d, J=7.4, H-3). All the other signals are compatible with the structure (3).

According to academic literature, the methylenic benzylic hydrogens of anacardic acid, due to the presence of the ortho carboxylic group, appear more deshielded (δ 2.94) than those in cardol (δ 2.56) and cardanol (δ 2.54) [24]. The ^{13}C NMR and GC-MS data confirm this analysis.

The cardanolic fractions (F4) were submitted to alkylthiolation reaction, while the methylated acid anacardic fraction (F-51) was submitted to methylation with diazomethane followed by alkylthiolation reaction, according to experimental. Both the phenolic hydroxyl and carboxylic group were methylated. Mass Spectra (Figure 2) were present as main fragments: m/z 374 (M+), m/z 314 (McLafferty), m/z 343 (M+ - CH$_3$O) and m/z 180 (benzylic rearrangement, cleavage at 1.2).
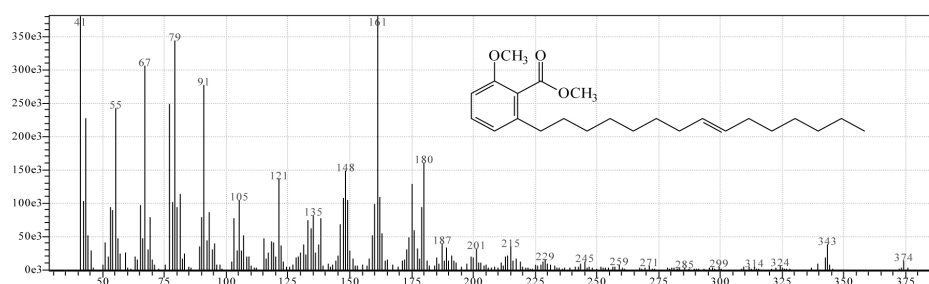
Table 1. ^{13}C NMR data for cardanols (1a + 1b), cardols (2a + 2b) and anacardic acid (3) (125 MHz, CDCl3).

C	1a + 1b*	2a + 2b**	3***
1	155.5	156.6	111.1
2	115.4	100.2	163.4
3	145.0	156.6	115.7
4	121.0	108.1	134.9
5	129.4	146.2	122.6
6	112.5	108.1	147.6
1'	----------	35.9	36.5
8'	129.9	129.9	129.9
9'	130.1	129.9	129.9
13'	31,7	31,7	31,7
14'	22,5	22,5	22,5
15'	14.0	14.1	14.1
C=O	----------	----------	175.2

* δ 130.1 (C8'); 130.2 (C9'); 128.2 (C-11'), 128,4 (C12'); 35.8 (C-13'); 23,1 (C-14) in 1b;
** δ 130.3 (C8'); 130.2 (C9'); 128.1 (C-11'); 128.0 (C12' in 2b)
*** δ 30,9 (C2'); 29.0 (C3'); 29.5 (C-4'); 29.5 (C5'), 29.5 (C5'), 29.7 (C6'), 33.4 (C7') in 3.

The determination of the positions of double bonds after the addition of dimethyl disulfide (DMDS) is described for cardanol (1a) and anacardic acid (3a) as ester. The mass spectra (electron impact 70 eV) show molecular ions (M+) and give key fragments that permit one to determine the position of the original double bond. Cleavage between the sulfur-substituted carbons leads to key fragments which allow one to determine the position of the double bond in the original mono-unsaturated phenolic compound. The spectrometer was set to scan ions of m/z 396, 381, 154 and 107 to cardanol fraction (with the double bond at C-8), while for methylated anacardic acid fraction we detected ions m/z 468 (M+), 453 (M+ - 15) 323

Figure 2. Mass spectrum of the methyl ester of the anacardic acid, GC/EI-MS (70 eV).
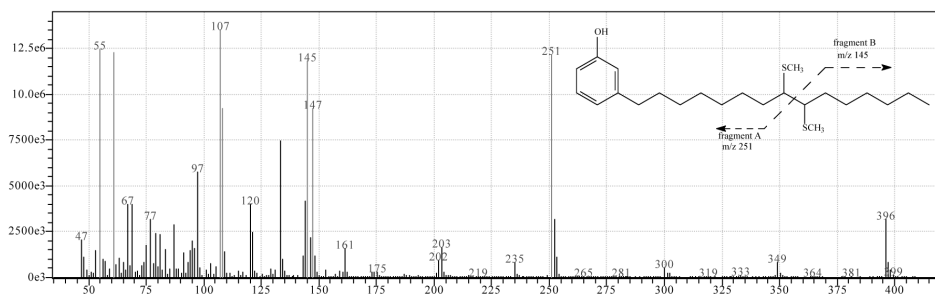
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Figure 3. Mass spectrum of the cardanol (1a), GC/EI-MS (70 eV), after lkylthiolation Reaction of the fraction F-4.

(fragment A), 145 (fragment B) as the main fragments. We presented typical mass spectra to cardanolic fraction (1a). This fraction gave a low relative abundance of cardol (2b), and it was not possible to determine the position of the double bonds in this experiment. According to other reports [14,22-23], if the double bonds are interrupted with more than four carbon atoms, an independent derivation of the alkenes occurs. When the double bonds are closer together, (such as in cardol, and alkene units interrupted by one methylene group) a complex product mixture is formed. Derivation reactions at higher temperatures (60 °C) and longer reaction periods (40 h) result in cyclizations and give complicated heterocyclic compounds.

The presence of anacardic acid derivatives in propolis has not been previously reported and may have occurred because of the presence of several cashew trees (*Anacardium occidentale*) in the apiary neighborhood. These results were confirmed by analysis of propolis samples collected in the same apiary, but in another season, which also contained these compounds. The plant source is of crucial importance for the chemical composition and thus, for the biological activity of propolis from a particular region [25].

The biological activities of the anacardic acid derivatives have attracted considerable attention for acting as molluscicides [26], anti-tumour agents [27-28], antimicrobes [29-32], antioxidants [33] and xanthine oxidase inhibitiors [34].

It was shown that the ethanolic extract has an antibacterial effect against *Escherichia coli* (zone of inhibition = 11 mm); *Pseudomonas aeruginosa* (zone of inhibition 12 mm, *Staphylococcus aureus* (zone of inhibition = 11 mm) and *Shigella* spp (zone of inhibition = 10 mm). Standard DMSO (dimethylsulfoxide) was used as a negative control.

This class of compounds, present in *Anacardium occidentale,* is also present in Ginkgo extracts; in the former they are called anacardic acids and in the latter ginkgolic acids, but both classes are 6-alkylsalicylic acids or 2-hydroxy-6-alkylbenzoic acids. Although the *Anacardium* extracts are reported to be antitumour agents [27,28,30,31] and non-carcinogenic, the Ginkgo extracts are reported to be cytotoxic agents [35,36,37]. However, it should be noted that there is no solid proof of a strong allergic reaction to these alkyl-phenols when taken orally [38].

Conclusion

The propolis samples obtained in different seasons from an *Apis mellifera* apiary at the Federal University of Piauí showed the unusual presence, among other terpenic compounds, of anacardic acid derivatives. The position of the double bonds of the cardanol and anacardic acid, in C-8, was confirmed by iodine-catalysed methylthiolation using dimethyl disulphide. The ethanolic extract had an antibacterial effect against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Shigella* spp.

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M. S. S. Silva, S. G. De Lima, E. H. Oliveira, J. A. D. Lopes, M. H. Chaves, F. A. M. Reis, A. M. G. L. Citó. Derivados de Ácido Anacárdico de Própolis Brasileira e sua Atividade Antibacteriana.

Resumo: A própolis é um produto resinoso que as abelhas recolhem de certas partes da planta e que apresenta excelentes propriedades medicinais. Este trabalho descreve a isolamento e identificação de triterpenóides e derivados de ácido anacárdico de própolies Brasileira e sua anti-atividade. Suas estruturas foram elucidadas por RMN ^{1}H e ^{13}C, incluindo técnicas uni e bidimensionais, e comparação com dados da literatura. Os compostos foram caracterizados como: cardanóis (1a + 1b), cardóis (2a + 2b), ácido anacárdico monoeno (3), a-amirina (4), b-amirina (5), cicloartenol (6), 24-metileno-cicloartenol (7) e lupeol (8). A determinação da posição da ligação dupla após reação com dissulfeto de dimetila (DMDS) é descrita para os derivados fenólicos. O extrato etanólico foi testado *in vitro* para atividade anti-bacterina pelo método de difusão em disco e mostrou resultados significativos contra *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* e *Shigella* spp.

Palavras-Chave: Ácido anacárdico; Atividade anti-bacteriana; Triterpenóides, Própolis brasileira, alquiltiolação.

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