**FT-RAMAN E GC/MS DE EXTRATO E CERAS
EPICUTICULARES DAS FOLHAS DE *ANACARDIUM
OCCIDENTALE* L. DA AMAZÔNIA**

**FT-RAMAN AND GC/MS OF EXTRACT AND EPICUTICULAR WAXES OF
ANACARDIUM OCCIDENTALE L. LEAVES FROM AMAZON.**

RAMOS, Glenda Quaresma¹, MOREIRA DE ALMEIDA, Sheylla Susan², AMARAL, Ana
Claudia Fernandes³, MAIA DA COSTA, Marcelo⁴, DA FONSECA FILHO, Henrique Duarte^{5*}

¹ State University of Amazonas, Programa de Pós Graduação em Medicina Tropical, 69040-000, Manaus, Brazil.

²Department of Health Sciences, Federal University of Amapa, 68903-419, Amapa, Brazil.

³ Laboratory of Medicinal Plants and Derivatives, Farmanguinhos, Fiocruz, 21040-900, Rio de Janeiro, Brazil.

⁴ Pontifical Catholic University of Rio de Janeiro, 22451-900, Rio de Janeiro, Brazil.

⁵ Federal University of Amazonas, Physics Department, 69077-000, Manaus, Brazil.

* Corresponding author
e-mail: hdfilho@ufam.edu.br

Received 25 October 2018; received in revised form 10 December 2018; accepted 13 December 2018

RESUMO

Anacardium occidentale é uma planta mundialmente conhecida e tem grande importância econômica na indústria de alimentos e resinas. Na medicina tradicional, diversos usos são feitos das folhas no tratamento de doenças gastrointestinais e de pele. Suas propriedades biológicas foram confirmadas em estudos antioxidantes, antimicrobianos e antiinflamatórios. Este trabalho teve como objetivo analisar a composição química do extrato etanólico e das ceras epicuticulares das folhas de *A. occidentale* por meio das técnicas de Cromatografia Gasosa (CG/EM) e Espectroscopia Raman (FT-Raman). A análise de FT-Raman mostrou um estiramento do C-H₂, banda característica em ceras epicuticulares de plantas. Na CG/EM foram observados um complexo de misturas alifáticas e cíclicas, entre elas os alcanos e triterpenos. No extrato etanólico os terpenos foram os compostos majoritários identificados por CG/EM. O conhecimento da composição química do *A. occidentale*, principalmente das ceras epicuticulares, mostra-se importante devido a sua relevância no mercado, e torna-se partida para novos estudos de conhecimento agroecológico, como as propriedades das ceras epicuticulares na interação com o meio ambiente, patógenos e herbicidas, e também para a busca de novas propriedades farmacológicas.

Palavras-chave: Anacardiaceae, espectroscopia, cromatografia, terpenos.

ABSTRACT

Anacardium occidentale is a globally known plant and has great economic importance in the food and resins industry. In traditional medicine, several uses are made of leaves in the treatment of gastrointestinal and skin diseases. Its biological properties have been confirmed in antioxidant, antimicrobial and anti-inflammatory studies. This work aimed to analyze the chemical composition of the ethanolic extract and the wax epicuticulares of the leaves of *A. occidentale* by means of the Gas Chromatography (GC/MS) and Raman Spectroscopy (FT-Raman) techniques. FT-Raman analysis showed a stretch of the C-H₂, characteristic band in plant epicuticular waxes. In GC/MS a complex of aliphatic and cyclic mixtures was observed, among them alkanes and triterpenes. In the ethanolic extract the terpenes were the majority compounds by GC/MS. The knowledge of the chemical composition of *A. occidentale*, mainly of the epicuticular waxes, is important due to its relevance in the market, and it becomes a starting point for new studies of agroecological knowledge, such

as the properties of epicuticular waxes in the interaction with the environment, pathogens and herbicides, and also for the search of new pharmacological properties.

Keywords: Anacardiaceae, spectroscopy, chromatography, terpenes.

INTRODUCTION

Anacardiaceae is a large family (Dicotyledonae) which comprises about 70 genera with approximately 875 species widely distributed in tropical regions. In Brazil, it can stand out, mainly, *Anacardium*, *Mangifera*, *Spondias* and *Schinus* genus (Mabberley, 1997). The *Anacardium* genus, described by Carl Linnaeus, is distributed in several regions of the world, showing adaptation in many ecosystems. Into the different species, it is interesting to highlight the *Anacardium occidentale* Lineus, or cashew, the nuts have great value in the international market of food, besides numerous uses in the plastics and resins industry (Fernandes *et al.*, 1993; Paramashivappa *et al.*, 2001).

Several studies have reported the use of various parts of the cashew in traditional medicine. The leaves are used to treat intestinal problems, sore throats, respiratory diseases, diabetes, hemorrhage, antiscorbutic, muscle weakness and urinary disorder (Kamtchouing *et al.*, 1998; Taylor, 2005). Pharmacognostic studies were carried out indicated the presence of flavonoids, anthocyanins, cardiac glycosides, tannins, sterols and triterpenes (Correa *et al.*, 1995; Di Stasi, 2002; Guerrero *et al.*, 2002; Yermakov *et al.*, 2010).

In recent years it has given a focus also on the leaf surface studies, that is a layer that allows interaction between the external and internal environment, this layer is called the cuticle, formed principally of cutin and waxes. The waxes are important to the structure and function of cuticle and are either deposited within the cutin matrix, or accumulate on its surface as crystals or films (Jefree, 2006; Yeats and Rose, 2013). In most cases, the majority of compounds comprising the cuticular wax is derived from very-long-chain fatty acids, including alkanes, aldehydes, primary and secondary alcohols, ketones, and esters. In some species, various lipophilic secondary metabolites, such as triterpenoids, flavonoids, and tocopherols (Tulloch, 1976; Barker, 1982; Bianchi, 1996).

This study aimed to analyze the chemical

constituents of the ethanolic extract and epicuticular waxes from the leaves of *A. occidentale* L. using spectroscopy FT-Raman (Fourier Transform Raman) and Gas Chromatography/Mass Spectrometry (GC/MS).

MATERIALS AND METHODS

2.1. Botanical Material

The fresh leaves (Figure 1) were collected in May 2014 at the Federal University of Amapá campus (0°0'22,9212", S 51°5.5896' W, altitude 6.0 m). The voucher specimens have been in the Amapaense Herbarium (HEMAB) located in IEPA (State of Amapá Institute of Scientific and Technological Research), under registration number 018684.

Figure 1 - *A. occidentale* leaf features. (a) Upper surface. (b) Lower surface.

2.2. Ethanol Extract

To obtain the alcohol extract from the leaves, they were separated, cleaned and dried in a circulating air oven at 40° C for 72 hours. Then, they were milled and the resulting powder, stored. The extract was prepared from five hundred grams (500 g) of leaf powder was

weighed using an electronic weighing balance and soaked in ethyl alcohol, at a ratio of 1:4 (powder/solvent). The powdered leaves were extracted using the ethanol through maceration for 72 h, at room temperature, and that process was repeated three times, and subsequently filtered using filter paper (Matos, 1995). The filtrate was concentrated using rotary evaporator.

2.3. Epicuticular Wax

For extraction of the waxes, after washed and drought, the leaves were placed in contact with Chloroform and agitated slightly by 30 seconds (Hamilton, 1996). The materials obtained were later kept for evaporation to remove the excess solvents.

2.4. Raman Spectroscopy

The analyses of Raman were made in the Coatings Guards Laboratory and Nanostructured Materials at Pontifícia Universidade Católica do Rio de Janeiro (PUC-RJ). For the measurements, a 473 nm laser and a confocal NT-MDT NTEGRA spectrometer were used. The spectra were recorded at 4 cm^{-1} spectral resolution over the wavenumbers 200-3500 cm^{-1} , for no sample degradation, we performed a single scan over a period of the 30s.

2.5. CG/MS

Both the ethanolic extract and the epicuticular wax were analyzed by HP6890 gas chromatograph interfaced with an HP5873, Mass Selective Detector (ionization energy 70 eV), equipped with a DB-5MS capillary column (30 m x 0.25 mm thickness; 0.25 μm film coating). The column temperature was programmed to 70-305 $^{\circ}\text{C}$ at 5 $^{\circ}\text{C}/\text{min}$ using helium as the carrier gas (1.0 mL/min). The injector and detector temperatures were 230 $^{\circ}\text{C}$ and 280 $^{\circ}\text{C}$ respectively. Identification of the components was done based on retention index, compared with reference spectra (Wiley, NIST databases) and MS literature data (Adams, 2009).

RESULTS AND DISCUSSION:

The FT-Raman spectra in ethanol extract showed no characteristic bands enough for interpretation (Figure 2).

Figure 2 – FT-Raman spectra of ethanolic extract.

This may be due to difficulties encountered when making measurements with Raman spectroscopy, for example, some biological samples may cause a strong fluorescence, another problem is the time of exposure to the laser that causes interference and can damage the samples; problems that make it difficult to read the spectra (Scharader *et al.* 1994; Reitzenstein *et al.*, 2007; Littlejohn *et al.*, 2015). The analysis of the wax samples showed peaks characteristic (Figure 3). The bands in 1063 cm^{-1} correspond to the stretching of the C-C ligation. The bands in 1443 and 1463 cm^{-1} are attributed with C-H deformation, the 1299 cm^{-1} is associated with the deformation of CH_2 .

Figure 3 – FT-Raman spectra of the epicuticular waxes.

Figure 4 shows a stronger band between 2700-3600 cm^{-1} . Similar bands were well

characterized in other studies with epicuticular waxes, which correspond to symmetric and nonsymmetric stretching modes CH₂ (Littlejohn *et al.*, 2015; Prinsloo *et al.* 2004; Weissflog *et al.*, 2010; Jetter *et al.*, 2007), and peak at 2930 cm⁻¹ is assigned to the terminal portion of the CH₃ groups (Prinsloo *et al.*, 2004).

Figure 4 – FT-Raman spectra of the epicuticular waxes.

GC/MS analyses of the epicuticular waxes revealed the presence of the 10 constituents, totaling 93.39 % of the composition (Table 1). The identified compounds and their respective percentages are shown in Table 1. According to CG-MS analyses, odd-numbered n-alkanes were the principal wax constituents, accounting for 83.52 % of the identified compounds. The majority of these alkanes are hentriacontane (36.74 %) and nonacosane (33.48 %). It is also verified the presence of triterpenes Moretenol (11.49%), α -amyrin (2.73 %) and Cycloartenol (1.13 %).

Table 1 - Chemical composition of epicuticular waxes by GC/MS.

Retention time (min)	Compound	%
41.790	Heptacosane	0,32
43.207	Octacosane	0,47
44.640	Nonacosano	33,48
45.916	Triacontane	2,27
47.267	Hentriacontano	36,74

48.455	Dotriacontano	1,27
49.694	Tritriacontane	3,49
50.637	α -Amyrin;	2,73
50.882	Cycloartenol	1,13
51.335	Moretenol	11,49

In studies with epicuticular waxes present high proportions of n-alkanes C₂₇ to C₃₃ (Jetter *et al.*, 2007). The terpenoid has been detected in the cuticular waxes of many plant species, and their derivatives too, with small amounts ranging from less than 50% of the total wax mixture (Oliveira and Salatino, 2000; Van Maarseveen and Jetter, 2009).

The GC/MS analyses of the ethanolic extract revealed the presence of the n-alkanes, fatty acids, phytosterols, and triterpenoids (Table 2). The identified compounds and their respective percentages are shown in Table1. The majority compounds were α -tocopherol (21%); sitosterol (17,13%), dammaradienol (12%) and phytol (8,70%). These show that the terpenes were the most prominent compounds in the ethanolic extract leaves.

Table 2 - Chemical composition of ethanolic extract by GC/MS.

Retention time (min)	Compounds	%
19,434	2,4-di-ter-butylphenol	0,49
28,886	Pinane;	0,39
29,291	Palmitic acid;	1,66
29,981	Palmític acid ethyl ester	1,19
32,163	Phytol	8,70
32,616	9,12,15-Octadecatrien-1-ol;	0,47
33,076	Stearic acid;	0,40
33,187	7,10,13-Hexadecatienoic acid, methyl ester	0,65
43.385	Squalene	1,16
44.580	Nonacosane	4,34
47.557	α -Tocopherol;	21,16

49.984	Sitosterol	17,13
50.630	α -Amyrin	2,84
51.305	Dammaradienol;	12,0

The presence of the terpenes and sterols are well detected in the leaves of the cashew and have contributed to the chemical knowledge of the plant (Maia *et al.*, 2000). Tocopherols are well known about their high antioxidant activity (Ammouche *et al.*, 2002; Kiron *et al.*, 2004). Sitosterols have been reported to anti-inflammatory (Dillard and German, 2000), antidiabetic (Ju *et al.*, 2004), antimicrobial and antioxidant (Sen *et al.*, 2012) activities. In general, the triterpenoids compounds have anti-inflammatory activity and cytoprotective effects. Phytol besides having significantly antimicrobial property (Bharathy and Maria, 2012) It is also precursors do tocopherol and related with developed of the the plant (Oyugi *et al.*, 2011; Vom Dorp *et al.*, 2015)

The analyses of the *A. occidentale* compounds revealed the presence of the general compounds with biological activities. Studies performed with leaves *A. occidentale* showing the hypoglycemic effect (Kamtchouing *et al.*, 2007; Alexander-Lindo *et al.*, 2004), antioxidant (Arul and Thangavel, 2011) and antimicrobial (Arul and Thangavel, 2011; Varghese *et al.*, 2013) related with presence that compounds.

CONCLUSIONS:

The data show that Raman that non-destructive technique can be used to identify the chemical composition of natural products. All gas chromatography analyses 21 compounds have been described, the majority n-alkanes, terpenes both the ethanolic extract and the epicuticular wax, respectively. Being the cuticle the first face contact with the external environment and the importance of it against the action of pathogens, chemical study of the epicuticular wax is shown to be necessary for the improvement of cultivars, such as applying herbicides to the leaves.

REFERENCES:

1. Mabberley, D.J. The Plant-Book: A Portable Dictionary of the Vascular Plants. Cambridge University Press, **1997**
2. Fernandes, A.M.A.P., Barata, L.E.S., Ferri, P.H. Lignans and a neolignan from *Virola oleifera* leaves. *Phytochemistry*, **1993**, 32, 1567.
3. Paramashivappa, R., Kumar, P.P., Vithayathil, P.J., Rao, A.S. Novel method for isolation of major phenolic constituents from cashew (*Anacardium occidentale* L.) nut shell liquid. *J Agric Food Chem*, **2001**, 49, 2548.
4. Kamtchouing, P., Sokeng, S.D., Moundipa, P.F., Watcho, P., Jatsa, H.B., Lontsi, D. Protective role of *Anacardium occidentale* extract against streptozotocin-induced diabetes in rats. *J Ethnopharmacol*, **1998**, 62, 95.
5. Taylor, L. The Healing Power of Rainforest Herbs: A Guide to Understanding and Using Herbal Medicinals. Square One Publishers, **2005**
6. Correa, S. M. V., Conserva, L. M., MAIA, J. G. S. Constituents of roots of *Inga edulis* var. *parviflora*. *Fitoterapia* **1995**, 66, 379.
7. Di Stasi, L.C., Hiruma-Lima, C. A. Plantas Medicinais da Amazônia e na Mata Atlântica. 2nd ed. São Paulo: Jézio Hernani Bomfim Gutierrez, **2002**
8. Guerrero, M.F., Puebla, P., Carrón, R., Martín, M.L., Arteaga, L., Román, L.S. Assessment of the antihypertensive and vasodilator effects of ethanolic extracts of some Colombian medicinal plants. *J Ethnopharmacol* **2002**, 80, 37.
9. Yermakov, A.I., Khlaifat, A.L., Qutob, H., Abramovich, R.A., Khomyakov, Y.Y. Characteristics Of The GC-MS Mass Spectra Of Terpenoids (C₁₀H₁₆). *Chem Sci J* **2010**, 2010.
10. Jeffree, C.E. The fine structure of the plant cuticle. In: *Biology of the Plant Cuticle*. Oxford: Riederer, M., Müller, C. (Eds.), **2007**, 11.
11. Yeats, T.H., Rose, J. K.C. The Formation and Function of Plant Cuticles | Plant Physiology. *Plant Physiol* **2013**, 163, 5.
12. Tulloch, A. P. Chemistry of waxes of higher plants. In: *Chemistry and biochemistry of natural waxes*. California: Kolattukudy, P.E., **1976**, 287.

13. Barker, E. A. Chemistry and morphology of plant epicuticular waxes. In: Chemistry and morphology of plant epicuticular waxes. London: D. F. Cutler, K. L. Alvin, C. E. Price (Eds), **1982**, 139–165.
14. Bianchi, G. Plant waxes. In: Waxes: Chemistry, Molecular Biology and Functions. Dundee: R. J. Hamilton (Ed), **1995**, 177–222.
15. Matos, F.J.A. Introdução à Fitoquímica Experimental. Fortaleza, **1988**.
16. Hamilton, R.J. Waxes: Chemistry, Molecular Biology and Functions. Oily Press, **1995**.
17. Adams, R.P. Identification of Essential oil components by gas chromatography/mass spectrometry. 4th ed. Carol Stream, Illinois: Allured Publishing Corporation, **2007**.
18. Riederer, M., Schreiber, L. Waxes—the transport barriers of plant cuticles. In: Waxes: Chemistry, Molecular, Biology and Functions. R. J. Hamilton (Ed), **1995**.
19. Reitzenstein, S., Rösch, P., Strehle, M.A., Berg, D., Baranska, M., Schulz, H., Rudloff, E., Popp, J. Nondestructive analysis of single rapeseeds by means of Raman spectroscopy. *J Raman Spectrosc* **2007**, 38, 301.
20. Littlejohn, G.R., Mansfield, J.C., Parker, D., Lind, R., Perfect, S., Seymour, M., Smirnoff, N., Love, J., Moger, J. In vivo chemical and structural analysis of plant cuticular waxes using stimulated Raman scattering microscopy. *Plant Physiol* **2015**, 168, 18.
21. Prinsloo LC, Plooy W du, Merwe C van der. Raman spectroscopic study of the epicuticular wax layer of mature mango (*Mangifera indica*) fruit. *J Raman Spectrosc* **2004**; 35: 561–567
22. Weissflog, I., Vogler, N., Akimov, D., Dellith, A., Schachtschabel, D., Svatos, A., Boland, W., Dietzek, B., Popp, J. Toward in vivo chemical imaging of epicuticular waxes. *Plant Physiol* **2010**, 154, 604.
23. Jetter, R., Kunst, L., Samuels, A.L. Composition of Plant Cuticular Waxes. In: Annual Plant Reviews Volume 23: Biology of the Plant Cuticle. Wiley-Blackwell, **2007**: 145–181 Available from: <https://onlinelibrary.wiley.com/doi/abs/10.1002/9780470988718.ch4>
24. Oliveira, A.F., Salatino, A. Major constituents of the foliar epicuticular waxes of species from the Caatinga and Cerrado. *Z Naturforschung C J Biosci* **2000**, 55, 688.
25. van Maarseveen, C., Jetter, R. Composition of the epicuticular and intracuticular wax layers on *Kalanchoe daigremontiana* (Hamet et Perr. de la Bathie) leaves. *Phytochemistry* **2009**, 70, 899.
26. Maia, J.G.S., Andrade, E.H.A., Zoghbi M. das, G.B. Volatile Constituents of the Leaves, Fruits and Flowers of Cashew (*Anacardium occidentale* L.). *J Food Compos Anal* **2000**, 13, 227.
27. Ammouche, A., Rouaki, F., Bitam, A., Bellal, M.M. Effect of ingestion of thermally oxidized sunflower oil on the fatty acid composition and antioxidant enzymes of rat liver and brain in development. *Ann Nutr Metab* **2002**, 46, 268.
28. Kiron, V., Puangkaew, J., Ishizaka, K., Satoh, S., Watanabe, T. Antioxidant status and nonspecific immune responses in rainbow trout (*Oncorhynchus mykiss*) fed two levels of vitamin E along with three lipid sources. *Aquaculture* **2004**, 234, 361.
29. Dillard, C.J., German, J.B. Phytochemicals: nutraceuticals and human health. *J Sci Food Agric* **2000**, 80, 1744.
30. Ju, Y.H., Clausen, L.M., Allred, K.F., Almada, A.L., Helferich, W.G. beta-Sitosterol, beta-Sitosterol Glucoside, and a Mixture of beta-Sitosterol and beta-Sitosterol Glucoside Modulate the Growth of Estrogen-Responsive Breast Cancer Cells In Vitro and in Ovariectomized Athymic Mice. *J Nutr* **2004**, 134, 1145.
31. Sen, A., Shukla, K. Analysis of IR, NMR and Antimicrobial Activity of β -Sitosterol Isolated from *Momordica charantia*. *Sci Secure J Biotechnol* **2012**, 1, 9.
32. Bharathy, V., Sumathy, B. M., Uthayakumari, F. Determination of

- phytocomponents by GC-MS in leaves of *Jatropha gossypifolia* L. *Sci Res Report* **2**, 286.
33. Oyugi, D.A., Ayorinde, F.O., Gugssa, A., Allen, A., Izevbigie, E.B., Eribo, E., Anderson, W.A. Biological activity and mass spectrometric analysis of *Vernonia amygdalina* Fractions. *J Biosci Technol* **2011**, 2, 287.
34. Vom Dorp, K., Hölzl, G., Plohm, C., Eisenhut, M., Abraham, M., Weber, A.P.M., Hanson, A.D., Dörmann, P. Remobilization of Phytol from Chlorophyll Degradation Is Essential for Tocopherol Synthesis and Growth of *Arabidopsis*. *Plant Cell* **2015**, 27, 2846.
35. Arul, V., Thangavel, K.P. Antioxidant and antimicrobial activity using different extracts of *Anacardium occidentale* L. *Int J Appl Biol Pharm* **2011**, 3, 436.
36. Varghese, J., Tumkur, V.K., Ballal, V., Bhat, G.S. Antimicrobial effect of *Anacardium occidentale* leaf extract against pathogens causing periodontal disease. *Adv Biosci Biotech* **2013**, 4, 15.