Artigo

Odontadenia lutea (Apocynaceae) LEAVES: PHYTOCHEMICAL STUDY AND INSECTICIDAL ACTIVITY AGAINST LEAF-CUTTING ANTS *Atta sexdens rubropilosa* Forel

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The present work describes the chemical constituents of *Odontadenia lutea* (Vell.) Markgr. leaves and the toxicities of its extract and fractions against *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae). Chromatographic procedures of the ethanolic extract resulted in the identification of the triterpene β-amyrin, the flavonoid rutin, two fatty acids palmitic and linolenic, and one glyceroglycolipid 3-*O*-(9,12,15-octadecatrienoyl)-glyceryl-β-D-galactopyranoside, which are known compounds, but they are described for the first time in the *Odontadenia* genus. The known triterpenes lupeol and α-amyrin were also identified. Structural identification of the compounds was performed by analysis of IR, ESI-MS, and 1D and 2D NMR spectra. The toxicity of its extract and fractions from *O. lutea* leaves was tested against leaf-cutting ants *Atta sexdens rubropilosa* Forel by employing ingestion bioassay procedures. The hexane fraction (2 mg mL^{-1}) decreases the average survival of ants from sixteen to six days, causing 98% mortality on the 14th day and 100% at the end of the experiment.

Keywords: triterpenes; cutting ants; *Odontadenia lutea*; monogalactosylmonoacylglycerol; Apocynaceae.

INTRODUCTION

The Apocynaceae family produces through their secondary metabolites a wide range chemical of compounds[1] among which are the alkaloids,[2-4] flavonoids,[5,6] triterpenoids,[7] cardenolides,[8,9] pregnanes, and iridoids.[10-12]

Odontadenia is an Apocynaceae genus composed of twenty species, whose occurrence is reported mainly in the territorial strip between Guatemala and Brazil.[13,14] However, despite the diversity of studies related to the Apocynaceae, few studies have examined the phytochemistry and biological activities of *Odontadenia*. Fractionation of the methanolic extract from *O. macranta* leaves resulted in the isolation of the pentacyclic triterpenes α-amirin and lupeol, and the limonoid odontadenin A; the latter showed a moderate cytotoxic effect against the tumor cell line A2780.[15] From the methanolic extract of *O. punticulosa* leaves, two saponins were isolated with antifungal activity against on *Candida albicans*: pulsatilla saponin D and 3β-O-[β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosylhederagenin.[16]

In view of the phytochemical potential of Apocynaceae as well as the scarcity of reports about *Odontadenia* genus, this research aimed to evaluate the insecticidal activity and to perform the phytochemical study of *O. lutea* leaves.

EXPERIMENTAL

General experimental procedures

Infrared (IR) spectra were acquired in a PerkinElmer Spectrum Frontier spectrophotometer. It operated from 4000 to 400 cm^{-1} for samples dispersed in KBr pellets, and when it was equipped with Attenuated Total Reflectance (ATR) device, it operated from 4000 to 700 cm^{-1}.

Nuclear Magnetic Resonance (NMR) spectra were acquired in the Bruker Avance III apparatus (11.75 T). A 5 mm broadband probe head with a z-gradient at 25 °C was used while operating at the frequency of 500.13 MHz for ^{1}H and 125.75 MHz for [13]C. Eventually, Heteronuclear Single-Quantum Correlation (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) experiments were performed. CDCl$_3$ and MeOD were used as solvents and tetramethylsilane (TMS) was used as an internal standard. The experiments were performed in 5 mm diameter tubes and were collected using Topspin 3.5 software (Bruker BioSpin).

Mass spectrometry (MS) was performed on the Bruker micrOTOF-Q III apparatus. The electrospray source (ESI) was operated in the negative and positive modes. High purity nitrogen (>98%) was used as the desolvating (200 °C; 4 L min^{-1}) nebulizer and collision gas (200 °C; 4 L min^{-1}). Nebulizer pressure was kept at 0.4 bar and the capillary voltage set at 4500 V. The Q-TOF conditions were as follows: Endplate offset: -500 V; Funnel 1: 200 Vpp; Funnel 2: 200 Vpp; Hexapole RF: 200 Vpp; Collision RF: 200 Vpp; Transfer Time: 70-85 μs; Pre Pulse Storage: 5-7 μs; Ion Energy Quadrupole: 5eV. The mass spectra were acquired and processed using a Bruker Compass Data Analysis Software (Bruker Daltonik, GmbH).

For extraction and isolation, ethanol, hexane, dichloromethane, ethyl acetate, and methanol P.A. (Anidrol, Dinâmcia, and Neon) were used. For the isolation by column chromatography, cellulose microcrystalline (Loba Chemie), Diaion HP-20 (Sigma-Aldrich), Sephadex LH-20 (Sigma-Aldrich) and Silica gel 230-400 mesh (Macherey-Nagel) were used.

Plant material

Leaves of *Odontadenia lutea* (Vell.) Markgr. were collected in August 2013 at the Campus de Ciências Exatas e Tecnológicas of

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the Universidade Estadual de Goiás (UEG), Anápolis, GO, Brazil (latitude 16º22'50.5"S, longitude 48º56'40.9"W). The specie was identified by Dr. Mirley Luciene dos Santos and a specimen voucher (HUEG 11381) was deposited in the Herbarium of the Universidade Estadual de Goiás. Number of SisGen: A7D29BD.

Extraction and isolation

The plant material (1.2 kg) was dried in an air circulation oven at 45 °C for 48 hours and pulverized in a Willey knife mill. The pulverized material (303 g) was extracted with ethanol (10 L) in a maceration process and then filtered. The filtrate was reduced using a rotatory evaporator, yielded the ethanolic extract (54.7 g). The crude ethanolic extract of *O. lutea* leaves (OLFE) was fractionated by vacuum filtration with the incorporation of microcrystalline cellulose (55 g) and passing of solvents in increasing order of polarity: hexane, dichloromethane, ethyl acetate, and methanol, 5 L each. After fractionation, the solvents were evaporated on a rotary evaporator, yielding the hexane (OLFEH, 9.9 g), dichloromethane (OLFED, 3.0 g), ethyl acetate (OLFEA, 10.6 g) and methanolic (OLFEM, 15.8 g) fractions.

The OLFEH fraction (8.7 g) was chromatographed using silica gel 60 (SiO_2) column chromatography (CC) (230-400 mesh, 5.0 x 15.0 cm, hexane/EtOAc, gradient, 9.9:0.1→0:10), yielding 174 fractions (15 mL each). Fraction 61-72 (110 mg) was chromatographed (SiO_2, 230-400 mesh, 2.0 x 22.0 cm, hexane/EtOAc, gradient, 9:1→0:10) yielding 91 fractions. Fraction 30 (5 mg) gave mixture **1**.

The OLFEM fraction (10 g) was chromatographed using Diaion HP-20 CC (5.0 x 14.0 cm, H_2O/MeOH, gradient, 10:0→0:10) and 145 fractions were obtained. Fraction 58-77 (1.9 g) was recrystallized with MeOH to yield **2** (375 mg). Fraction 136-144 (77 mg) was chromatographed (Sephadex LH-20, 2.0 x 48.0 cm, MeOH, isocratic) yielding the mixture of **3a** and **3b** (16 mg) and **3b** and **4** (28 mg).

Insecticidal activity

Bioassays were used to study the effect of the extract and fractions of *O. lutea* on ants *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae). The worker ants used in the assays, whose body mass was about 20-25 mg, were randomly picked from a laboratory nest kept at Centro de Estudos de Insetos Sociais, UNESP, Rio Claro, São Paulo, Brazil. The ants were fed with leaves of *Eucalyptus* sp., oat flakes and occasionally *Hibiscus* sp., *Ligustrum* sp. or leaves and rose petals.

Ants isolated from the anthill were kept on a solid artificial diet, which was prepared with 1.25 g of glucose, 2.5 x 10^{-1} g of bacteriological peptone, 2.5 x 10^{-2} g of yeast extract, 2.5 x 10^{-1} g of bacteriological agar, and 25 mL of distilled water.[17] After being solubilized in a microwave oven and autoclaved for 15 minutes at 120 °C and 1 atm, the diet was poured hot into Petri dishes of 10 cm diameter, previously sterilized. Upon cooling and solidification, it was wrapped in PVC film and kept in a refrigerator for use during the experiment period.

The insecticidal activity on the ants was verified by the ingestion of the extract and fractions of the leaves of *O. lutea* incorporated into the solid artificial diet in three concentrations (0.2, 1.0, and 2.0 mg mL⁻¹). First, a diet preparation was made as described and after being autoclaved, poured into a Petri dish, previously sterilized, and mixing the extract when the temperature was close to 40 °C. And after cooling and solidification, it was wrapped in PVC film and kept in a refrigerator for use during the experiment period.

Fifty ants were put into five Petri dishes (ten ants per dish) for each treatment. The control diet, or diet plus test compounds, was placed on aluminum foil in the approximate amount of 4 x 10^{-1} to 5 x 10^{-1} g plate⁻¹. These plates were placed in oven B.O.D. at 24 ºC (± 1) with relative humidity > 70%. The experiments were examined daily for the removal and annotation of the number of dead ants, diet renewal, and exchange of the filter paper during a maximum period of 25 days, considering the premise of the normal survival period of ants kept with artificial diet.

The analysis was performed by determining the accumulated mortality per day of treatment. Subsequently, the median survival time was determined, and the survival curves were compared using the log-rank non-parametric test (p < 5 x 10^{-2}) through Graph-Pad Prism 3.0 software.

RESULTS AND DISCUSSION

Phytochemical study

The phytochemical investigation of the ethanolic extract of *O. lutea* leaves provided seven compounds (Figure 1). A hexane-soluble fraction of the ethanolic extract yielded lupeol (**1a**), α-amyrin (**1b**), and β-amyrin (**1c**). These compounds could not be separated after successive chromatographic analyses, and they were identified on the basis of IR, and ¹H, and ¹³C NMR (Figures 1S-3S). They have been previously isolated from Apocynaceae genera;[7,18,19] lupeol (**1a**), and α-amyrin (**1b**) have been reported from *O. macrantha*,[15] and from species of genera *Hoya* and *Mandevilla*.[20,21] For these triterpenes are reported antioxidant, anti-inflammatory and cytotoxic activities.[22-24]

A methanol-soluble fraction of the ethanolic extract yielded the flavonoid rutin (**2**), the glyceroglycolipid 3-*O*-(9,12,15-octadecatrienoyl)-glyceryl-β-D-galactopyranoside (**3a**), and the two fatty acids palmitic (**3b**) and linolenic (**4**). The flavonoid was identified on the basis of spectroscopic analysis, which showed close agreement with published data for rutin (**2**) (IR; ¹H and ¹³C NMR; HMBC; Figures 4S-7S).[25] Rutin (**2**) was isolated from several species of Apocynaceae, among them *Hancornia speciosa* Gomes and *Alstonia boonei* De Wild, which exhibited anti-inflammatory,[26] antioxidant and antimicrobial activity.[6]

Compounds **3a** and **3b** were obtained in a mixture, which exhibited spectral data (Figures 8S-13S) indicating the presence of a glyceroglycolipid and a fatty acid. The ¹H NMR spectrum displayed signals for hydrogens of oxygenated carbons at δ_H 3.56 (m), 3.67 (m), 4.09 (dd), and 4.17 (dd) resembling the glycerol system. From the HSQC and HMBC experiments the observed correlation between the ¹H signals at δ_H 4.09 and 4.17 and the ¹³C signal at δ_C 174.0, whose hydrogens were coupled to each other and to ¹H signals at δ_H 3.67, and the latter to ¹H signals at δ_H 3.56 led to their assignments as acyl group (C-1''), 2H-4, H-3, and 2H-1. The hydrogens at δ_H 3.56 showed correlation with the ¹³C signal at δ_C 103.9, which showed one-bond correlation (HSQC) with the ¹H signals at δ_H 4.24 (d, 7.8). A comparison of the ¹³C spectrum with those of β-D-galactopyranoside confirmed the glycosyl structure, and the assignments were made using HSQC and HMBC (Table 1).[27]

These correlations resulted in a 1-β-D-galactopyranosyl-3-acyl-glycerol system. In addition, the presence of signals for olefinic hydrogens (δ_H 5.30-5.39; by HSQC, δ_C 131.3; 129.6; 127.8; 127.4; 126.8) and the presence of a triplet (δ_H 0.99, J = 7.5) of a terminal methyl group suggested an unsaturated fatty acid as a substituent at C-3 of glycerol. The methylene hydrogens at δ_H 2.37 (t, J = 7.6) were coupled to the methylene ¹H signals at δ_H 1.63, which were coupled to the methylene ¹H signals at 1.31-1.35 (m). The methylene hydrogens at δ_H 2.37 showed correlation with the ¹³C signal at δ_C 174.0, suggesting the presence of a unit $-OCOCH_2(CH_2)_n$ at C-3.
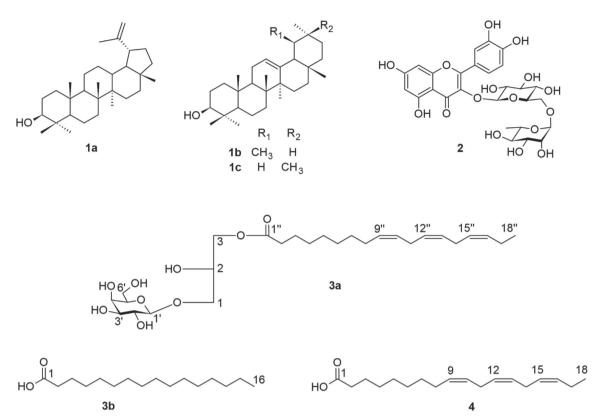
Figure 1. Compounds identified from the leaves of Odontadenia lutea

Table 1. ¹H (MeOD, 500 MHz) and ¹³C (MeOD, 125 MHz) NMR data of 3a

Position	3a	
	δ_H (mult., *J* in Hz)	δ_C
1	3.56 (m)	62.6
2	3.67 (m)	69.7
3	4.09 (dd, 11.3 and 6.2)	65.0
	4.17 (dd, 11.9 and 4.5)	
1'	4.24 (d, 7.8)	103.9
2'	3.47-4.01 (m)	71.1
3'	3.47-4.01 (m)	75.3
4'	3.47-4.01 (m)	68.8
5'	3.47-4.01 (m)	73.4
6'	3.47-4.01 (m)	61.0
1''	-	174.0
2''	2.37 (t, 7.6)	33.5
3''	1.63 (m)	24.5
4''	1.31-1.35 (m)	29.3
5''	1.31-1.35 (m)	29.3
6''	1.31-1.35 (m)	29.2
7''	1.31-1.35 (m)	29.2
8''	2.10 (m)	26.7
9''	5.30-5.39 (m)	127.8
10''	5.30-5.39 (m)	126.8
11''	2.83 (t, 6.0)	25.1
12''	5.30-5.39 (m)	127.4
13''	5.30-5.39 (m)	127.4
14''	2.83 (t, 6.0)	29.4
15''	5.30-5.39 (m)	129.6
16''	5.30-5.39 (m)	131.3
17''	2.10 (m)	26.7
18''	0.99 (t, 7.5)	13.0

The terminal methyl group at δ_H 0.99 was coupled with the methylene ¹H signals at δ_H 2.10 (m), which showed a long-range correlation with the olefinic signals at δ_C 126.8-131.3. The two methylene hydrogens at δ_H 2.83 showed also a correlation with the olefinic signals, indicating that the double bonds are at the end of the chain. The ESI-MS in the negative mode of the fraction containing **3a** indicated the presence of an ion fragment at *m/z* 277.2122 [M-H]⁻, which corresponds to the fatty acid ($C_{18}H_{30}O_2$) esterifying the hydroxyl at C-3 in glyceryl group in **3a**. The positions of the double bonds in the side-chain at C-9'', C-12'', and C-15'' were deduced by HMBC. The ESI-MS in the positive mode confirms the molecular formula $C_{27}H_{46}O_9$ for **3a** with *m/z* 537.3068 [M+Na]⁺. Thus, the spectroscopic data supported the structure of **3a** as 3-*O*-(9,12,15-octadecatrienoyl)-glyceryl-β-D-galactopyranoside. It was isolated previously from *Euphorbia nicaeensis* (Euphorbiaceae) as (2S)-3-*O*-(9,12,15-octadecatrienoyl)-glyceryl-β-D-galactopyranoside, and it displayed significant anti-inflammatory activity.[27]

Compound **3a** is reported for the first time from the *Odontadenia* genus, however, monogalactosylmonoacylglycerols are not generally found in Apocynaceae. The ¹H NMR spectrum showed a second triplet at δ_H 0.92 (J = 7.0) of a terminal methyl group, and ¹³C NMR showed signal for a second acyl function at δ_C 176,9, suggesting the presence of saturated fatty acid. It was confirmed by ESI-MS in the negative mode of the fraction containing **3a**, which indicated the presence of **3a** and palmitic acid (**3b**) *m/z* 255.2347 [M-H]⁻.[28]

In another mixture, compound **4** could not be separated from a small amount of **3b**. The ¹H and ¹³C NMR spectra (Figures 14S-17S) in addition to signals described for **3b**, revealed the presence of six olefinic carbons. The HSQC and HMBC experiments were consistent with the structure of linolenic acid. This was supported by the ESI-MS in the negative mode, which showed *m/z* 277.2191 [M-H]⁻ (Figure 18S) for $C_{18}H_{30}O_2$. These data were consistent with the structure of (9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid.[29] Compounds **3b** and **4** have been reported for *Calotropis procera*

(Aiton) Dryand,[30] and **3b** for *Catharanthus roseus* (L.) G. Don and in species of *Plumeria*, Apocynaceae, genera.[31,32] A great number of fatty acids were found to act as antimicrobial, as compound **4** against *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Bacillus subtilis* and *Helicobacter pylori*, viruses such as hepatitis C (HCV) and fungi *Rhizoctonia solani* and *Crinipellis perniciosa*.[29]

Additionally, the review of the literature points out that the isolation of the limonoid Odontadenin A from *O. macranta* previously mentioned[15] does not correspond to the expected for Apocynaceae. Apparently, this is the only report of the isolation of a metabolite of this class to the family.[33] They are metabolites whose occurrence is well described in Rutaceae and Meliaceae, and less frequently in Cneoraceae and Harrisonia (Simaroubaceae).[34]

In vitro insecticidal activity

The survival of the worker ants *Atta sexdens rubropilosa* was significantly reduced in the diets containing the crude ethanolic extracts of *O. lutea* (Table 2). All the fractions obtained from ethanolic extracts using hexane, dichloromethane, ethyl acetate, and methanol were toxic to workers. However, the hexane-soluble fraction (OLFEH) at 2 mg mL^{-1} was responsible for the highest mortalities, 98% in 14 days, and 100% in 25 days of treatment. In the control experiment, 58% of mortality occurs in 25 days, a usual number of ants that survive with an artificial diet. OLFEH at 2 mg mL^{-1} decreased the survival period of ants from sixteen days, as in control, to six days. Lupeol (**1a**) found in *Didymopanax vinosum* E. March. (Araliaceae)

and known for its effect on ant *A. sexdens rubropilosa*,[35] was obtained in this fraction. Lupeol (**1a**), and β-amyrin (**1c**) isolated from *Inula japonica* Thunb. (Asteraceae) were found to act as acaricidal against *Tetranychus cinnabarinus* (Boisduval) (Acari: Tetranychidae).[36] Extracts of *Senecio salignus* DC. (Asteraceae) containing **1a** and **1c** showed insecticidal activity against *Spodoptera frugiperda* (Lepidoptera: Noctuidae).[37]

Apparently, lupeol (**1a**) might be acting synergistically with β-amyrin (**1c**), known for their insecticidal action, and α-amyrin (**1b**) emphasizing the excellent results obtained with a hexane-soluble fraction (OLFEH).

CONCLUSIONS

This work describes the isolation and identification of seven compounds in addition to the evaluation of the insecticidal action of the extract and fractions of *O. lutea* leaves. Compounds **1c**, **2**, **3a**, **3b,** and **4** are described for the first time for *Odontadenia* genus. The bioassay with *A. sexdens rubropilosa* suggested that the triterpenes lupeol (**1a**), α-amyrin (**1b**), and β-amyrin (**1c**) contributed to the insecticidal activity of the hexanic fraction at 2 mg mL^{-1}.

SUPPLEMENTARY INFORMATION

Supplementary data of the compounds **1**, **2**, **3a/3b**, and **3b/4** (NMR, IR, and ESI-MS spectra) is available free of charge at http://quimicanova.sbq.org.br/.

Table 2. Cumulative mortality and median survival (MD) of *A. sexdens rubropilosa* workers submitted to the bioassay with artificial diet plus extract and fractions of *O. lutea* at concentrations 0.2, 1.0 and 2.0 mg mL^{-1}

Treatment (mg mL^{-1})		Accumulated percentage of mortality per day										MD * (days)
		1	2	3	6	8	10	14	17	21	25	
OLFE	Diet Control	0	0	0	4	4	10	14	16	20	26	>25*a*
	0.2	0	2	2	6	14	18	26	30	50	52	22*b*
	1.0	0	0	2	14	16	18	20	28	42	42	>25*a*
	2.0	2	2	2	16	28	40	42	44	50	52	20*b*
OLFEH	Diet Control	0	0	0	8	18	28	46	52	56	58	16*a*
	0.2	0	0	0	10	16	20	32	34	40	42	>25*a*
	1.0	0	0	0	16	30	42	64	78	82	84	12*b*
	2.0	0	0	0	52	66	90	98	98	98	100	6*b*
OLFED	Diet Control	0	0	0	2	2	14	38	44	46	52	24*a*
	0.2	0	0	4	8	12	20	30	50	52	64	17*a*
	1.0	0	0	8	22	26	30	54	58	60	78	14*b*
	2.0	0	0	10	26	28	36	58	74	76	80	13*b*
OLFEA	Diet Control	0	0	0	8	18	28	46	52	56	58	16*a*
	0.2	0	0	2	12	20	24	32	38	42	44	>25*a*
	1.0	0	0	0	2	22	32	60	66	68	68	14*b*
	2.0	0	0	0	8	20	38	54	64	68	70	13*b*
OLFEM	Diet Control	0	0	0	2	2	14	38	44	46	52	24*a*
	0.2	0	2	2	8	10	14	24	30	32	48	>25*a*
	1.0	0	2	2	10	10	18	36	58	60	72	15*a*
	2.0	0	2	2	8	12	18	46	58	58	66	15*a*

"MD" – Median Survival; " * " – Different letters in relation to the control indicated a significant difference according to the "log rank" test (p < 0,05); "OLFE" – Ethanolic extract from leaves of *O. lutea*; "OLFEH" – Hexanic fraction of leaves of *O. lutea*; "OLFED" – Dichloromethane fraction of leaves of *O. lutea*; "OLFEA" – Acetate-ethylic fraction of leaves of *O. lutea*; "OLFEM" – Metanolic fraction of leaves of *O. lutea*.

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