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Deguelia duckeana in vitro establishment and triterpenes callus production

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Abstract

Deguelia duckeana (Fabaceae) is an endemic plant from the Brazilian Amazonian Rain Forest which is producer of flavonoids, stilbenes and lignans which showed antituberculosis, antibacterial, antifungal and antitumoral activities. In order to find out if *in vitro* plant cell cultures are able to produce the same kind of substances as the mother plant, the present work was proposed. So, the present study deals with the *in vitro* establishment and callus induction from *D. duckeana*. Callus were dried and extracted with hexane/ethyl acetate (1:1) and methanol and further subjected to chemical fractionation. The phytochemical study of the hexane/ethyl acetate and methanol extracts allowed the isolation of the triterpenes: lupeol, lupen-3-al-3β-hidroxi-20(29)-lupen-28-al (betulinaldehyde) and betulinic acid, which structures were elucidated by Nuclear Magnetic Resonance (NMR) analysis. This is the first report of callus induction from *D. duckeana*, which showed to be a producer of important bioactive substances but different from the mother plant collected in nature.

Introduction

Plants species of the Fabaceae family have global relevance for human and animal food and nutrition, presenting high protein content, high rates of peptides when compared to cereals, and fruits rich in carbohydrates and dietary fibers which provide micronutrients such as vitamins, fatty acids, folic acid and minerals, being highly beneficial to health (Jacob et al. 2016). In addition, more than 4000 flavonoids have been described for vascular plants, where most of them were described in Fabaceae species which many are involved in the metabolic processes associated with nitrogen-fixing bacteria (Perret et al. 2000).

The genus *Deguelia*, which belongs to the Fabaceae family, contains only 20 species in tropical South America, and in Brazil they are concentrated mainly in the states of Amazonas and Pará. Chemically, the genus *Deguelia* is a promising source of flavonoids and isoflavonoids, with a predominance of rotenoids, chalcones, flavonoids, isoflavonoids, stilbenes and triterpenes (Magalhães et al. 2001; Cursino et al. 2016).

The species *Deguelia duckeana* Azevedo is a large vine, mainly of non-flooded and dense *capoeira* forests of wide ecological amplitude ranging from riversides to secondary forests. The species is endemic to the Brazilian Amazon, in the states of Amazonas and Pará, and is known as *cipó-cururu* or *timbó* (Tozzi 1989).

Lima et al (2012) identified in the dichloromethane extract of the roots of *Deguelia duckeana* the stilbene (3,5,4'-trimethoxy-4-prenylstilbene), the chalcones (4-hydroxylonchocarpine, 4-hydroxiderricin and 4-methoxyderricidine), flavanone (4'-hydroxy-isolonchocarpine) and flavone (3',4'-methylenedioxy-7-methoxy-flavone). In the hexane extract of the branches, chalcones (lonchocarpine and derricidine), and stilbene (3, 5, 4'-trimethoxy-stilbene) were isolated as well as a mixture of steroids (β-sitosterol and stigmasterol). Studies conducted by Cursino et al (2016) identified stilbene (3,5,4'-trimethoxy-4-prenilstilbene), chalcones (4-methoxillonchocarpine and 4-methoxiderricidine), flavones (5-hydroxy-4',7-

dimethoxy-6-prenylflanone, 4'-hydroxyisolonocarpine), flavones (3',4'-methylenedioxy-7-methoxyflavone, 3',4',7-trimethoxyflavone and racemoflavone) and a lignan (yangabine).

Studies carried out with the dichloromethane extract (DCM) from the roots, showed antibacterial activity against *Salmonella enteritidis*, *Enterococcus faecalis*, *Mycobacterium tuberculosis* and *Staphylococcus aureus*; and the hexane extract of the leaves showed activity against *Escherichia coli* (Carrion et al. 2013; Cursino et al. 2016). Chalcone 4-hydroxylonchocarpine and flavone 4-hydroxy-isolonchocarpine isolated from extracts of *D. duckeana*, showed cytotoxic activity against the tumor cell line of the neuron SK-N-SH. The flavonoids lonchocarpine, 4-hydroxylonchocarpine, 4-hydroxy-isolonchocarpine and 3',4'-methylenedioxy-7-methoxy flavone inhibited the eukaryotic stretching factor 2 (e-EF2) active in the catalysis of the movement in the ribosome (stretching) corresponding to the second stage of protein synthesis in SK-N-SH neural cells (Cursino et al. 2016).

In order to meet industrial needs medicine manufacturing, the cultivation of tissues, cells, and plant organs *in vitro*, has become a tool for the production of vegetable biomass on a large scale. This technique was developed in view of the growing demand for products of natural origin and growing concern about the threats of population loss and plant genetic diversity, together with the awareness of the current degradation of the environment (Roberts et al. 2007; Yue et al. 2016; Isah 2016).

The present work deals with *in vitro* establishment of *D. duckeana*, calli induction and chemical fractionation of their extracts. Calli were extracted with hexane/ethyl acetate (1:1) and methanol. The phytochemical study of the hexane/ethyl acetate and methanol extracts allowed the isolation of the triterpenes: (3β)-Lup-20(29)-en-3-ol (lupeol), lupen-3-al-3β-hidroxi-20(29)-lupen-28-al (betulinaldehyde) and (3β)-3-Hydroxy-lup-20(29)-en-28-oic acid (betulinic acid).

Materials and Methods

Plant material

Leaves from different internodes of young plants of *Deguelia duckeana* were collected in 2012 at Praia Dourada, located in the city of Manaus, AM, Brazil, and used as explants. An individual was kept in the nursery of the Laboratory of Bioprospecting and Biotechnology (LABB) of the National Institute of Amazonian Research - INPA. A voucher was deposited in the herbarium of INPA, under number 50410. The study was registered in the Genetic Heritage and Associated Traditional Knowledge Management System (SISGEN/MMA) under number: A5B34C1.

Disinfection

For the initial phase of tissue culture, aseptic *in vitro* cultures were established, where the explants were subjected to 21 disinfection treatments (Table 1) in the presence or absence of a fungicide solution containing Mancozeb® and Tween 20 detergent, 70% ethanol, calcium hypochlorite ($Ca(OCl)_2$) 4%,

sodium hypochlorite (NaClO) in concentrations 0.5, 1.0 and 2.0%, in different combinations and immersion times, with three washes in distilled and sterile water between each disinfectant. The explants underwent pre-disinfestation which consisted of gentle washing with mild soap and running water. These disinfecting treatments were named as DD01 to DD21.

Table 1
Combinations and immersion times in disinfesting agents used in leaf explants of *Deguelia duckeana*.

Treatment	Fungicide (t, h)*	Ethanol 70% (t, min)	NaClO 0.5% (t, min)	NaClO 1.5% (t, min)	NaClO 2.5% (t, min)	$Ca(OCl)_2$ 4% (t, min)
DD 01	-	3	15'	-	-	
DD 02	-	3	-	-	-	15
DD 03	-	3	7'	-	-	7
DD 04	-	3	-	-	10	10
DD 05	-	3	-	-	-	10
DD 06	-	3	30	-	-	-
DD 07	-	4	20	-	-	10
DD 08	-	4	-	-	40	8
DD 09	17	3	-	-	6	10
DD 10	17	3	-	-	15	-
DD 11	17	3	-	-	5	5
DD 12	17	3	-	-	10	-
DD 13	17	3	-	-	-	10
DD 14	17	3	-	-	5	-
DD 15	17	3	-	-	10	-
DD 16	17	3	-	-	-	15
DD 17	17	3	-	-	10	10
DD 18	48	3	-	-	10	-
DD 19	48	3	-	15	-	-
DD 20	23	3	-	15	-	-
DD 21	04	8	-	10	-	-

Legend: *(t) = time; DD = Disinfection of explants of the species *Deguelia duckeana*.

Subsequently, the explants were inoculated in Murashige and Skoog (1962) (MS) with 100% of nutrients, commercial powder preparation (Sigma Chemical Co.) supplemented with 87.6 mM of sucrose, using 8 $g.L^{-1}$ of agar as a gelling agent.

Callus induction and multiplication

After the establishment of aseptic cultures, the explants were transferred to MS culture media with 100% of the nutrients of commercial powder preparation of MS (Sigma-Aldrich), added with growth regulators in varying combinations and concentrations (Table 2), aiming to promote the induction and multiplication of the calluses. They were subsequently supplemented with 87.6 mM of sucrose by using a gelling agent 8 $g.L^{-1}$ of agar, and later autoclaved at 121°C, at a pressure of 0.1 MPa for 20 min.

Table 2

Combinations and concentrations ($mg.L^{-1}$) of auxins and cytokinins added to the MS culture medium for the induction of calluses in *Deguelia duckeana*.

Treatment	[Auxin] ($mg.L^{-1}$)	[Cytokinin] ($mg.L^{-1}$)
DDI 01	2 NAA	3 BAP
DDI 02	10 Pic	2 Kin
DDI 03	15 Pic	2 Kin
DDI 04	2 NAA	6 BAP
DDI 05	2 2,4-D	-
DDI 06	4 2,4-D	2 Kin
DDI 07	4 2,4-D	-
DDI 08	2 NAA	3 BAP + 2 Kin

Legend: DDI = Callus induction of the *Deguelia duckeana* species. NAA: α-naphthalene acetic acid; BAP 6-benzylaminopurine; Kin: Kinetin; Pic: picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid); 2,4-D: 2,4-dichlorophenoxyacetic acid.

The cultures were kept in a growth room with a controlled temperature of 25 ± 2 ºC, under a 16:8 h photoperiod (light/dark), provided by fluorescent lamps and subcultured at 30-day intervals.

Extracts Obtention

Calluses from the 11th, 12th and 15th subcultures were collected, weighed, and placed separately in glass flasks, where they were then frozen, lyophilized and macerated in a mortar and pestle.

The vegetable material obtained was collected and extracted first with the mixture of the solvents hexane/ethyl acetate (EtOAc) 1:1 (v/v) followed by extraction with methanol (MeOH) where, for each 1 g of dry mass, 10 mL was used of the solvent. Five extractions were performed for the hexane/EtOAc 1:1 system using ultrasound for 20 minutes for each extraction, afterwards the material was filtered and dried in a controlled laboratory environment +/- 24 ºC, the same procedure was performed to obtain the methanolic extract. For the concentration of the extracts, rotary evaporator was used under reduced pressure, at a temperature < 50 ºC.

Hexane/EtOAc 1:1 extract fractionation

In the hexane/EtOAc extract fractionation, 1.5 g of sample was fractionated by open chromatographic column (CC) using silica gel (SiO_2) 60 (200–400 Mesh, 26 x 2.5 cm, 105 g SiO_2) as stationary phase and gradient of hexane/DCM, 100% DCM, DCM/MeOH gradient to 100% MeOH as mobile phase. The volume of each elution system added to the chromatographic column was 250 mL and 66 fractions were obtained. The fractions were analyzed by thin layer chromatography (TLC)

From the observation of chromatoplates eluted and developed with ultraviolet light developers at wavelengths of 254 nm and 365 nm, ceric sulphate ($Ce(SO_4)_2$), and iodine vapor), the DdC0F35 fraction was analyzed by NMR and Mass Spectrometry (MS).

Fractions 25–30 (44.8 mg) were collected and refraction by open CC in SiO_2 (200–400 Mesh, 12 x 1.5 cm, with 448 mg SiO_2), eluting in gradients of hexane/DCM, 100% DCM, DCM/MeOH gradient to 100% MeOH. The volume of each elution system was 30 mL, yielding 49 fractions. Due to the similarity shown by the fractions DdC2F11-12 and DdC2F16-17 when developed with the same physical and chemical developers in the chromatoplates, they were collected and sent for analysis of EM and ^{1}H and ^{13}C NMR.

MeOH extract fractionation

The methanolic extract of the calluses (14 g) was fractionated by liquid-liquid partitioning where two procedures were performed. In the first, 6 g of methanolic extract solubilized in 400 mL of solution with MeOH and distilled water (6: 4) were used, and extracted four times with EtOAc in the proportion of 1:1. In the second, 8 g of extract solubilized in 500 mL of MeOH and distilled water (6: 4) were used, being extracted four times with EtOAc in a 1:1 ratio.

The EtOAc phases obtained were combined (1.3 g) and fractionated in open CC using Sephadex LH-20 (50 x 3 cm), as a stationary phase and elution in an isocratic system in MeOH, generating 49 fractions. The fractions obtained were analyzed by Comparative Thin-Layer Chromatography (CTLC) using physical

developers 254 nm and 365 nm UV light and chemical sulfuric anisaldehyde, NP-PEG, $Ce(SO_4)_2$, $FeCl_3$ and iodine vapor.

Purification of pooled fractions 5–7 (161 mg) was performed on an open chromatographic column on SiO_2 60 gel (200–400 Mesh, 40 x 1 cm, 16 g of SiO_2) eluting with a gradient of DCM, DCM/acetone, acetone and 100% MeOH. The volume of each elution added to the chromatographic column was 110 mL, yielding 64 fractions.

The DDFAfr4-6 and DDFAfr19-21 fractions of the DDFACSL5-7 column fractionation were analyzed by NMR (300 MHz) with ^1H one-dimensional analyzes and the two-dimensional experiments of COSY, HSQC and HMBC were performed.

Compounds identification

The NMR spectra were recorded in a Bruker BioSpin AG, Model Fourier 300 UltraShield, with a frequency of 300 MHz, equipped with EASYPROBE DUL 300MHz S1 5mm Z-GRADIENT probe, and the structure clarification was performed by interpreting the 1D and 2D spectra, following experiments 1D: ^1H, ^{13}C and 2D: COSY, HMBC, HSQC. The spectra were processed using the Bruker® TopSpin program (v3.6.2).

The mass spectra were recorded using an Amazon Speed Model mass spectrometer, ESI Source in positive mode - coupled to a Model Prominence UFLC UPLC system (Shimadzu), equipped with LC-20AT binary pump, SPDM diode array detector (DAD) -20A and SIL-20a automatic injector, with a Shim-pack XR-ODS column (2 mm × 50 mm, Shimadzu), Ion Trap.

Results and Discussion

Deguelia duckeana explants disinfection

The *in vitro* establishment of *D. duckeana* was possible thereby yielding callus cultures obtained from young leaves. To obtain aseptic cultures, 21 asepsis tests were performed. Among them, DD21 treatment were the best, with previous immersion in fungicide Mancozeb® for 4 hours, followed by immersion in 70% ethanol for 8 minutes and 10 minutes in 1.5% NaClO. The treatment (DD20) presented decontamination of 90%. This treatment was repeated to confirm the results.

The use of fungicide was crucial to obtain aseptic cultures, some fungicides cause damage to the fungal cell membrane by inactivating essential enzymes or proteins, others have specific impacts on fungal metabolism pathways, such as the production of sterol or chitin. As is the case with Mancozeb, a fungicide of the class of alkylenebis (dithiocarbamate), which causes general cellular dysfunction by action on the binding of the hydrogen-sulfur (SH) group of proteins such as cysteine, an amino acid present in the cell wall of fungi, and methionine, an essential amino acid (Russell 2009; Nelson & Cox. 2014).

In tissue culture, the use of 70% ethanol in asepsis is very widespread, since the degree of alcohol hydration is ideal for allowing this agent to penetrate the membrane of microorganisms and denature proteins, leading to death. Higher concentrations of ethanol can denature the cell and have a bacteriostatic effect. In addition to its germicidal action, 70% ethanol removes surface waxes from the tissues and, if applied initially, facilitates the action of other disinfesting agents, for this reason, it is recommended that ethanol be used in conjunction with other disinfesting agents, mainly to chlorine-based, such as sodium hypochlorite and calcium hypochlorite (Pelczar et al. 1980; Estrela et al. 2002).

Hypochlorite has antimicrobial activity due to its complex reaction when in contact with microorganisms present in the place to be cleaned. These first act as an organic solvent, degrading fatty acids into fatty acid salts (detergent) and glycerol (alcohol), which reduces the surface tension of the solution, allowing it to penetrate the plant tissue and then into the cytoplasmic membrane (Estrela et al. 2002).

Treatments for disinfestation with hypochlorite (sodium and calcium) must be designed experimentally at different times and concentrations, being carefully tested and evaluated, as demonstrated in this study (Estrela et al. 2002).

The use of sodium and calcium hypochlorite is widespread in tissue culture, and the main reason for using $Ca(OCl)_2$ is the possibility of obtaining more aggressive treatments, with higher concentrations of chlorine, as some cultures do not respond satisfactorily to NaClO with commercial concentrations.

The efficiency in disinfestation depends on the phytosanitary condition of the matrix plant, determining the difficulty in decontaminating the explant during isolation. The explants used that went through pre-disinfestation with water and soap, but were not immersed in fungicide solution, were not successful in obtaining from aseptic cultures or treatments that used higher concentrations of sodium and calcium hypochlorite, and longer immersion times.

Callus induction and multiplication

Cultures that remained aseptic were transferred to the culture media with different growth regulators, as shown in Table 3, the prospect of better results led to the combination of growth regulators where DDI 08 (3 $mg.L^{-1}$ BAP + 2 $mg.L^{-1}$ NAA + 2 $mg.L^{-1}$ Kin) induced calluses in 75% of the explants evaluated, after 2 weeks of cultivation. being repeated every 30 days to obtain biomass for later extraction. The vegetal material collected from the 11th, 12th and 15th subcultures were used for extraction, fractionation, and substances isolation.

Table 3

Combinations and concentrations ($mg.L^{-1}$) of auxins and cytokinins and percentage of callus induction in leaf explants of *Deguelia duckeana*, after 30 days of *in vitro* cultivation (n = 20).

Treatment	[Auxin] ($mg.L^{-1}$)	[Cytokinin] ($mg.L^{-1}$)
DDI 01	2 NAA	3 BAP
DDI 02	10 Pic	2 Kin
DDI 03	15 Pic	2 Kin
DDI 04	2 NAA	6 BAP
DDI 05	2 2,4-D	-
DDI 06	4 2,4-D	2 Kin
DDI 07	4 2,4-D	-
DDI 08	2 NAA	3 BAP + 2 Kin

Although callus induction is possible only with the addition of an auxin to the culture medium, often the use of an auxin combined with one or two cytokinins may be necessary to obtain better results, as is the case presented. This condition may be related to the maintenance of the appropriate endogenous balance between auxins and cytokinins, which can act synergistically in the regulation of cell division, an essential process for the formation of callus (Johri et al. 2001; Abd Rahman et al. 2021).

The calluses obtained were multiplied in the culture medium that showed the greatest success in induction (3 $mg.L^{-1}$ BAP + 2 $mg.L^{-1}$ NAA + 2 $mg.L^{-1}$ Kin), being subcultured every 30 days to obtain biomass for later extraction.

Substances identification

It was possible to isolate two substances from the hexane/EtOAc extract fractionation: the triterpenes betulinaldehyde (substance **I**) and lupeol (substance **II**) (Fig. 1). Where the fraction DdC0F35, obtained from the hexane/EtOAc extract showed the signals between δ_H 0.75 and 1.14 in the ^1H (**S1**) and ^{13}C NMR spectra, referring to the triterpene methyls, with typical signs of lupeol molecules being observed, two singles with displacement in δ_H 4.61 and 4.74 referring to the two hydrogens of the double terminal bond (H-29)(**S2**) which was confirmed by the HSQC spectrum where it is possible to observe that the methylene carbon (C-29) with displacement at δc 110.18 ppm is coupled with the hydrogen double bond (**S3**).

In the ^{13}C NMR spectrum, in addition to the δc 110.18 ppm (C-29) signal, a quaternary carbon with δc displacement at 149.7 ppm corresponding to C-20 was also observed, both characteristic of the olefinic group (**S4**).

Another characteristic signal observed was in the ^{1}H NMR spectrum, a singlet in δ_H 9.68 ppm correlating with the quaternary carbon signal with displacement in δ_C 206.77 (**S5**) which indicates the presence of an aldehyde bound to C-28 in the molecule, confirmed by the HSQC contour map. In combination, these data allowed for the identification of this molecule as 3β-hydroxy-20(29)-lupen-28-al (betulinaldehyde) (I) (Fig. 1).

When analyzing the COSY (**S6**), HMBC and HSQC two-dimensional contour maps of this substance, it was possible to observe that it is necessary to correct the ^{13}C NMR data assignment of the substance 3β-hydroxy-20(29)-lupen-28-al (betulinaldehyde) between carbons 15 and 22, and 25 and 26 (Table 4). Monaco and Previtera (1984) previously reported the possibility that these carbons are in exchanged positions, as marked with the letters a and b (Table 4). This statement is made based on the observations of the contour maps.

Table 4

^{13}C NMR data of 3β-hydroxy-20(29)-lupen-28-al (betulinaldehyde, substance I) isolated from *Deguelia duckeana* calluses, in $CDCl_3$ (75 MHz) and comparison with literature data (Mahato and Kundu 1994; Monaco and Previtera 1984).

C	Observed	Multiplicity	Literature
1	38.85	CH_2	38.7
2	27.38	CH_2	27.3
3	78.99	CH	78.9
4	38.72	C	38.8
5	55.29	CH	55.5
6	18.26	CH_2	18.2
7	34.31	CH_2	34.3
8	40.81	C	40.8
9	50.46	CH	50.4
10	37.16	C	37.1
11	20.74	CH_2	20.7
12	25.52	CH_2	25.5
13	38.69	CH	38.7
14	42.55	C	42.5
15	33.23	CH_2	29.2[a]
16	28.8	CH_2	28.8 [a]
17	59.35	C	59.3
18	48.04	CH	48.0b
19	47.53	CH	47.5b
20	149.7	C	149.7
21	29.24	CH_2	29.8
22	29.85	CH_2	33.2

C	Observed	Multiplicity	Literature
23	27.98	CH_3	27.9
24	15.36	CH_3	15.4
25	16.15	CH_3	15.9[b]
26	15.89	CH_3	16.1[b]
27	14.27	CH_3	14.2
28	206.77	C	205.6
29	110.18	CH_2	110.1
30	19.0	CH_3	19.0

Fractions DdC2F11-12 and DdC2F16-17 were pooled (44.8 mg) and analyzed by MS and ^{1}H and ^{13}C NMR, where typical lupeol triterpene signals were observed, with δ_H 4 displacement terminal double hydrogens (Fig. 8), 57 and 4.69 which in HSQC couples with the carbon in δ_C 109.32 (**S7**), in addition to the presence of the signals between δ_H 0.7 and 1.25 (**S8**), typical of triterpene methyls, by the contour maps and compared with the data found in the literature according to Table 5 (Mahato and Kundu 1994) it was possible to determine the structure as being (3β)-Lup-20(29)-en-3-ol (lupeol) (II) (Fig. 1).

Table 5

^{13}C NMR data of lupeol (substance II) isolated from *Deguelia duckeana* calluses, in $CDCl_3$ (75 MHz) and comparison with literature data (Mahato and Kundu 1994).

C	Observed	Multiplicity	Literature
1	38.69	CH_2	38.7
2	27.4	CH_2	27.4
3	78.99	CH	78.9
4	38.85	C	38.8
5	55.28	CH	55.3
6	18.31	CH_2	18.3
7	34.26	CH_2	34.2
8	40.82	C	40.8
9	50.42	CH	50.4
10	37.15	C	37.1
11	20.92	CH_2	20.9
12	25.12	CH_2	25.1
13	38.03	CH	38
14	42.82	C	42.8
15	27.43	CH_2	27.4
16	35.57	CH_2	35.5
17	42.99	C	43
18	48.28	CH	48
19	47.98	CH	47.9
20	150.99	C	150.9
21	29.83	CH_2	29.8
22	39.99	CH_2	40
23	27.98	CH_3	28

C	Observed	Multiplicity	Literature
24	15.37	CH_3	15.4
25	16.12	CH_3	16.1
26	15.97	CH_3	15.9
27	14.54	CH_3	14.5
28	17.99	CH_3	18
29	109.32	CH_2	109.3
30	19.3	CH_3	19.3

The EtOAc phase of the methanolic extract of the calluses of *D. duckeana* also presented betulinaldehyde (subs. I) and lupeol (subs. II), in mixture, in addition to (3β)-3-Hydroxy-lup-20(29)-en-28-oic acid (betulinic acid) (III) (Fig. 2).

The ^1H NMR spectrum showed signs of double-bonded vinyl hydrogens at δ_H 4.68 and 4.65 (**S9**), characteristic of triterpenes of lupane structure. Signals between δ_H 0.65 and 1.70 were also observed for methyls. The two-dimensional contour maps of HSQC (**S10**) and HMBC (**S11**) of the NMR spectra, showed signs of 30 carbons, characteristic of triterpenes correlating with hydrogens. It was possible to observe the presence of a carbon with displacement in δ_C 177 which was attributed to carbonyl group in C28, characteristic of the acid group, in addition to carbons in δ_C 109.3 and 150.8 referring to carbons in position C29 and C20, as well as a carbon with displacement at δ_C 77.2 that would correspond to hydroxyl at position C3. The comparison of the data obtained (Table 6) with those described in the literature (Wenkert et al., 1978) allowed us to conclude that this was the substance (III) (Fig. 2).

Table 6

^{13}C NMR data of betulinic acid (substance III) isolated from the EtOAc phase of *Deguelia duckeana* calluses, in DMSO-d_6 (75 MHz) and comparison with literature data (Wenkert et al. 1978).

C	Observed	Multiplicity	Literature
1	38.7	CH_2	38.2
2	27.65	CH_2	27.1
3	77.2	CH	76.7
4	38.7	C	38.5
5	55.3	CH	54.8
6	18.42	CH_2	17.9
7	34.33	CH_2	33.9
8	38.7	C	38.5
9	50.38	CH	49.9
10	39.9	C	36.7
11	20.87	CH_2	20.4
12	25.54	CH_2	25
13	37.95	CH	37.5
14	42.2	C	41.9
15	30.62	CH_2	29.2
16	32.37	CH_2	31.7
17	55.3	C	55.4
18	47.13	CH	48.5
19	50.37	CH	49.9
20	150.8	C	150.3
21	30.57	CH_2	30.09
22	36.9	CH_2	36.3
23	28.56	CH_3	28.1

C	Observed	Multiplicity	Literature
24	16.24	CH_3	15.73
25	16.27	CH_3	15.9
26	16.44	CH_3	15.8
27	14.8	CH_3	14.3
28	177.7	C	177.2
29	109	CH_2	109
30	19.4	CH_3	18.9

To our knowledge, most studies with plant extracts in which the presence of betulinaldehyde has been reported appear with its analogs, lupeol, betulin and betulinic acid (Rios et al. 2001; Ziegler et al. 2004; Dorr et al. 2011).

Triterpenes are widely distributed in plants, from the roots to the fruits. Studies show that there are many molecules derived from the lupeol triterpene occurring naturally in several plant species. In addition, triterpenes such as lupeol and lupeol derivatives, such as betulinic acid and betulinaldehyde, have shown a wide range of relevant clinical activities, such as anti-inflammatory drugs, for the treatment of both autoimmune and non-autoimmune inflammations, reducing interaction adenyl cyclase and T-cell proliferation, making it possible to use it as an antihistamine and immunosuppressant (Shahlaei et al. 2012). Anti-plasmodic activity has been reported (Ziegler et al. 2004) as well.

Betulinaldehyde as well as betulinic nitrile and morholic acid were used as precursors in the synthesis of drugs and all of them showed anti-HIV activity by inhibiting the late phase of virus replication, and probably its maturation, as well as showing promise as an alternative source for anti- retroviral drugs (Dorr et al. 2011).

Lupeol is widely present in human food such as vegetables like soy (*Glycine max*), cabbage (*Brassica oleracea*), carrots (*Daucus carota*), peppers (*Capsicum annuum*), cucumbers (*Cucumis sativus*), tomatoes (*Lycopersicon esculentum*), olives (*Olea europa*), figs (*Ficus carica*), mangos (*Mangifera indica*), strawberries (*Fragaria* sp.), grapes (*Vitis vinifera*), and hazelnuts (*Corylus avellana*), being one of the triterpenes found in vegetable oils, cereals and fruits. This substance stands out for its biological potential, mainly for its anti-cancer action, and for this reason drawing the attention of the pharmaceutical industry in recent years (Guevara et al. 1996; Saleem et al. 2001; Li et al. 2022; Saleem et al. 2005; Prasad et al. 2009; Laszczyk 2009; Siddique and Saleem 2011; Mannowetz 2017). Studies have revealed the curative potential of this substance on skin (Saleem et al. 2001; Li et al. 2022), prostate (Saleem et al. 2005), tongue, head and neck cancers (Lee et al. 2007). Recent studies report the effectiveness of lupeol as a male contraceptive (Mannowetz et al. 2017) as well. In addition to being

present in well-known medicinal plants such as *Aloe vera*, *Calendula officinalis, Panax ginseng*, *Camellia sinensis*, *Allanblackia monticola, Himatanthus sucuuba, Celastrus paniculatus, Zanthoxylum riedelianum, Leptadenia hastata, Crataeva nurvala, Bombax ceiba* e *Sebastiania adenófora* (Li et al. 2022; Siddique and Saleem 2011). Since lupeol presents interesting activities as an anti-inflammatory (Rathinavel et al. 2021) and apoptotic (Prasad et al. 2009), new sources of them are welcome to the pharmaceutical sciences.

The ubiquitous presence of pentacyclic triterpenoids in plants across multiple phylogenetic lineages suggests that these secondary compounds perform essential functions in plants, perhaps acting as a chemical defense against insects or pathogen attacks. In addition, they have several proven biological activities, such as anti-tumor, antimicrobial, anti-inflammatory activities among others (Zang et al. 2022; Ren and Kinghorn 2019).

Conclusion

Deguelia duckeana was shown to be successfully established *in vitro* and proven to be a producer of triterpenes from the lupane skeleton: lupeol, betulinaldehyde and betulinic acid, compounds showed to have several pharmacological activities.

Declarations

Competing Interests

The authors declare that they have non-financial interests to disclose.

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Author contributions

CVN designed and supervised the whole study; PMP and CLSR performed the experiments and contributed to data analysis and interpretation; PMP and CLSR wrote and edited the first draft of manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability statements

The main data are available as supplemental material. Further data are available under request to authors.

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Figures

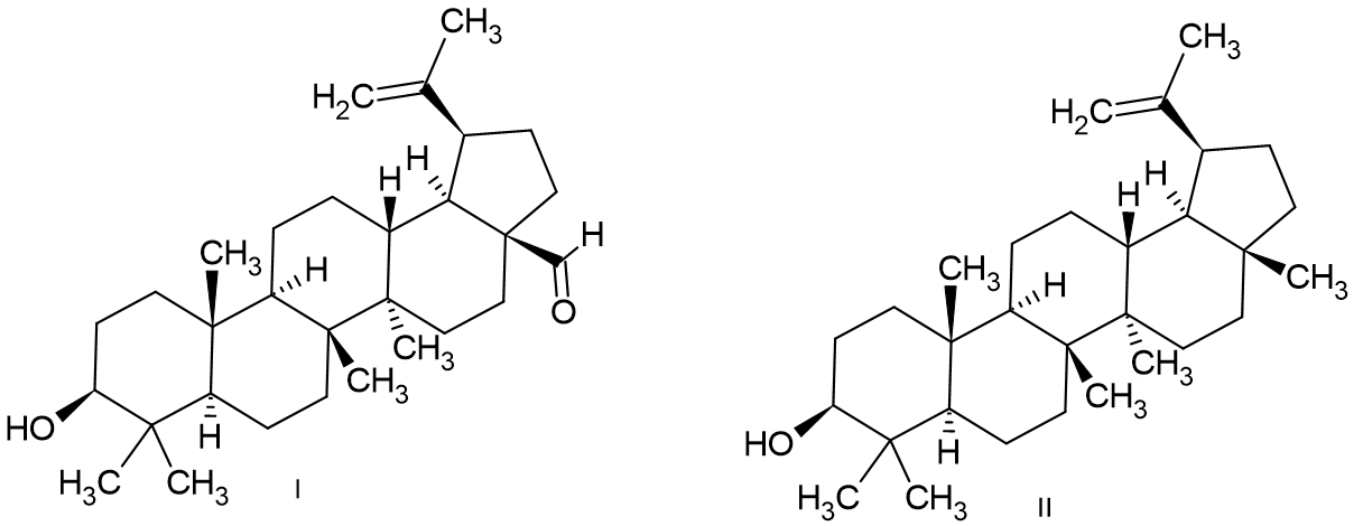


Figure 1

Chemical structures of betulinaldehyde (**I**) and lupeol (**II**)

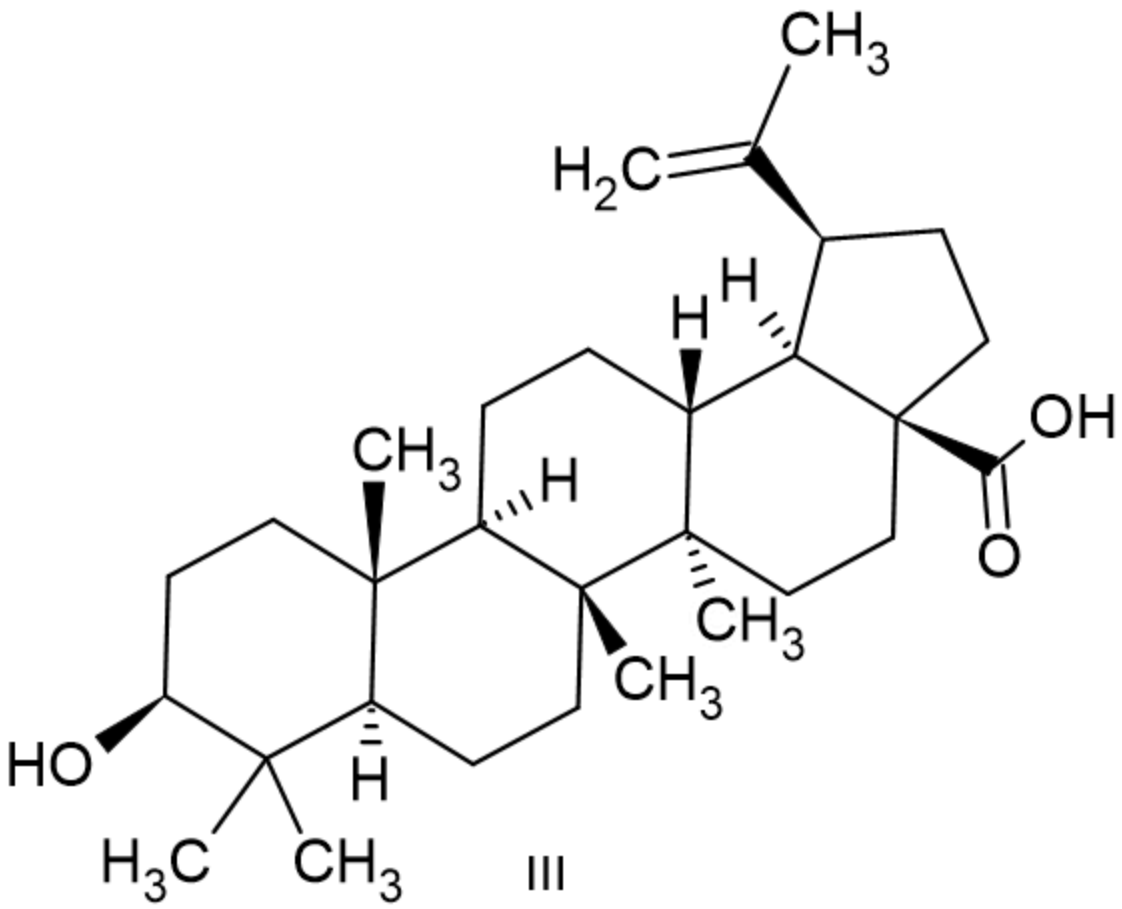


Figure 2

Chemical structure of betulinic acid

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