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Article

Royleanine A, an Antitumor Dihydro-β-agarofuran Sesquiterpene Pyridine Alkaloid from *Maytenus royleanus*

Ala Ud Din *,[a] ***and Bina S. Siddiqui***[b]

[a]*Department of Chemistry, University of Buner, Buner 17290, KPK, Pakistan*

[b]*H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan*

Genus *Maytenus* (Celastraceae) has proved to be a good source of new bioactive sesquiterpene pyridine alkaloids. *Maytenus royleanus* is a medicinal plant used in folk medicine for different ailments in a variety of ways around the world. Investigation of the roots of *M. royleanus* resulted in the isolation of an undescribed antitumor sesquiterpene pyridine alkaloid: Royleanine A (**1**). Its structure was established by analysis of spectroscopic data and comparison with reported data. Royleanine A (**1**) was evaluated for its antitumor potential against several cancer cell lines and was found to be significantly active against OVC-5 cells (ovarian cancer cell line) with half maximal inhibitory concentration (IC_{50}) 28.9 μg mL^{-1}, cervical (Hela) IC_{50} 0.064 μg mL^{-1} and prostate (PC-3) cancer lines with 0.034 μg mL^{-1}.

Keywords: *Maytenus royleanus*, pyridine alkaloid, Royleanine A, antitumor activity

Introduction

Since the very dawn of medicine, plant secondary metabolites have been investigated as a source of new therapeutic agents.[1] Species of *Maytenus* (Celastraceae) proved as promising source of new bioactive agents. Species of *Maytenus* have been widely used in folk medicine[2,3] for different ailments like influenza, rheumatism, gastrointestinal disorders and tumors in South America and Asia.[4]

A variety of bioactive secondary metabolomes, over time, have been reported from species of *Maytenus*, including maytansinoids, spermidine, terpenoids, cardinolids, flavonoids and dihydro-β-agarofuran sesquiterpene pyridine alkaloids. Dihydro-β-agarofuran sesquiterpene pyridine alkaloids displayed a broad range of biological activities like antitumor,[5] insecticidal,[6] immunosuppressive,[7,8] anti-human immunodeficiency virus (HIV),[9] multi drugs resistant-reversing activity,[10] cytotoxicity,[11] anti-tubercular[12] and anti-inflammatory effects.[13]

The genus *Maytenus* is represented in Pakistan by three species: *M. willichiana*, *M. senegalensis* and *M. royleanus*. *M. royleanus* is locally known as "*soor azghee*" (red thorn) in Pakistan and has been used in folk medicine of the locality. A prelude to this research work was our visit to a very popular local traditional physician (Hakeem) in district Buner of Pakistan, who used this plant in combination with other plant extracts, for the treatment of '*Nasoor*' which is a local name for complicated diseases like cancer and up till now, many patients have been cured. The results were even confirmed by follow up test in a hospital. As part of our search to discover new antitumor agents from local medicinal plants,[14-16] we investigated the roots of *M. royleanus*. In a previous article,[16] we reported the gas chromatography mass spectrometry (GC-MS) analysis of seed oil and isolation of triterpenes from roots of *M. royleanus*.

Herein, we report the isolation and structural determination of a new sesquiterpene pyridine alkaloid, Royleanine A (**1**) and its *in vitro* antitumor activity against different cancer cell lines.

Experimental

General experimental procedure

All chemicals and solvents used were purchased from commercial suppliers (Sigma-Aldrich, Karachi, Pakistan). Melting point was measured with Buchi B-535 digital device (Flawil, Switzerland). Optical rotation was taken on

*e-mail: allauddin4763@gmail.com
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Jasco-P2000 digital polarimeter (Easton, Maryland, USA) in MeOH at room temperature. For infrared (IR) spectral data Bruker VECTOR-22 (Billerica, Massachusetts, USA) spectrometer was used; ν_{max} in cm^{-1}. UV spectrum was recorded with the help of analytical LC-MS 996 photodiode array detector. ^1H (600 MHz, CD_3OD) and ^{13}C (125 MHz, CD_3OD) nuclear magnetic resonance (NMR) data were recorded by Varian Inova 600 instrument (California, USA), values of δ in ppm and J in Hz. For chemical profiles of fractions and compounds purity, analytical Shimadzu-high performance liquid chromatography (HPLC) (Kyoto, Japan) was used (MeOH/H_2O with 0.1% formic acid), having reverse phase (RP)-C_{18} (5 µm diameter, 15 cm × 4.6 mm length, Phenomenex Luna®, CA, USA) column, Sedex 55 evaporative light scattering detector (ELSD), 996 photodiode array detector and electrospray ionization (ESI-MS) in + ve mode equipped with time-of-flight (TOF) analyzer. Both low resolution ESI and high-resolution ESI mass spectra were recorded on Mariner ESI-TOF-MS (Connecticut, USA) instrument.

Plant materials

M. royleanus roots were collected in northern area (Buner) of Pakistan in May 2018. The selected specie was identified by Prof Ambara Khan and Dr Fazle Rahim. A voucher specimen (HBKU-825) was deposited in the Herbarium of Department of Botany, Bacha Khan University Charsadda, Khyber Pukhtoonkhwa, Pakistan. Fresh plant material was washed with distilled water to remove dust particles and was shade dried. The plant material was grinded to fine particles and extracted with methanol.

Extraction and isolation

Dried roots (13 kg) of *M. royleanus* were extracted with commercial MeOH at 25 °C after 24 h and the process was repeated three times. The filtered extract after concentration at 40 °C resulted into brownish thick methanolic extract (100 g) which was stored in refrigerator at 4 °C. According to our reported fractionation procedure, crude methanolic extract was partitioned on the basis of increasing solvent polarity, into seven fractions (FH1, FH2, FD1, FD2, FD3, FM1 and FM2) (Supplementary Information (SI) section Figure S2).

The liquid chromatography mass spectometry (LC-MS) profiles of all crude fractions were developed and all showed complex peaks cluster. Dichloromethane fraction (FD2, 25 g) was partitioned into five subfractions (FD2A-E) by column chromatography, the LCMS chemical profile of all five fractions (FD2A-E) were developed. In chromatogram of FD2B peak at *m/z* 909 become prelude for future study, after dereplication of known data. The selected peak was isolated with preparative HPLC, using reverse phase C_{18} column (5 µm, 15 cm × 4.6 mm), gradient solvent system MeCN/H_2O (flow rate of 10-100 mL in 30 min) 0.1% acidic (formic acid), resulted into pure compound **1** (14 mg).

Royleanine A (**1**)

$[\alpha]_D^{27.9}$ −115; UV (MeOH) λ / nm 240; IR (KBr) ν / cm^{-1} 2922, 1731, 1592; ^1H NMR (600 MHz, CD_3OD) δ 5.62 (d, *J* 3.6 Hz, H-1), 5.20 (dd, *J* 3.6, 1.8 Hz, H-2), 4.65 (d, *J* 1.8 Hz, H-3), 7.00 (brd s, H-5), 2.25 (d, *J* 4.2 Hz, H-6), 5.54 (dd, *J* 6.0, 4.2 Hz, H-7), 5.40 (d, *J* 6.0 Hz, H-8), 5.22 (d, *J* 13.4 Hz, H-11a), 4.41 (d, *J* 13.4 Hz, H-11b), 1.51 (s, H-12), 1.61 (s, H-14), 5.91 (d, *J* 11.4 Hz, H-15a), 3.93 (d, *J* 3.6 Hz, H-15b), 7.42 (d, *J* 7.1 Hz, H-3'), 8.11 (dd, *J* 7.1, 1.8 Hz, H-4'), 8.68 (d, *J* 1.8 Hz, H-6'), 4.65 (q, *J* 7.0 Hz, H-7'), 2.54 (q, *J* 7.1 Hz, H-8'), 1.42 (d, *J* 7.0 Hz, H-9'), 1.61 (d, *J* 7.1 Hz, H-10'), 2.91 (s, Ac-1), 2.61 (s, Ac-2), 1.84 (s, Ac-5), 2.16 (s, Ac-7), 1.98 (s, Ac-8), 2.13 (s, Ac-11); ^{13}C NMR (125 MHz, CD_3OD) δ 74.8 (C-1), 69.8 (C-2), 77.6 (C-3), 71.9 (C-4), 75.4 (C-5), 51.6 (C-6), 70.2 (C-7), 72.1 (C-8), 53.5 (C-9), 95.0 (C-10), 61.0 (C-11), 23.6 (C-12), 85.8 (C-13), 18.5 (C-14), 71.3 (C-15), 165.8 (C-2'), 123.0 (C-3'), 139.0 (C-4'), 127.0 (C-5'), 152.5 (C-6'), 36.6 (C-7'), 46.4 (C-8'), 12.1 (C-9'), 9.9 (C-10'), 175.6 (C-11'), 170.2 (C-12'), 20.9 (Ac-1), 20.8 (Ac-2), 21.2 (Ac-5), 20.8 (Ac-7), 20.9 (Ac-8), 21.8 (Ac-11), 171.8 (CO-1), 170.5 (CO-2), 171.0 (CO-5), 171.4 (CO-7), 171.2 (CO-8), 172.0 (CO-11); HR-ESI-MS *m/z*, calcd. for $C_{45}H_{51}NO_{19}$ $[M]^+$: 909.3171, found: 909.3078.

Anti-proliferative disk diffusion assay

In vitro anti-proliferative disk diffusion assay was performed in Josephine Ford Cancer Centre (JFCC), Henry Ford Hospital, Detroit (USA).[17] Royleanine A (**1**) was screened for determination of its potency and selectivity against twelve cancer cell lines. Measure of the differential inhibition of solid tumor against normal or leukemia cell line is considered as selectivity, while positive activity is greater than 250 zone units (200 zone units = 6.5 mm) inhibition. The sample was first dissolved in dimethyl sulfoxide (DMSO), then transferred onto a paper disk, applied to an agar plate which was then seeded with a specific tumor cell line. For cell growth, the agar plates were incubated and the activity of the sample was measured from the size of zone of inhibition in zone units or mm on agar plate. In order to determined half maximal inhibitory concentration (IC_{50}) value for the sample, human tumor cells were cultured in

T25 tissue culture flasks in concentration of 5×10^4 cells, then 5 mL of media Roswell Park Memorial Institute (RPMI) 1640 provided with 15% bovine calf serum, 5% penicillin and 5% glutamine. After 72 h of incubation, sample was transferred to culture flasks in order to achieve concentrations from 10^1-10^{-5} μg mL^{-1}. After three days incubated of flasks, the cells were cleaned, trypsinized, rotated and the number of both viable and dead cells were counted by using 0.08% trypan blue stain. The number of living cells was plotted as function of concentration and the IC_{50} value determined by interpolation. Each point was performed in triplicate and then standard deviation was calculated.

Anti-proliferative assays

For compound **1**, the antiproliferative potential was determined by the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) assay on Hela (cervical) and PC-3 (prostate) cancer lines. Cells have grown in DME (Dulbecco's Modified Eagle) medium for PC-3 and ME (minimal essential) medium for Hela, containing 10% fetal bovine serum (FBS) and 2% penicillin/streptomycin and maintained at 37 °C with 5% CO_2 level for 24 h in flask. Cells (1×10^5 cells mL^{-1}) were placed in a 96 well plates for 24 h incubation, to allow cell attachment. Various concentrations of sample compound varying from 100-1 μM are added into the well and incubated for 48 h. The IC_{50} values were calculated and at least three independent experiments were carried out for the sample. Doxorubicin was used as positive control in this assay for both PC-3 and Hela.[18]

Results and Discussion

After dereplication of known data by using available chemical database,[19] no hits were observed for peak at *m/z* 909 in LC-MS profile of fraction FD1B (see Experimental section). FD1B was subjected to reversed-phase high-performance liquid chromatography (RP-HPLC), which resulted in the isolation of an undescribed dihydro-β-agarofuran sesquiterpene pyridine alkaloid, Royleanine A (**1**), Figure 1.

Compound **1** was purified as white amorphous powder, its molecular formula ($C_{45}H_{51}NO_{19}$) was assigned on the basis of NMR data and HR-ESI-MS which showed molecular ion peak at *m/z* 910.3128 $[M + H]^+$. The UV spectrum showed maximum absorption at 240 and 274 nm indicating the presence of aromatic moiety in compound **1**. In the IR spectrum absorption bands at 1735 and 1592 cm^{-1} indicated ester carbonyl and C=C of aromatic group. The ^{13}C NMR spectra of **1** indicated total 45 carbon atoms,

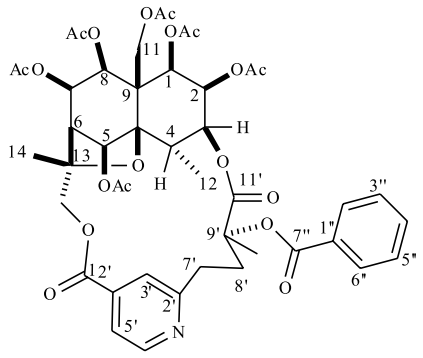

Figure 1. Chemical structure of compound **1**.

including nine CH_3, four CH_2, sixteen CH and sixteen non-hydrogenated carbons. The NMR data further suggested the presence of nine esters including six acetate esters, one benzoate and one evoninate ester.

In the ^{13}C NMR (125 MHz, CD_3OD) spectra ester carbonyls appeared at δ 171.5, 170.0, 171.0, 171.9, 171.2, 171.7, 172.6, 168 and 172.8, being assigned to C-1, 2, 5, 7, 8, 11, 11', 12' and 7'', respectively, by heteronuclear single quantum coherence spectroscopy (HSQC) correlations (Table 1). The six acetate protons in ^1H NMR (600 MHz, CD_3OD) spectra resonated at δ 2.22 (s, OAc-1), 1.85 (s, OAc-2), 1.74 (s, OAc-5), 2.06 (s, OAc-7), 2.01 (s, OAc-8), 2.28 (s, OAc-11) were assigned to respective positions by 2D NMR data. The ^1H NMR spectra of **1** further showed the presence of three *sec*-methyls at δ 1.38 (d, *J* 7.8 Hz, H-12), 1.63 (s, H-14), 1.97 (s, H-10'), two methine protons at δ 2.76 (d, *J* 7.8 Hz, H-4), 2.76 (d, *J* 3.6 Hz, H-6) and six oxygenated methine protons at δ 5.79 (d, *J* 3.6 Hz, H-1), 5.47 (dd, *J* 2.4, 3.6 Hz, H-2), 4.80 (d, *J* 2.4 Hz, H-3), 6.41 (brd s, H-5), 5.71 (dd, *J* 9.6, 3.6 Hz, H-7) and 5.73 (d, *J* 9.6 Hz, H-8) which were characteristic peaks of dihydroagarofuran polyester sesquiterpene skeleton in compound **1**.[20] In ^1H NMR spectrum downfield signals at δ 8.87 (brd s, H-3'), 7.50 (d, *J* 4.8 Hz, H-5'), 8.61 (d, *J* 4.8 Hz, H-6') were assigned to 2',4'-disubstituted pyridine moiety, present in majority of *Maytenus* alkaloids,[20] while protons at δ 8.07 (dd, *J* 1.2, 7.8 Hz, H-2'', 6''), 7.54 (t, *J* 7.2 Hz, H-3'', 5'') and 7.67 (t, *J* 7.8 Hz, H-4'') were assigned to benzoate moiety. The presence of pyridine and benzoate moieties were supported by ^{13}C NMR spectra, the pyridine carbons resonated at δ 153.6 (C-2'), 152.0 (C-3'), 128.7 (C-4'), 127.5 (C-5'), 153.0 (C-6') and six phenyl carbons at δ 166.6 (C-1''), 131.1 (C-2'', 6'') and 130.1 (C-3'', 5''). Analysis of 2D NMR data confirmed that pyridine ring was 2,4-disubstituted and benzoate group was attached at position C-9' (Figures 2 and S1, SI section). The heteronuclear multiple bond correlation (HMBC) spectrum of **1** exhibited long range correlations between H-1/C-11, COO-1; H-2/C-9, COO-2; H-3/C-10, 12, 11'; H-4/C-2, 5;

Table 1. ^{1}H (600 MHz) and ^{13}C NMR (125 MHz) data of Royleanine A (**1**) in CD_3OD

C No.	δ_C / ppm	C-type	δ_H (multi, integral, *J*) / ppm	HMBC
C-1	74.8	CH	5.62 (d, *J* 3.6 Hz)	
C-2	69.8	CH	5.20 (dd, *J* 3.6, 1.8 Hz)	C4, 11, COO-2
C-3	77.6	CH	4.65 (d, *J* 1.8 Hz)	C1, 4, 10
C-4	71.9	C	–	–
C-5	75.4	CH	7.0 (brd. s)	–
C-6	51.6	CH	2.52 (d, *J* 4.2 Hz)	C7, 8, 10
C-7	70.2	CH	5.54 (dd, *J* 6.0, 4.2 Hz)	C9, COO-7
C-8	72.1	CH	5.40 (d, *J* 6.0 Hz)	C1, 7, 11
C-9	53.5	C	–	–
C-10	95.0	C	–	–
C-11	61.2	CH_2	5.22 (d, *J* 13.4 Hz, H_a)	C8, 9, COO-11
			4.41 (d, *J* 13.4 Hz, H_b)	C8, 9, 10
C-12	23.6	CH_3	1.51 (s)	C3, 4, 10
C-13	85.8	C	–	–
C-14	18.5	CH_3	1.61 (s)	–
C-15	71.3	CH_2	5.91 (d, *J* 11.4 Hz, H_a)	C12’
			3.93 (d, *J* 11.4 Hz, H_b)	C12’, 13, 14
C-2’	165.8	C	–	–
C-3’	123.0	CH	7.42 (d, *J* 7.1 Hz)	C7,5’
C-4’	139.0	CH	8.11 (dd, *J* 7.1,1.8 Hz)	C2’,5’,12’
C-5’	127.0	C	–	–
C-6’	152.5	CH	8.68 (d, *J* 1.8 Hz)	C2’,4’,12’
C-7’	36.6	CH	4.65 (q, *J* 7.0 Hz)	C2’, 9’, 11’
C-8’	46.4	CH	2.54 (q, *J* 7.1 Hz)	C9’, 10’
C-9’	12.1	CH_3	1.42 (d, *J* 7.0 Hz)	C2’, 8’
C-10’	9.9	CH_3	1.16 (d, *J* 7.1 Hz)	C7’, 8’, 11’
C-11’	175.6	C	–	–
C-12’	170.2	C	–	–
1-Ac	20.9	CH_3	2.19 (s)	COO-1
2-Ac	20.8	CH_3	2.16 (s)	COO-2
5-Ac	21.2	CH_3	1.84 (s)	COO-3
7-Ac	20.8	CH_3	2.16 (s)	COO-4
8-Ac	20.9	CH_3	1.98 (s)	COO-5
11-Ac	21.8	CH_3	2.13 (s)	COO-6
1-CO	171.8	–	–	–
2-CO	170.5	–	–	–
5-CO	171.0	–	–	–
7-CO	171.4	–	–	–
8-CO	171.2	–	–	–
11-CO	172	–	–	–

HMBC: heteronuclear multiple bond correlation.

H-5/C-7; H-6/C-10; H-7/C-9, 13; H-11/C-1, 8, COO-11; and H-15/C-13, COO-12’, which confirmed the presence of dihydroagarofuran sesquiterpene skeleton. The relative stereochemistry of **1** was confirmed by nuclear overhauser effect spectroscopy (NOESY) spectrum and comparison with spectral data of related reported compounds.[20,21]

The relative stereochemistry of compound **1** was determined on the basis of NOESY correlations, chemical

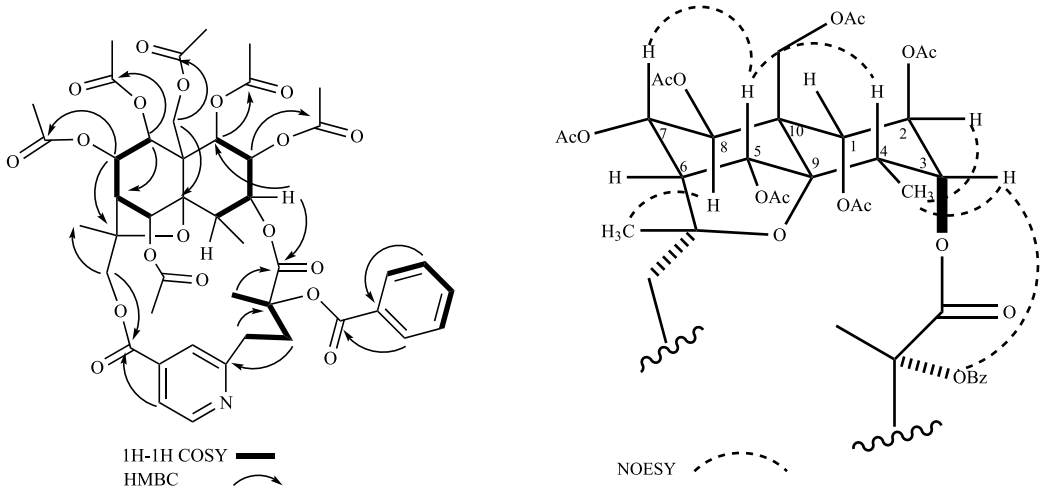

Figure 2. Selected COSY, HMBC and NOESY correlations for compound **1**.

shift (δ) and coupling constant (J) values. Acetyl groups at C-1 and C-2 were assigned as axial configuration on the basis of J value for coupling of H_{eq}-1 with H_{eq}-2 (J 3.6 Hz) and H_{eq}-2 with H_{eq}-3 (J 1.8 Hz). This was further confirmed by NOESY correlations between H-1/H-2 and H-2/H-3. Proton at C-3 also showed NOESY correlations with equatorial methyl at C-12 and with OBz protons at C-9' which were assigned α configuration. Acetyl groups at C-5, 7 and 8 were assigned axial configuration on the similar way with the help of chemical shift, coupling constant values and NOESY correlations. All spectral data analysis and comparison with related reported compounds were in agreement that the structure of compound **1** was 1β,2β,5α,7α,8α,11-hexaacetoxy-3α,15-[2'-methyl-2'α-benzoate-4'(4''-carboxy-2''-pyridyl)-butanoic acid] dicarbolactone-dihydroagarofuran, which was given a trivial name Royleanine A.

Royleanine A (**1**) was tested in Josephine Ford Cancer Centre, Henry Ford Hospital, Detroit, Michigan (USA), against twelve cancer cell lines (Table 2). Compound **1** was found active against OVC-5 cells (ovarian cancer cell line), with IC_{50} values of 28.9 μg mL^{-1}, but did not show any significant activity against other cell lines.

Compound **1** (Royleanine A) was also tested against two available cancer cell lines; Hela (cervical cancer cells) and PC-3 (prostate cancer cells) for its anti-proliferative activity (Table 3) at International Center for Chemical and Biological Sciences (ICCBS), university of Karachi, Pakistan. Compound **1** showed excellent activity against both cell lines even more than the standard used, with IC_{50} values of 0.064 and 0.034 μg mL^{-1} for Hela and PC-3, respectively.

The selectivity of compound **1** for ovarian, cervical and prostate cancer cell lines were very surprising and interesting results.

Table 2. Anti-proliferative disk diffusion assay of Royleanine A (**1**)

Tumor cell line	IC_{50} / (μg mL^{-1})
Murine lyphocytic leukemia (L1210)	> 100
Murine granulocyte macrophage colony formy unit (CFU-GM)	> 100
Murine colon adrenocarcinoma (Colon38)	> 100
Human lung adrenocarcinoma (H125)	> 100
Human colon adrenocarcinoma (H116)	> 100
Androgen sensitive prostate cancer (LNCaP)	> 100
Hormone responsive breast cancer (MCF-7)	> 100
Melanoma (MDA)	> 100
Ovarian cancer (OVC-5)	28.9
Glioblastoma (U251N)	> 100
Murine pancreatic solid tumor (PANC-1)	> 100
Humane leukemic lymphoid (CEM)	> 100

IC_{50}: half maximal inhibitory concentration.

Table 3. Antiproliferative activity of Royleanine A (**1**) against Hela and PC-3

Compound	IC_{50} ± SD / (μmol L^{-1})	
	Hela	PC-3
Royleanine A	1.616 ± 0.04 (0.064 μg mL^{-1})	0.845 ± 0.01 (0.034 μg mL^{-1})
Doxorubicin	3.10 ± 0.20	0.91 ± 0.12

Doxorubicin used as standard drug; IC_{50} for compound **1** reported both in μmol $L^{-1}$and μg mL^{-1}. IC_{50}: half maximal inhibitory concentration; SD: standard deviation.

Conclusions

The phytochemical study of *Maytenus royleanus* roots resulted into a new compound **1**: 1β,2β,5α,7α,8α,11-hexaacetoxy-3α,15-[2'-methyl-2'α-benzoate-4'(4''-carboxy-2''-pyridyl)-butanoic

acid] dicarbolactone dihydroagarofuran (Royleanine A). Royleanine A (**1**) was tested for their anti-proliferative potential against fourteen cancer cell lines. The excellent cytotoxic selectivity against ovarian cancer cell line was in harmony with ethnomedicinal uses of this plant. Royleanine A (**1**) is a good candidate for anti-tumor drug development after extensive further work on it. *M. royleanus* not only will have greater use in folk medicine but is a promising source of new bioactive secondary metabolomes for future research work.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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