Supplementary materials

LC-ESI-MS/MS profiling of alkaloids and antiproliferative activity of
Pachypodium lamerei Drake leaves

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Running title:
Biological and chemical profiling of Pachypodium lamerei

Abstract
In the present study, evaluation of the antiproliferative activity of Pachypodium lamerei Drake leaves (family Apocynaceae) against human breast cancer cell lines MDA-MB-231 was done for the total methanolic extract, crude alkaloidal mixture and ursolic acid using the MTT colorimetric assay. The methanolic extract showed the strongest antiproliferative activity followed by ursolic acid and crude alkaloidal fraction with an IC50 equal to 6.2, 14.55 and 56.3 µg/ml respectively compared to oleocanthal. It is the first record for the LC/ESI-MS/MS alkaloidal profiling of the leaves of P. lamerei. Seven alkaloids were tentatively identified according to their fragmentation patterns. Four alkaloids were related to the parent indole class and two alkaloids belong to the quinoline class in addition to one steroidal alkaloid with a pregnan nucleus. Phytochemical investigation of the methanolic extract lead to the isolation of three triterpenoidal compounds including ursolic acid, 11,12-didehydrousolic acid lactone and ursolic acid lactone.
Table S2. $^1$H & $^{13}$C NMR chemical shifts of compound 1 in (CDCl$_3$) and compounds 2 & 3 in (MeOD)

| No. | $^{13}$C ($\delta$) | $^1$H ($\delta$) | $^{13}$C($\delta$) | $^1$H ($\delta$) | $^{13}$C($\delta$) | $^1$H ($\delta$) |
|-----|---------------------|------------------|-------------------|------------------|-------------------|------------------|
| 1   | 38.83               | 0.85, s          | 38.59             | —                | 38.44             | —                |
| 2   | 27.07               | 1.52, m          | 27.19             | —                | 26.95             | —                |
| 3   | 77.45               | 3.15(dd, $J=10.0,4.0$ Hz) | 78.18 | 3.06 (dd, $J=12.0,5.0$ Hz) | 78.08 | 3.06 (dd, $J=12.0,5.0$ Hz) |
| 4   | 38.65               | —                | 38.81             | —                | 38.59             | —                |
| 5   | 55.25               | 0.69, s          | 55.15             | —                | 54.62             | —                |
| 6   | 18.36               | 1.27, m          | 16.57             | —                | 16.50             | —                |
| 7   | 29.76               | 1.26, m          | 30.9              | —                | 31.09             | —                |
| 8   | 39.13               | —                | 43.04             | —                | 42.20             | —                |
| 9   | 49.47               | 1.47, m          | 52.97             | —                | 51.06             | —                |
| 10  | 30.72               | —                | 36.78             | —                | 36.07             | —                |
| 11  | 23.33               | 1.87, m          | 128.44            | 5.46 (1H,dd,$J=10.0, 3.0$ Hz) | 26.59 | —                |
| 12  | 125.61              | 5.20, brs        | 133.63            | 5.93 (1H, d, $J= 10.0Hz$) | 26.68 | —                |
| 13  | 138.18              | —                | 94.22             | —                | 90.64             | —                |
| 14  | 42.09               | —                | 41.72             | —                | 41.53             | —                |
| 15  | 27.29               | 0.87, s          | 26.26             | —                | 25.21             | —                |
| 16  | 24.24               | 1.55, m          | 22.54             | —                | 22.46             | —                |
| 17  | 47.85               | —                | 45.83             | —                | 45.26             | —                |
| 18  | 52.80               | 2.15, d, $J=10.1$ Hz | 61.03 | —                | 60.36             | —                |
| 19  | 38.76               | 1.30, s          | 40.06             | —                | 39.66             | —                |
| 20  | 38.92               | 0.91, s          | 38.01             | —                | 37.84             | —                |
| 21  | 28.14               | 1.36, m          | 30.37             | —                | 30.34             | —                |
| 22  | 33.05               | 1.58, m          | 31.70             | —                | 31.34             | —                |
| 23  | 28.07               | 0.82, s          | 29.37             | 0.83, s          | 29.34             | 0.93, s          |
| 24  | 15.67               | 0.73, s          | 14.74             | 0.92, s          | 14.30             | 0.92, s          |
| 25  | 15.51               | 0.94, s          | 18.56             | 0.81, s          | 18.41             | 1.06, s          |
| 26  | 16.99               | 0.76, s          | 17.61             | 1.12, s          | 17.53             | 1.01, s          |
| 27  | 23.59               | 1.04, s          | 15.58             | 1.06, s          | 15.20             | 1.18, s          |
| 28  | 180.65              | —                | 181.50            | —                | 181.38            | —                |
| 29  | 17.07               | 0.80 (d, $J=8.0$ Hz). | 17.06 | 0.84, d, $J=8.0$ Hz | 16.87 | 0.84, d, $J=8.0$ Hz |
| 30  | 21.26               | 1.25 (d, $J=4.0$ Hz). | 17.43 | 0.86, d, $J=4.0$ Hz | 17.43 | 0.86, d, $J=4.0$ Hz |
Figure S1 [A,B,C,D] Cytotoxic effect of different samples against (MDA-MB-231) cells using MTT assay n=(3), data expressed as the mean value of IC$_{50}$ ± S.E., statistical significance at P $\leq$ 0.05, compared to both DMSO and oleocanthal.
Figure S 2. LC/ESI/MS-MS spectrum of Aspidospermidine (1).

Figure S3. Fragmentation pattern of Aspidospermidine (1)
Figure S4. LC/ESI/MS-MS spectrum of Desacetyl vindoline (7).

Figure S5. Retro Diels-Alder type fragmentation of Vindoline
Figure S6. LC/ESI/MS-MS spectrum of Conessidine (2).

Figure S7. LC/ESI/MS-MS spectrum of Camptothecin (3)
Figure S8. LC/ESI/MS-MS spectrum of 10-Hydroxycamptothecin (4)

Camptothecin 3 (m/z, 349) \(-\text{CO}_2 [-44]\) \(\rightarrow\) (m/z, 305)

11-Hydroxycamptothecin 4 (m/z, 365) \(-\text{CO}_2 [-44]\) \(\rightarrow\) (m/z, 321)

Figure S9. Characteristic fragment ion peaks of compounds (3 & 4)
Figure S10. LC/ESI/MS-MS spectrum of 11-hydroxyrhazimanine (5)

Figure S11. LC/ESI/MS-MS spectrum of Bleekerine (6)
3. Experimental section

3.1. Plant material

*Pachypodium lamerei* Drake leaves were collected from Shebin Al Qanater, Al Qalyubiyah, Egypt in April 2015. The identity of the plant was confirmed by Dr. Therese Labib, senior specialist of Plant Taxonomy at El-Orman Botanical Garden, Giza, Egypt. A voucher specimen (Reg. No. 1-Pl-1115) has been deposited in the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Cairo, Egypt.

3.2. Preparation of crude alkaloid fraction

The dried powder of *P. lamerei* leaves (1kg) was extracted with 80 % methanol (10X6 L) at 55°C. The dried methanolic residue (80gm) was defatted with petroleum ether to yield (60gm) residue. The residue was shaken with 1.5L 5% acetic acid for 2hrs, then the solution was extracted with chloroform (3X500ml). Neutralization of the aqueous layer was done by 10% Na₂CO₃ solution followed by extraction with 3X500 ml chloroform. The collected chloroform layer, after washing with distilled water, was dried under reduced pressure to yield 200 mg of crude alkaloidal fraction (Hadi and Bremner 2006). The alkaloid fraction was monitored on TLC plate, solvent system CHCL₃: EtOH: NH₃ (85:10:4 v/v), dragendorff's spraying reagent. The alkaloidal fraction was kept for both LC/MS and biological investigation.

3.3. LC/ESI-MS/MS analysis:

Chromatographic separation of analytes was carried out on Hypersil Gold column (C18 bonded ultrapure silica based column), (50 mmx2mmx1.9µm) from Thermo scientific (New York, USA). The ionization energy was 60 ev. Elution was performed isocratically at room temperature using freshly prepared mobile phase of acetonitrile (A): 0.2% formic acid aqueous solution (B) (90:10 v/v). The flow rate was 250µl/min. TSQ Quantum Access MAX triple stage quadrupole mass spectrometer, Thermo Scientific (New York, USA) equipped with electrospray ionization ESI operated in the positive ionization mode. Chromatography was carried on Accela U-HPLC system which composed of Accela 1250 quaternary pump and Accela open autosampler (New York, USA) operated at 25°C. X-caliber software version
2.2 was used to control all parameters of UPLC and MS and analysis of the obtained data. Qualitative identification of the different constituents was performed by comparison of their mass spectral fragmentation patterns with those stored in Mass Bank online library (Mass Bank version 2.10, supported from National Bioscience Database Center, Japan Science and Technology Agency (2011-2013) and National Institute of Standards and Technology (NIST spectral search program for the NIST/EPA/NIH mass spectral library version 2.0, 2011) in addition to the comparison to literature data.

**3.4. Isolation of three tritepenes from *P. lamerei* leaves:**

The air dried powder of *P. lamerei* leaves (2 kg) was extracted with 80% Methanol (10X5L) at 55°C to yield (350 g) dry residue. 170 g of defatted methanolic extract was obtained after defatting. The defatted methanolic extract was subjected to Mitsubishi Diaion HP-20 column. Fractionation was done using distilled water/MeOH, 25%, 50%, 75% and finally with 100% methanol. The 75% and 100% fractions were collected together and concentrated yield a residue (10 g) based on monitoring on TLC. The obtained residue was fractionated using VLC on silica gel elution started with CH2Cl2 and increasing polarity with MeOH. Three collective fractions (A- C) were obtained. Fraction A (2.2 gm) eluted with 1% MeOH in CH2Cl2 afforded one spot which was further purified on Sephadex column using MeOH for elution to yield Compound 1 (1.8 gm). Fraction B (65 mg) eluted with 3-5% MeOH: CH2Cl2 showed one spot giving violet color upon spraying with 20 % H2SO4. This fraction was repeatedly subjected to preparative TLC plates to yield an inseparable mixture of compounds 2 and 3 (50 mg). all characteristic 1H &13C NMR chemical shifts of compound 1, 2 & 3 are recorded in ( Table  S2).

**3.5. MTT proliferation assay:**

The IC₅₀ value for each compound was calculated by non-linear regression (curve fit) of log (concentration) versus the % survival, implemented in Graph Pad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA). The % cell survival was calculated as follows: % cell survival = (Cell No. treatment/Cell No. DMSO) x 100% and the assay was carried out following reference (Akl et al. 2014). MDA-MB-231, in exponential growth, was plated at a density of 1×10⁴ cells per well (6 wells/group) in 96-well culture plates and maintained in
RPMI-1640 media supplemented with 10% FBS and allowed to adhere overnight at 37 °C under 5% CO₂ in a humidified incubator. The next day, cells were washed with PBS, divided into different treatment groups and then fed serum-free defined RPMI-1640 media containing 40 ng/mL of HGF as a mitogen and experimental treatments (containing various doses of the specific tested compound) or vehicle-treated control media and incubation resumed at 37 °C under 5% CO₂ for 72 hr. Cells in all groups were fed fresh treatment media every other day during the 72 hr. treatment period. Control and treatment media were then removed, replaced with fresh media, and 50 µL MTT solutions (1 mg/mL) was added to each well and plates were re-incubated for 4 hr. At the end of the incubation period, the color reaction was stopped by removing the media and adding 100 µL DMSO to dissolve the formazan crystals formed. Incubation at 37 °C was resumed for up to 20 minutes to ensure complete dissolution of crystals. Absorbance was determined at λ 570 nm using an ELISA plate reader (BioTek, VT, USA).

3.6. Culture conditions:

The human breast cancer cell lines MDA-MB-231 (ER−, PR−, HER2−) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). A stock solution was prepared by dissolving each tested compound and diff extracts in sterilized DMSO at a concentration of 20 mM for all assays and stored at 4 °C. Working solutions at their final concentrations for each assay were prepared in an appropriate culture medium immediately prior to use. The vehicle (DMSO) control was prepared by adding the maximum volume of DMSO, used in preparing test compounds, to the appropriate media type such that the final DMSO concentration was maintained as the same in all treatment groups within a given experiment and never exceeded 0.1% and the procedure was done following reference method (Akl et al. 2014). This cell lines were maintained in RPMI-1640 (GIBCO-Invitrogen, NY) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G, 100 µg/mL streptomycin and 2 mmol/L glutamine in a 5% CO2 containing humidified atmosphere at 37 °C. For sub-culturing, cells were rinsed twice with sterile Ca²⁺ and Mg²⁺-free phosphate buffered saline (PBS) and incubated in 0.25% trypsin containing 0.025% EDTA in PBS for 10 min at 37 °C. The released cells were centrifuged, re-suspended in fresh media and
counted using hemocytometer. All cells were maintained at 37 °C in an environment of 95% air and 5% CO2 in humidified incubator.

**3.7. Viable cells Measurement:**

Viable cell count was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay following reference method (Mosmann 1983). The optical density of each sample was measured at 570 nm on a microplate reader (BioTek, VT). The number of cells per well was calculated against a standard curve prepared at the start of each experiment by plating various concentrations of cells (1,000-60,000 cells per well), as determined using a hemocytometer.

References

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