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Isolation of Alkaloids and Anti-tumor Activity of the Crude Methanolic Extract of Algerian *Cytisus purgans*

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ABSTRACT

In this study, two known quinolizidine alkaloids which are sparteine and lupanine were isolated from the methanolic extract of the plant *Cytisus purgans* of Algerian flora by open column chromatography. These two compounds were identified on the basis of their spectral data (GC/MS, IR, MS, ^{1}H and ^{13}C). The anti-tumor activity of the crude methanolic extract of the aerial parts of the plant was also evaluated *invitro* against human breast cancer (MDA-MB-231) and human lung cancer (A549) cell lines using MTT assay.

Key words: Quinolizidine alkaloids, *cytisus purgans*, Fabaceae, Algerian flora, anti-tumor activity.

INTRODUCTION

The Family of *Fabaceae* is a cosmopolitan family with about 700 genera and 18000 species, and is one of the most important families, is characterized by a large number of derived traits[1]. This large Family is characterized by an impressive phytochemical diversity. Polyphenols (especially flavonoids and tannins) are common, but from a pharmaceutical perspective various types of alkaloids are probably the most interesting and pharmaceutically relevant groups of compounds[2]. In the genera *Genista* and *Cytisus* (both commonly called broom), as well as *Laburnum*, quinolizidine alkaloids, including cytisine and sparteine, are common[3].

Cytisus is an ornamental genus, it is presented by about 50 species mainly Mediterranean, is native to central and south Europe, Canaries Isles and North Africa. The common name ' broom ' may have been given to the plant because of its growth habit. Six species represented in Algeria, the more abundant is *Cytisus trifolus* L'her. Other rare species which are *Cytisus balansae* Boiss.Et Reut.(*Cytisu spurgans*), *Cytisus fontanesi* Spach (Ouarsenis, Bibans…), *Cytisus sessilifolius* L. (Babor) and *Cytisus boeticus* Webb.(North of Oran).*Cytisus balansae* Boiss. (*Cytisus purgans*) is a rare species of Algeria (Aures, Mahdids, and Lella Khadidja)[4].

Cytisus purgans is a shrub always more or less ramifies, a trifoliolate and persistent leaves and a yellow flowers, native to central France, north central Spain and Portugal, Algeria and southern Morocco[5].

The antibacterial activity of the MeOH extract of the plant *Cytisus purgans* was assayed *in vitro* by agar disc method against three bacterial species. *Cytisus purgans* exhibited antibacterial activity towards allbacteria strains. The maximum antibacterial activity was shown by *Staphylococcus aureus* followed by *Pseudomonas aeruginosa* and *Escherichia coli*, respectively[6].

Quinolizidine alkaloids represent about 2% of the known alkaloids from plants[7], and most of them were initially noticed as constituents of plants poisonous to humans and livestock. However, accumulated evidence indicates that some of them exhibit potentially useful pharmacological properties like anti-cancer, anti-bacterium, anti-virus, anti-inflammation and pain relief[8].

The sparteine has been used as an oxytocic drug and is of interest because of itsantiarrhythmic effect and inhibitory effect of natural killer cell growth[9].

Species which contain lupanine must consider potentially toxic, and unsuitable for forage[10].

Many plants of the genus *Cytisus* are consumed as infusions due to its beneficial effects, for example, *Cytisus multiflorius* have terapeutic properties enclosing diuretic, anti-inflammatory, anti-hypertension and antidiabetic effects[11].

EXPERIMENTAL

General

Toluene, ethyl acetate, acetone, diethylamine,dimethyl sulfoxide, methanol, diethyl ether, chloroform, hydrochloric acid, ammonium hydroxide, anhydrous sodium sulfate,Dulbecco's Modified Eagle's Medium (DMEM),penicillin, streptomycin, L-glutamine,trypsin and ethylenediaminetetra acetic acid (EDTA) were obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany.

Silica gel 60 (Merck, 70-230 mesh) was used for column chromatography.Preparative TLC was performed by using silica gel P/UV_{254} with $CaSO_4$ (Machery-Nagel, Germany, 2 mm layer of adsorbent). Analytical TLC was performed on silica gel (Silica gel 60, layer 0.20 mm, Alugram Sil G, Mashery-Nagel, Germany). Visualization of TLC plates was performed by using UV lamp at 254 nm and 365 nm (VL-4.LC, 365/254, Vilber Lourmat, France), as well as in iodine vapors.

Infrared spectra were run on Thermo Scientific Nicolet 6700 FT-IR spectrometer (4000-400 cm^{-1}). The NMR spectra were recorded on a BrukerAvance III 500 spectrometer,^{1}H NMR at 500.26 MHz and ^{13}C NMR at 125.80 MHz, solvent DMSO d_6, TMS internal standard. Chemical shifts were given in d (ppm). Mass spectra were recorded on a Agilent 6890/5973 GC/MS system (Agilent, Santa Clara, CA). GC/MSconditions: HP-5ms column (30 m, id 0.25 mm, film thickness 0.25 mm); split mode of injection (split ratio:40.8:1); injector temperature 270 ºC; pressure 17.18 psi, MSD Transfer Line Heater 280 ºC; flow rate of He 1.3 mL/min, temperature program: initial temperature 150 ºC with increasing of temperature at 10 ºC/min to 290 ºC. Temperature of: MS quadruple detector 150ºC, ion source 230ºC. Mass scan range 35-700 amu at 70 eV. Multiplate reader used for MTT cell viability assay, Zenith 3100(Anthos Labtec Instruments GmbH, Austria).

Plant collection and identification

Cytisus purgans was collected on June 2013 at Aures Mountains (2328m of altitude) at Khenchela province in the north east of Algeria,and identified by ProfessorMohamed Kaabech, Laboratory of biodiversity and phytogenetic resources, University of Ferhat Abbas, Sétif, Algeria. A voucher specimen is deposited at the herbarium of laboratory of organic chemistry and phytochemistry of University Mohamed Boudiaf of M'sila, Algeria.

Extraction and isolation of alkaloids

The air-dried aerial parts of plant were cut into small pieces and were extracted with 99% methanol three times at room temperature. The combined extracts were concentrated, acidified with 5% hydrochloric acid and then, extracted with diethyl ether three times. The aqueous layer was made alkaline with 25% ammonium hydroxide to pH (9-10) and extracted with chloroform six times. The chloroform extracts were combined and dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo* to give crude alkaloid mixture[12].

Column chromatography was performed on a classic 30 cm long x 4 cm diameter glass column packed with 130 g of silica gel 60 (70-230 mesh). The ethyl acetate solution of 1 g of alkaloid fraction was applied to the top of column by use of a pipette. Elution was started with toluene, then with solvent mixtures with increasing polarities: toluene: ethylacetate:acetone and toluene: acetone: diethylamine. Finally, all 145 fractions (obtained by column chromatography) were monitored by analytical TLC (above mentioned solvent mixtures).

Fraction 141 was rechromatographed on preparative TLC. Mobile phase used for elution was chloroform:methanol:acetone=85:10:5 w/w. After elution and drying of preparative TLC plate, by scraping the layers and additional desorption from methanol, sparteine and lupanine were isolated. Purity of compounds was confirmed on analytical TLC with the same mobile phase mentioned above. Also, gas chromatographic/mass spectrometric analyses onAgilent 6890/5973 GC/MSsystem confirmed purity of isolated compounds.

Ant-tumor activity of the crude methanolic alkaloids extract

Preparation of stock solutions

Stock solutions of the alkaloids plant extract was made in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL, filtered through a 0.22 mm Millipore filter before use, and diluted by a nutrient medium to various working concentrations, so the final concentration of DMSO in culture medium never exceeded 0.5% (v/v).

Cell cultures

Human breast cancer (MDA-MB-231) and human lung cancer (A549) cell lines, obtained from Human Tumor Cell Bank (HTB) of the American Type Culture Collection (ATCC), were used in this study. MDA-MB-231 cells were grown in RPMI1640 and A549 cells were grown in DMEM medium. Both media were supplemented with 10 % fetal bovine serum, penicillin/streptomycin and L-glutamine. Cells were maintained in a monolayer culture into the 25 cm^2 tissue culture flasks (Nunclon™). The cells were prepared for experiments using trypsinization procedure with trypsin/EDTA.

MTT cell viability assay

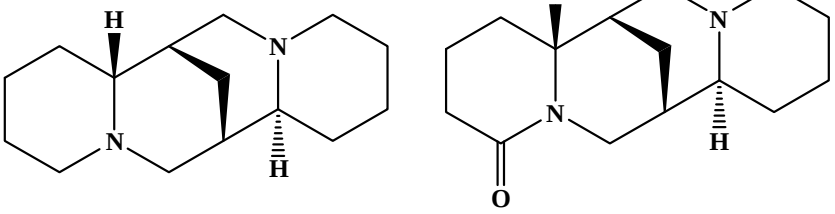
Plant alkaloids extract was tested for it effect on viability of two different human tumor cell lines. MDA-MB-231 and A549 cells were seeded in 96-well plates (Sarstedt™) at $3x10^3$ cells in 200 µL of appropriate tissue culture medium per well. Every concentration of examined compound was tested in triplicate, and incubated at 37ºC in a humidified 5% CO_2 atmosphere. Control cells contained the appropriated amount of DMSO. Culture medium with corresponding concentration of investigated plant extract, but without cells, was used as a blank.

The viability of cultured cells was determined by assaying the reduction of MTT to formazan. In brief, cells were treated with different dilutions of alkaloids (0.001, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25 and 0.5 mg/mL) or cultivated in the cell culture medium containing the appropriate amount of DMSO (control). After 6h, 24 h and 48h of incubation, the culture medium was removed, and MTT solution was added. After additional 4 h of incubation, medium with MTT was removed and DMSO (150µl/well) was added to dissolve the

formazan crystals. The plates were shaken for 10 minutes. Absorbance was measured at 595 nm with a multiplate reader. The results are presented as relative to the control value (untreated cells)[13].

RESULTS AND DISCUSSION

The crude MeOH extract of aerial parts of *Cytisus purgans* was subjected to open column chromatography; two known quinolizidine alkaloids were isolated which are sparteine and lupanine (figure 1).These two compounds were identified on the basis of their spectral data(GC/MS, IR, MS, ^{1}H and ^{13}C).



1 *2*

Scheme 1:

Spectroscopic data for sparteine *1*:$C_{15}H_{26}N$, colorless oil,M_w=234g/mol

IR (KBr) n cm^{-1}: 2935, 2816, 2765, 2745 (CH).

^{1}H NMR (DMSO d_6, 500 MHz): d (ppm) 1.26 (2H, m, 5beq and 8b ax), 1.35 (1H, m, 12aeq),1.38 (1H, m, 5a ax), 1.43 (2H, m, 12b ax and C-H-9), 1.60 (3H, m, 14aeq, 14b ax, 5a ax), 1.68 (4H, m, 13a ax, 13beq, 4aeq and C-H-6), 1.80 (4H, m, C-H-7, 2b ax, 15a ax and C-H-7), 2.01 **(4H, m, 3a ax, 3beq, 8aeq and C-H-11), 2.19-2.89 (5H, 17a ax, 15beq, 17beq, 10aeq, 2aeq)**

^{13}C NMR (DMSO d_6, 125 MHz): d (ppm) 22.88 (C-4), 24.86 (C-13), 29.33 (C-3), 29.74 (C-14), 34.31 (C-8), 36.21 (C-5), 36.97 (C-7), 37.11 (C-12), 37.25 (C-9), 53.74 (C-17), 53.78 (C-15), 56.81 (C-2), 61.9 (C-10), 65.21 (C-11), 65.23 (C-6).

MS (70 eV, m/z): 234 (14), 193 (22), 137 (94), 136 (26), 122 (9), 110 (14), 98 (100), 97 (29).

Spectroscopic data for lupanine *2*: Colorless oil, $C_{15}H_{24}N_2O$, M_w=248 g/mol.

IR (KBr) n cm^{-1}: 2931, 2857 and 2765 (CH), 1634 (C=O).

^{1}H NMR (DMSO d_6, 500 MHz): d (ppm) 1.12 (1H, m, 13a ax), 1.15 (1H, ddd, 8b ax, J=10.19 Hz, J=3.98 Hz, J=2.35 Hz), 1.26 (1H, m, 12b ax), 1.38-1.43 (2H, m, 14aeq and 14b ax), 1.45 (1H, m, 5a ax), 1.47 (1H, m, 11 ax), 1.48 (1H, m, 12aeq), 1.51 (1H, m, 4aeq), 1.55 (1H, m, 9 eq), 1.64 (1H, m, 13beq), 1.70 (1H, m, 4b ax), 1.72 (1H, m, 5beq), 1.80 (1H, m, 15a ax), 1.86 (1H, dd, J=10.97 Hz, J=3.89 Hz, 17a ax), 1.97 (1H, m, 7 equiv), 2.03 (1H, m, 8aeq), 2.20 (2H, m, 3a ax and 3beq), 2.44 (1H, m, 10b ax), 2.68 (1H, d, 15beq, J=9.89 Hz), 2.72 (1H, dd, 17beq, J=10.97 Hz, J= 3.89 Hz), 3.32 (1H, m, 6ax), 3.70 (1H, m, 10aeq).

^{13}C NMR (DMSO d_6, 125 MHz): d (ppm) 19.74 (C-4), 22.83 (C-13), 24.86 (C-14), 28.53 (C-5), 29.74 (C-7), 33.02 (C-3), 33.77 (C-12), 34.00 (C-9), 38.11 (C-8), 48.52 (C-10), 53.76 (C-17), 56.83 (C-15), 61.08 (C-6), 65.23 (C-11), 168.54 (C=O). MS (70 eV, m/z): 248 (45), 209 (19), 150 (51), 149 (33), 136 (100), 98 (25).

The IR spectrum of compound **1** showed multiple bands at ~2935 $cm^{-1}$and at ~2816 cm^{-1}suggested quaternary nitrogen and combination overtones, embodying the C-H stretching the Bohlman's bands characteristic of quinolizidine-type alkaloids[14].

The peaks at ~1650 $cm^{-1}$and at ~1400 cm^{-1}indicating the presence of a conjugated carbonyl group and C-N stretching band, respectively[15].

The IR spectrum revealed, also, the presence of an intense peak at 1022 cm^{-1}due tothe C-O stretching band.

The mass spectrum of compound 1 (Mw = 234g/mol) showed a molecular ion $[M]^+$at m/z 234 (23.77 % rel. int.). A base peak at m/z 193 (22.13%) corresponding to $[M - 41]^+$indicating the loss of C3H5 which also showed at m/z 41 (10.65 %) (allylic cation). Another base peak at m/z 137 (100%) indicating the cleavage of the compound. The peaks at m/z 110 (18.85 %), m/z 98 (64.75 %) and m/z 55 (9.01 %) corresponding to fragmentation of lupinine ion respectively[16].

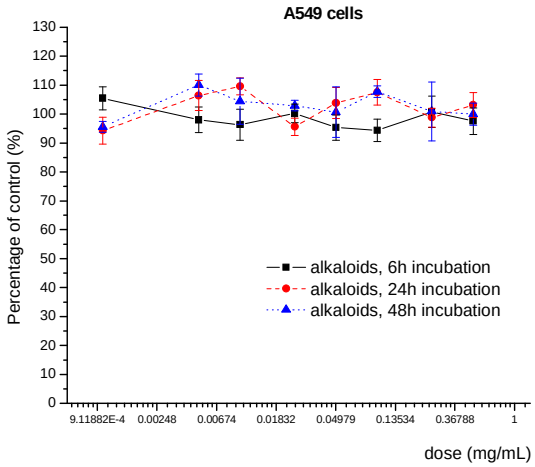


Fig. 1: Structure of sparteine1 and lupanine 2

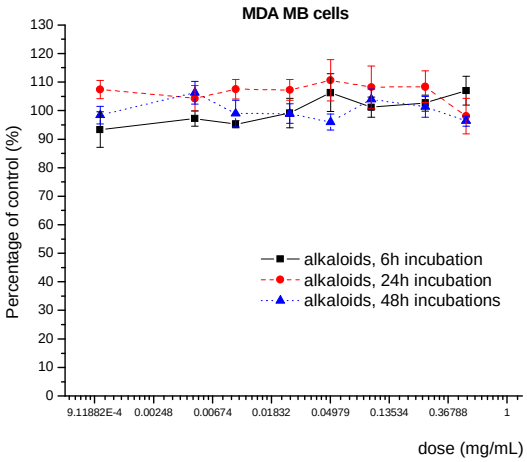


Fig. 2: Cell viability of MDA-MB 231 and A549 cell lines, 6h, 24h and 48h after treatment different concentrations of crude methanolic extract of alkaloids estimated by MTT assay. Results from three separate experiments are presented as percentage of control (Mean+S.E.)

It is known also that the fragment at m/z 98 (64.75 %) arises from ring A following the fragmentation pattern of the sparteine-type alkaloids[17].

The mass spectrum of compound 2 (Mw = 248g/mol) showed a molecular ion $[M]^+$at m/z 248 (69.67 %). A base at m/z 247 (50.81 %) corresponding of $[M - H]^+$, another peak at m/z 219 (11.47 %) indicating the loss of 28 units of mass $[M - CO]^+$. The predominant ions at m/z 149 (57.37 %), 136 (100 %), 110 (19.67 %), 98 (20.49 %), 84 (10.65 %), 55 (16.39 %) and m/z 41 (9.83 %) are characteristic of lupanine[18, 19, 20]. Results of NMR spectra were compared with literature data[21, 22].

Anti-tumor activity

We investigated *in vitro* anti-tumor activity of the crude methanolic extract of alkaloids of *cytisuspurgans* on two different cell lines (human breast cancer, MDA-MB-231 and human lung cancer, A549 cell lines). The cell viability of studied human cancer cells, 6h, 24h and 48h after treatment with increasing concentrations of the plant extract is presented in Figure 2. Numerical data illustrating cell viability were obtained from MTT assays and presented as percentage of control. Tested plant extract is showed no anti-tumor effects toward of both human cancer cell lines. Neither increasing the dose as well as prolongation of the incubation time with the tested plant extract did not lead to a statistically significant increase of anti-tumor effect.

It is known that *cytisus* species accumulate sparteine-type quinolizidine alkaloids as basic constituents[12], this phenomenon is interesting from theviewpoints of chemotaxonomy of leguminous plants and biosynthesis of quinolizidine alkaloids[23].

Quinolizidine alkaloids can be divided into more than six structural groups, although, previous studies demonstrate that matrine-type quinolizidine alkaloids, for example,mediate growth inhibition in different types of cancer cells[13], but sparteine-type quinolizidine alkaloids showed more antibacterian and antiviral effects[24, 25].

To the best of our knowledge, for the first time, we herein report the alkaloid profile of *Cytisus purgans* of Algerian flora, as well as the anti-tumor activity.

CONCLUSIONS

Cytisus purgans is a rare species of Algerian Flora. The phytochemical study of this plant indicate the presence of two known quinolizidine alkaloids: sparteine and lupanine, these two compounds were isolated from the methanolic extract of the plant and identified using different spectroscopic methods.

In the other side the test of the crude methanolic extract of alkaloids against two human cancer cells which are: breast and lung cancer, showed that the plant not exhibited any anti-tumor effect.

Future studies may show more about the benefits of this plant which accumulate quinolizidine alkaloids.

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