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Research Article

Identification of Esterified Oleanolic Acid in *Cestrum Parqui* Leaves and its Apoptotic Induction on HT-29 Cell Line

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ABSTRACT

In nowadays, there is a continuous and urgent need to discover natural anticancer products because there is an increase in the toxic sides effects associated with current chemotherapeutic agents. For this reason, we evaluated in this study, the antiproliferative effect of 8 fractions obtained from methanolic extract of *Cestrum parqui* leaves on three different cells lines (HT-29, HL-60, Molt-3 cells). After selection of the most active fractions, a bioactive purified compound noted "S" was isolated by chromatography methods (HPLC-DAD-UV; HPLC-PAD-ELSD and HPLC-UV-preparative). We used HT-29 as model cells to determine the effect of purified compound S on growth cell, induction of apoptosis death and in cell cycle disruption.

The MTT test showed that compound S inhibited the growth of HT-29 cells with an IC50 of 6µg/ml. The analysis of treated- HT-29 cells by flow cytometry after staining of cells with Annexin/FITC and propidium iodide (PI), demonstrated that this compound induces cell cycle arrest in G1 phase and induce apoptotic cell death. The structure of "S" was elucidated base on one and two NMR spectroscopic procedures and mass spectrometry as esterified oleanolic acid ((8E,11E)-tetradeca-8,11-dienoate of oleanolic acid) .

In conclusion, this study is the first investigation that shows that *Cestrum parqui* contains an esterified oleanolic acid which possesses anticancer activity against HT-29.

Key words: *Cestrum parqui*, anticancer activity, HT-29 cell line, esterified oleanolic acid.

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INTRODUCTION:

Despite many therapeutic advances in the understanding of the processes in carcinogenesis, cancer mortality worldwide remains unacceptably high. Owing to the side-effects of chemotherapy, there have been increasing studies of natural's products or herbal medicines that are considered safe and some have even been proven to be able to block, reverse or prevent the development of invasive cancers (1). Triterpénoïdes, either natural or synthetic, are known to have various properties, including anti-inflammatory, anticarcinogenic, antiproliferative

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and differentiating activities (2,3,4). Among these triterpenoid , oleanolic acid. It has been shown that oleanolic acid act at various stages of tumor development to inhibit tumor initiation and promotion (5) as well as to induce tumor cell differentiation (6) and apoptosis (7). Recently, it is demonstrated that the derivative of OA and its synthetic are more potent than OA. Indeed, The 2-a-OH derivative of OA, maslinic acid, induced apoptosis and suppressed COX-2 expression at very low concentrations compared to OA in HT-29 cells (8,9). In addition, synthetic derivative of oleanolic acid 2-cyano-3, 12-dioxoolean-1,9-dien-28-oic acid (CDDO), and its C-28 methyl ester (CDDO-Me) and C28-imidazolz (CDDO-Im) demonstrated potent antitumor activity (10,11).

Cestrum parqui is a member of solonacae family and it contains terpenoids (12). In recent years, there has been considerable attention on biological effects of cestrum parqui: spermicidal, anti-inflammatory, insecticidal activities (13,14,15); however until now, the anticancer activity of *Cestrum parqui* was not study. We report here the isolation of bioactive compound and study its antiproliferative and anticancer activities.

METHODS:

Plant material:

The leaves of Cestrum parqui are obtained from the National Institute of Agronomy Garden, Tunisia. The methanol extract of leaves of *Cestrum parqui* was prepared as fellow. The leaves were dried in a steam room at 40°C for 3 days and then it was ground to produce powder. The powdered material was extracted in methanol for 7 days and it was repeated for three times. Then the collected methanolic phases were combined and the residue was removed by filtration. The filtrate was concentrated under reduced pressure by rotary evaporator to obtain methnolic Extract. Then, the methanolic extract was subjected to silica gel column chromatography (CC, Ø 65mmxL1900mm) using CH2Cl2/MeOH mixtures of increasing polarity (40:1; 30:1; 20:1; 10:1; 8:2; 7:3; 6:4; 1:1 and finaly with methanol only) to give 8 fractions.

Chromatographic Analysis:

Analytical HPLC

The fractions were analyzed by HPLC-DAD using a Hitachi (San Jose, CA USA) Lachrom Elite HPLC system equipped with a quaternary pump, auto sampler, in-line degassing unit, temperature control unit, photo-diode array UV detector and fitted with an analytical column Kromasil C18 (Teknokroma, Barcelona, Spain) (25 × 0.4 cm i.d., 100-Å, 5-µm particle size). Acquisitions were made using EZChrom Elite 3.1.3 from Scientific Software Inc. (Pleasanton, CA, USA). Load: 40 µl, 10 µg. Elution: [A] 0.1% (v/v) aqueous TFA, [B] 0.08% (v/v) TFA in water/CH3CN 1:4, gradient 12 to 30 % [B] over 30 min at a flow rate of 1 ml/min. DAD detection was performed from 210 to 380 nm. Data were acquired in triplicate.

LC-PDA-ELSD analysis

The HPLC work was performed on an Alliance 2695 apparatus coupled with a 996 UV diode array detector (Waters Corporation, Milford, MA, USA) together with Polymer Laboratories (Amherst, MA, USA) PL-ELS 1000 evaporative light scattering (ELS) detector. Fractions were collected on a Waters fraction collector III (Waters Corporation). A column Kromasil C18 (Teknokroma, Barcelona, Spain) (25 × 0.4 cm i.d., 100-Å, 5-µm particle size) was used as stationary phase. The mobile phase consisted of water (0.1% acetic acid) (A) and methanol (B) at a flow rate of 1 ml/min. Analysis was performed using the following gradient elution: 0-20min:80%B, 20-40min:100%B. The detection wavelength was 254 nm and the injection volume was 100µl. The method of ELS detector was set up as follows: Gas flow was 1.5 l/min, Nebulizer temperature: 90° C, Evap. temperature: 120° C.

Prep-HPLC

Preparative RP-HPLC chromatography was performed on a Waters (Milford, MA) Prep LC pumping system equipped with a Waters 2489 UV/Vis detector and fitted with a X-Terra Prep MS C18 column (Waters).

Separation was performed using the same gradient elution as described in HPLC-PDA-ELSD conditions. Flow rate was set up at 10 ml/min, detection wavelength was 254 nm, and injection volume was 20 ml.

Spectroscopic analysis

^{1}H, ^{13}C NMR and two-dimensional NMR spectra were obtained with Bruker WP500 spectrometer at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. Measurements were made in $CDCl_3$ at 25 °C. In ^{1}H NMR spectra, chemical shifts are reported using TMS as an internal standard. In the ^{13}C NMR spectra, chemical shifts are reported as (ppm) values relative to the carbon signals (77.0 ppm) $CDCl_3$. Proton-detected heteronuclear correlations were measured using HMQC (optimised for $^{1}J_{HC}$ = 145 Hz) and HMBC (optimised for $^{n}J_{HC}$ = 8 Hz) experiments. Homonuclear correlation was measured using Cosy experiment. EIMS spectra (70eV) was

obtained respectively with Sciexand HP59712 spectrometer.

Biological analysis

Cell proliferation analysis:

Analysis of cell proliferation was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells with and without treatment were plated in 96- well-plates for 72h and the MTT method was carried out as described previously (16).

Cell cycle analysis:

After treatment of HT-29 cells with purified compound for 72h cells were detached from culture flask with trypsine. The cells were then collected together with floating cells and then centrifugated at 1800 rpm for 5 min. After removed of supernatant we added to cells 50µl of PBS and 500µl of staining solution (Vindelov/PI) and we incubated the cells for 30 min in room temperature before analysis by FACscan flow cytometry.

Annexin V binding assay:

Annexin V is phospholipids-binding protein have high affinity for phosphatidylserine which are translocated from the inner to the outer layer of the cell membrane in apoptotic cell death.

However, marking cells using the nucleic acid labeling coumpound PI indicates that a part of the cell is penetrable by PI; this is considered to be a sign of necrosis. So the double staining by annexin v and PI permet us to define the type of cell death.

After treatment of HT-29 cells with purified compound for 72h, cells were detached from culture flask with trypsine. The cells were then collected together with floating cells and then centrifugated at 1800 rpm for 5 min. after removed of supernatant we resuspended the cells in binding buffer. Then, the cells were incubated with Annexin V/FITC in darkness for 30 min and 400µl of buffer binding and 15 µl of PI were added before flow cytometry analysis.

STATISTICAL ANALYSIS:

Statistical comparison between the groups was done by student's *t* test using software STATISTICA. Differences between the groups were considered significant when $p<0.05$.

RESULTS:

Identification of purified compound by spectroscopic of resonance magnetic nuclear:

Compound S: ((8E,11E)-tetradeca-8,11-dienoate of oleanolic acid), was obtained as an amorphous solid with a molecular peak at m/z 663. The aliphatic moiety of compound S was established via the chemical shifts of C1' (177.5 ppm), C-2' (33.4 ppm), C-8' (131.1 ppm), C-9' (127.9 ppm), C-10' (41.5 ppm), C-11' (129.7 ppm) and C-12' (137.4 ppm).

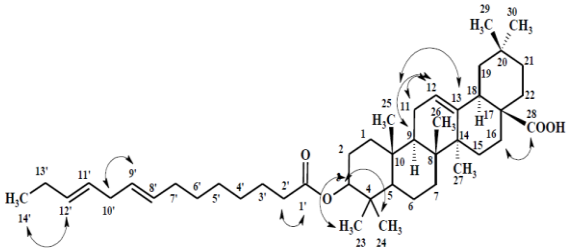
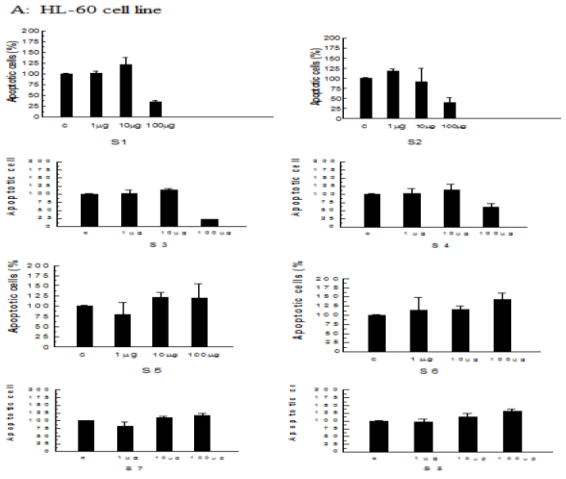


Figure 1: Compound "S": (8E,11E)-tetradeca-8,11-dienoate of oleanolic acid

The oleanolic structure (Fig 1) was proven via the chemical shifts of methyl groups: CH_3-25 (15.1 ppm); CH_3-26 (16.6 ppm); CH_3-29 (23.1 ppm) ; CH_3-27 (26.6 ppm); CH_3-23 (27.1 ppm); CH_3-24 (27.2 ppm) et CH_3-30 (32.5 ppm). Carbons C-12 (125.3 ppm); C-13(143.1 ppm); C-3 (78.6 ppm) and C-28 (182.1 ppm) were also characteristic groups of the oleanolic structure. Heteronuclear correlations H-12'/C-14'; H-9'/C-10'; H-2'/C-1'; CH_3-23/C-3; H-12/C-9; H-11/C-12 and H-16/C-28 observed on the HMBC spectrum approve well the proposed structure. Homonuclear correlations (COSY spectrum) were in agreement with the heteronuclear one.

Cytotoxicity of 8 fractions obtained from methanolic extract of *Cestrum parqui* leaves on various cancer cells:

The inhibitory effect of 8 fractions obtained after fractionation of methanolic extract of *Cestrum parqui* leaves on the growth of various cancer cells lines was measured by MTT cell proliferation assay. The MTT assay has been widely used to determine the cell viability. In culture, the metabolic activity of cells results in the chemical reduction of the yellow tetrazolim MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) to purple formazan.



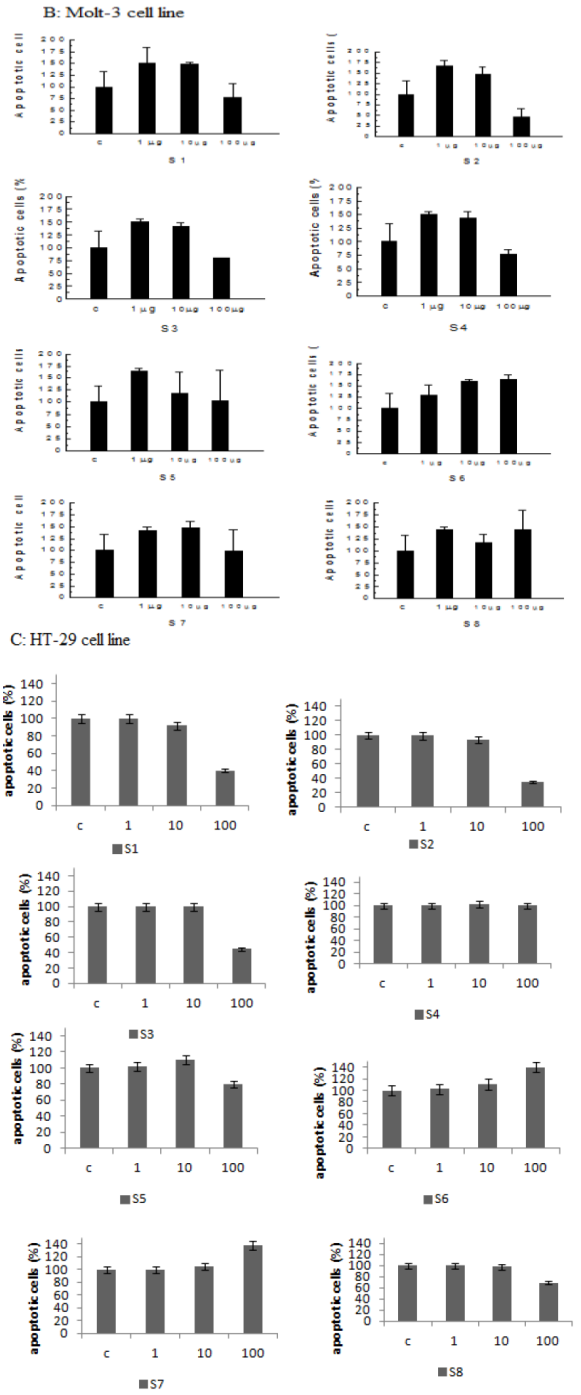


Figure 2: Antiproliferative effect of the 8 fractions of methanolic extract of *Cestrum parqui* leaves on human cancer cells lines: A: HL-60 cancer cell line, B: Molt-3 cancer cell line and C: HT-29 cancer cell line

The results (Fig.2) showed that only the fraction S2 at the concentration of 100µg/ml was effective in suppressing the growth of all cancer cells lines utilized in this study. However, the fractions S1 and S3 were effective in suppressing the growth of HL-60 and HT-29 cells at some concentration 100µg/ml and the fraction S4 was active only on the HL-60 cell line. While, all the others fractions from S5 to S8 were not active on the three cell lines.

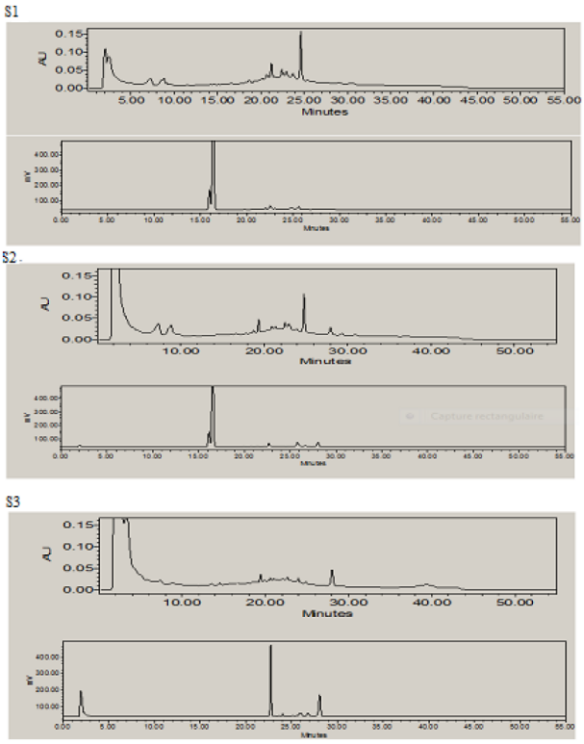


Figure 3: HPLC-PDA –ELSD profile of three active fractions (S1, S2 and S3)

The results of HPLC –PDA-ELSD (Fig3) demonstrated that the two fractions S1and S2 were nearly similar and we found that their existed an importantly compound not visible in UV and eluted in the same time (16.5 min) in this two fractions. However, the fraction S3 contained an importantly compound which is eluted at 22.5 min. For this reason we mixed only the two first fractions S1 and S2. The mixtures of two fractions were analyzed by preparative HPLC at the same protocol used in HPLC-PDA-ELSD to isolate the compound which is eluted at time of 16.5 min. After purification of this compound we tested in the first it anti-proliferative activity.

For testing the anti-proliferative effect of purified compound isolated from fraction S1 and S2 we choose as model cells HT-29 because we find in the Fig 2 that this cell line was the most sensitive to fraction S1 and S2. The result demonstrated that this compound has anti-proliferative effect on HT-29 cells line and the IC 50 value was 6 µg/ml (Fig4).

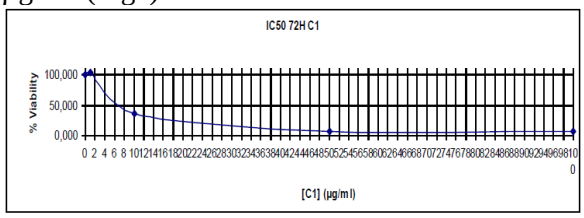


Figure 4: Antiproliferative effect of purified compound on HT-29 cells

Effect of purified compound on cell cycle profile in HT-29 cells:

Defects in cell cycle regulation and mutations in genes controlling the cell cycle are common phenomena in cancer. Many anticancer drugs act by blocking one or more stages of the cell cycle. Thus, studying the cellular DNA content and cell cycle distribution has become an important part of the drug development process.

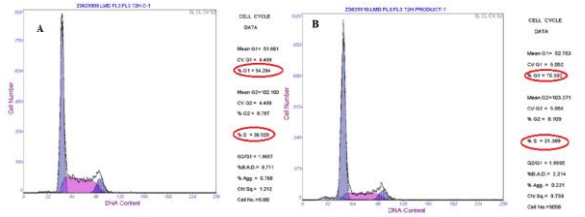


Figure 5: analysis of cell cycle of HT-29 cells treated with 6 µg/ml of purified compound : A: control and B: treated cells with purified compound

Analysis by flow cytometry of the cell cycle after treatment of HT-29 cells purified compound at concentration of 6µg/ml showed that the percentage of G1 cells reaches 70,5%, however the percentage of G1 cells in the control (cells no treated with the purified compound) is 54,28 (Fig.5). The same treatment reduced the percentage of cells in S phase from 36,9 to 21,28% and has no effect on the percentage of cells in G2 phase. The plot of flow cytometry, converted into histogram shows these results (Fig.5).

Purified compound induced apoptotic death of HT-29 cells:

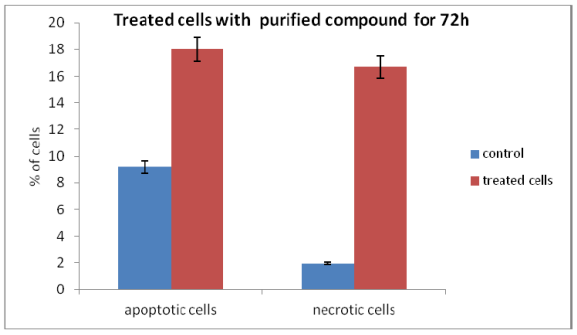


Figure 6: Effect of purified compound on the induction of apoptotic death of HT-29 cells

Apoptosis is one of the major processes leading to cell death. In recent years, cell cycle – mediated apoptosis has been receiving considerable attention due to its profound effect on controlling the growth of various malignancies. To investigate whether purified compound induced growth inhibition or cell cycle arrest, we used annexin V/PI fellow by flow cytometry which permit us to measure the apoptotic cells death. The result (Fig 5) showed that the purified compound induced both modes of cell death in HT-29 cells. This result is not surprised because we found that methanolic extract of *cestrum parqui* leaves induce the spermatozoa death by apoptotic and necrotic pathway (17) and many data demonstrated that some compounds induced both modes of cell deaths (18,19, 20) and Leist and Nicotera propose that the appearance of necrotic cells may be a result of the incomplete apoptosis (21).

DISCUSSION:

Chenni *et al.*, showed that a methanolic extract of *Cestrum parqui* leaves caused DNA fragmentation of spermatozoa leading it to die by apoptotic pathway (17). Other work carried out, in our laboratory, on the HL-60 cells showed that a fraction called fraction II could stop the cell cycle in G1 phase, induct DNA fragmentation and possible role in the initiation of apoptosis in HL-60 cells. As it is well known that any substance has apoptotic property may be indicated as an anticancer agent. To our knowledge, no studies have been conducted to verify the possible anti-cancer activity of substances extracted from *cestrum parqui* leaves.

In our study we showed that cestrum containing, after chromatographic separation processes, some substances regrouped in three fractions (S1,S2 and S3) and its anti-proliferative activity on three cell types is sufficiently clear. HT-29 cells seems the most sensitive to cestrum extract and for this reason that we used it in our tests. After analysis HPLC-PDA-ELSD we found that only the fraction S1 and S2 appear to be formed by similar compounds and we showed that the obtained compound (S) purified by preparative – HPLC was not detectable by UV. Spectroscopic means show that "S" is an esterified oleanolic acid named (8E,11E)-tetradeca-8,11-dienoate of oleanolic acid. This compound has shown significant inhibition of tumor growth which might be a promising chemotherapy candidate in treating cancers in clinic. Further experiments will focus in the mode of action of this compound.

We have shown that this compound caused an increase in the percentage of cells blocked in G1 phase of the cell cycle of HT-29 cells that means that our compound caused disruption of the DNA of treated cells, leading them to block their cycle in the G1 phase. This cycle arrest conduct cells to die by apoptosis as illustrated by our results obtained by double labeling with annexinV-PI.

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