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IN VITRO ANTIPROLIFERATIVE EFFECT AND INDUCTION OF APOPTOSIS BY *Retama monosperma* L. EXTRACT IN HUMAN CERVICAL CANCER CELLS

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Abstract

The antiproliferative effect of different extracts obtained from *Retama monosperma* L. was investigated on human SiHa and HeLa cervical cancer cell lines using a MTT colorimetric assay. The *Retama monosperma* L. dichloromethane fraction (Rm-DF) was the most active extract, exhibiting a significant cytotoxic activity on both cell lines in a dose-dependent manner, after 72h of treatment. IC_{50} values obtained were 14.57±4.15µg/ml and 21.33±7.88µg/ml, for SiHa and HeLa cell lines respectively. The morphological features assessment of apoptosis in Rm-DF-treated cells showed a condensation of chromatin and apoptotic bodies, accompanied by a decrease in mitochondrial membrane potential ($\Delta\Psi_m$) and an increase in reactive oxygen species in both cell lines. The induction of apoptosis was further confirmed by Western blotting pro-caspase 3, Bcl2 and PARP; caspase 3 activity assay; and Annexin V labelling. Analysis of Rm-DF by CG/MS revealed the presence of five known quinolizidine alkaloids as well as, sparteine (10,97%), L- methyl cytisine (9.11%), 17- oxosparteine (3.49%), lupanine (0.93%) and anagyrine (39.63%). This study shows that *Retama monosperma* L. extract exhibits a potential anticancer activity against cervical cancer cell lines *in vitro* through the inhibition of proliferation and induction of apoptosis, which may involve a mitochondria-mediated signaling pathway.

Key words: *Retama monosperma* L., *in vitro* cytotoxicity, apoptosis, cervical cancer cell lines, quinolizidine alkaloids.

Article information's

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Abbreviations: **Rm**: Retama monosperma; **Rm-HE**: Retama monosperma hexanic extract; **Rm-ME**: Retama monosperma methanolic extract; **Rm-DF**: Retama monosperma dichloromethane fraction; **Rm-AF**: Retama monosperma ethyl acetate fraction

INTRODUCTION

Natural products have been shown to be an important source for the development of new drugs. Cancer continues to be one of the major causes of death worldwide. To date, there is an increasing interest in screening medicinal plants and identification of active components that are effective against cancer cells. Approximately 50% of a total of 155 clinically approved anticancer drugs were either unmodified natural products or in the forms of their semi-synthetic derivatives, or synthesized molecules based on natural product compound pharmacophores (29). The emergence of multidrug resistance in several tumors requires a continuing need for the development of new anticancer drugs and

new chemotherapy strategies. Targeting apoptosis is one of the most important and promising mechanisms against uncontrolled growth of tumor cells (17, 36). A wide variety of natural substances found in medicinal plants such as curcumin, artemisinin have been recognized to induce apoptosis in various human tumor cells (14,22).

Retama monosperma L. (Boiss.) or Genista monosperma L. (Lam.), locally named as "R'tam", is an annual and spontaneous plant belonging to the Fabaceae family. The genus Retama includes four species with a geographic distribution in the Mediterranean area, North Africa, and the Canary islands (13). In Morocco, Retama genus is largely located in desert regions and the Middle Atlas (26). This plant is used in traditional medicine in many countries, as a purgative, vermifuge, antihelmintic, and abortive (3). Moreover, several studies investigated Retama Genus for various pharmacological effects, including hypoglycemic and diuretic (26,27), cytotoxic (6,12,25), antioxidant and antiviral (11), antihypertensive (10), and anti-inflammatory (4) activities.

The aim of this study was to investigate the antiproliferative effects of extracts obtained from Retama monosperma L. against two cervical cancer cells lines, SiHa and HeLa. Further experiments were carried out to determine whether this antiproliferative effect was associated with apoptotic signaling. We evaluated alterations in morphology, phosphatidyl serine distribution, the activation of pro-caspase-3, the cleavage of PARP, and Bcl2 expression in SiHa and HeLa cells treated with Retama monosperma extract. We also investigated the preliminary chemical analysis of the most active extract, using GC/MS.

MATERIALS AND METHODS

Plant material and extraction procedures

R*etama monosperma* L. (Boiss.) leaves were collected in March 2009 from Sidi Boughaba reserve in Mehdia-Rabat (Morocco). The plant was identified at the Scientific Institute of Rabat by Pr. M. Fennane, and the specimen was deposited in the Scientific Institute herbarium under the voucher specimen reference N° RAB78140. The powder of the dried plant was extracted successively using a Soxhlet apparatus with *n*-hexane and methanol to obtain hexanic extract (Rm-HE) and methanolic extract (Rm-ME). The resulting extracts were then evaporated by a Rotavapor to give dried extracts. The methanol concentrated extract was dissolved in distilled water and was successively extracted with dichloromethane and ethyl acetate to obtain dichloromethane fraction (Rm-DF) and ethyl acetate fraction (Rm-AF). All extracts were evaporated and kept at -20°C until use.

Cell culture

Human cervical adenocarcinoma cells lines, HeLa and SiHa, used in this work were kindly provided by Dr. P. Coursaget (INSERM, University François Rabelais, Tours, France). Cells were cultured in modified eagle medium (MEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% penicillin–streptomycin. Cells were maintained at 37°C and 5% CO_2 in a humid environment. All experiments were performed with cells in exponential growth phase.

Cell proliferation and cytotoxicity assay

HeLa and SiHa cells were seeded in a 96-multiwell plate at a density of 5. 10^3 cells/well for 24h. The cells were then treated with plant extracts and fractions at concentrations between (5-80µg/ml) for 72h. Vinblastin was used as positive control. After incubation, 10µl MTT (5mg/ml) were added to each well and cells were incubated at 37°C for 4h. The supernatant was then removed and replaced by 150µl DMSO to dissolve insoluble formazan crystal. The absorbance was measured with a plate reader (Genesys 10 UV scanning, Thermo Electron Corporation) at 550nm. The absorbance of untreated cells was considered as 100%. The IC_{50} (concentrations which reduce the viability of treated cells by 50%) values were graphically obtained from the dose–response curves.

Assessment of apoptosis (Hoechst 33342 staining)

Morphology alterations due to apoptosis were detected by *Hoechst 33342* staining. HeLa and SiHa cells were cultured on glass chamber slides in 2 well plates and were treated with the Rm-DF for 24, 48 and 72h at a concentration of 20µg/ml. After incubation, cells were washed twice with PBS and fixed with (4% paraformaldehyde and 0.1% Triton X-100) for 5min. The cells were then washed with PBS and incubated with 10µg/ml Hoechst 33342 (Sigma) at 37°C for 30min. Cells were visualized using fluorescence inverted microscope (Axiovert 200M Zeiss, Germany) equipped with an LD achroplan 40X objective. The 360nm and 460nm filters were used respectively for excitation and emission. The images were collected with a CCD cooled camera (Coolsnap HQ, Ropper Scientific).

Annexin V-FITC/propidium iodide flow cytometric analysis

Analysis of phosphatidylserine externalization in apoptotic cells was determined by an ApoTarget Annexin-V-FITC Apoptosis kit (Invitrogen, Cergy Pontoise, France), according to the manufacturer's instructions. Cells (2.10^5 cells) were seeded in 6-well plates and treated with 20µg/ml Rm-DF for 48h. They were then collected and suspended in 100µl of Annexin V binding buffer. 5µL of Annexin-V-FITC and 10µL of propidium iodide were added and then incubated 15min at room temperature in the dark. Flow cytometry analysis was carried out using a FACScalibur (BD Biosciences) flow cytometer.

Mitochondrial membrane potential ($\Delta\Psi_m$) measurement

Analysis of mitochondrial membrane potential was carried out using the lipophilic cationic probe, JC-1 (Molecular Probes, Eugene, OR) whose monomer emits at 530nm (green) after excitation at 500nm. Depending on the mitochondrial membrane potential, JC-1 is able to form J-aggregates which shifts the fluorescence emission from green to yellow-orange (590nm) as mitochondrial membrane becomes more polarized. Therefore, the I_{590}nm/I_{530}nm emission ratio value allows observation of mitochondrial dysfunction. Residual mitochondrial potential as a percentage of control was expressed as follows: (R treated/R control) x 100; R = I_{590} nm/I_{530} nm. SiHa and HeLa cells were treated with Rm-DF for 24h and 48h. JC-1 reagent (10µM) was added for 20min at 37°C in the dark. Cells were then washed with PBS and centrifuged at 1500 rpm, for 5min at 4°C. The obtained pellet was resuspended in 1ml ice-cold PBS and the measurements were performed using the Spectrofluorometer (RF-5301PC, Shimadzu, Tokyo, Japan).

Measurement of ROS production

Production of ROS (reactive oxygen species) was monitored via oxidation of the carboxydichlorofluorescein analog probe, C2938. SiHa and HeLa cells (2 x10^5) were seeded into 6-well plates and treated with the appropriate concentration of the extract for 24h. Control and treated cells were washed and stained with 10µM C2938 (30 min, 37˚C). Fluorescence emission from the oxidized probe was quantified with a Spectrofluorophotometer (RF-5301PC, Shimadzu) (excitation: 488±1 nm; emission: 518±1 nm).

Western blot analysis

Cells were treated with 20µg/ml of Rm-DF for 24, 48 and 72h, scraped, washed with PBS and lysed in ice-cold lysis buffer [(10mM Tris pH 7.4, 150mM NaCl, 5mM EDTA, 1mM Na_3VO_4, 1mM dithiothreitol, 10 µg/ml Leupeptin , 10µg/ml aprotinin, 10% glycerol, 1%Brij (v/v)], placed on ice for 20min and centrifuged at 14,000g for 15min at 4 °C. The amount of protein was determined using the Bio-Rad protein quantification kit. Equal amounts of proteins (25-30µg/ml) were subjected to electrophoresis on SDS-polyacrylamide gels and, transferred to a nitrocellulose membrane by electroblotting. After blocking non-specific sites, the membrane was incubated overnight with appropriate primary antibodies: monoclonal anti- pro-Caspase 3 (1/700), monoclonal anti-β actin (dilution 1/5000), monoclonal anti-BCl_2 (1/700), and polyclonal anti-PARP (1/1000). Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG were used as secondary antibodies and proteins were detected using an enhanced chemiluminescence (ECL) kit.

Gas chromatography/mass spectrometry (GC/MS) analysis

The identification of the compounds from *Retama monosperma* dichloromethane fraction (Rm-DF) was performed by GC/MS analysis using (TRACE CG TRA) Chromatograph, equipped with (Polaris Q MS) Mass selective detector and VB5 (5% phenyl ; 95% methylpolisyloxane) capillary column (30m, 0.25mm, film thickness 0.25µm). Injection volume was 1 µl with a splitless; the injector and detector temperatures were held constant at 250 °C. For GC/MS detection an electron ionization system with ionization energy of 70eV was used. Helium was used as the carrier gas with an inlet pressure of 10.48 psi, corresponding to a flow rate of 1.0ml/min. The analytical conditions were as follows: oven temperature from 60 to 280°C at rate of 16°C min^{-1} and the final temperature of 300°C was held for 10min. Identification of the compounds was based on the comparison of their relative retention time and spectral mass with those of Nist and Wiley7 library data of the GC/MS system.

Statistical analysis

Data are presented as means ± SD of at least triplicate determinations of three different assays. Statistical analysis was performed by Student's-test with Microsoft excel software. Significant differences are indicated by $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

RESULTS

Effect of Rm extracts on SiHa and HeLa cell growth

In order to investigate the effects of *Retama monosperma* extracts and its fractions on cell proliferation, SiHa and HeLa cervical cancer cells were treated with the extracts at different concentrations (5-80µg/ml) for 72h. Cell growth was assessed using MTT assay. IC_{50} values were calculated from the dose–response curves obtained by plotting percentage of cell growth as a function of extracts concentrations. As displayed in Fig.1, cell growth curves show that only Rm-DF exerts a dose-dependent effect on cell proliferation. SiHa cells were relatively more sensitive to Rm-DF than HeLa cells with IC_{50} value of 14.57±4.15µg/ml and 21.33±7.88µg/ml respectively (Table.1). However, the ethyl acetate extract showed weak activity while hexanic and methanolic extracts exhibited no effect. Rm-DF at a dose of 20µg/ml was chosen for further mechanistic studies.

Morphological features associated with apoptosis

To determine whether cell growth inhibition by Rm-DF was associated with apoptosis, SiHa and HeLa cells were treated with Rm-DF for 24, 48 and 72h, and Hoechst 33342 staining was performed. Cells with nuclear chromatin condensation or apoptotic bodies, which are the typical characteristic of apoptosis, were observed after treatment. The percentage of apoptotic cells was calculated and data showed that apoptotic cells significantly increased in a time-dependent manner in both SiHa and HeLa cells after exposure to Rm-DF (Fig.2).

Plasma membrane redistribution of Annexin V

During apoptosis, the plasma membrane changes include a redistribution of phosphatidylserine from the cytoplasmic face to the outer leaflet making it available for Annexin-V binding. To differentiate cells undergoing

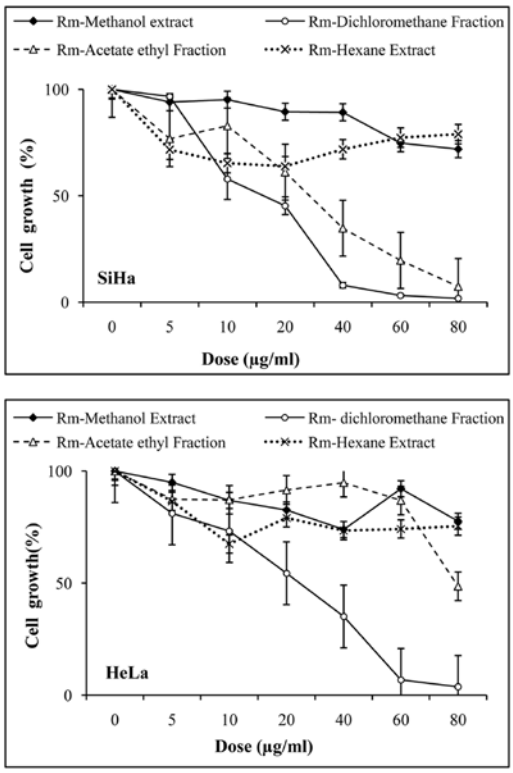

Figure 1. Cytotoxic activity of *Retama monosperma* L. extracts in cervical cancer cells. SiHa and HeLa cells were incubated with different concentrations of extracts (5-80µg/ml) for 72h. Cell growth was determined by MTT assay. IC_{50} values (µg/ml) were determined graphically from viability curves. Vinblastin was used as positive control. Data are expressed as means ± SD of quadruplicate determinations.

necrosis or apoptosis, Rm-DF treated cells were analyzed by flow cytometry using PI and Annexin V labeling. After treatment with Rm-DF (20µg/ml) for 48 h, early apoptosis (annexin labeling) was seen in 28.34% and 57.68% in SiHa cells and HeLa cells respectively (Fig.3). While, late apoptosis (annexin and PI labeling) was observed in 5.84% of SiHa cells and 10.35% of HeLa cells.

Effects of Rm-DF on the expression of proteins involved in apoptosis

In order to elucidate the mechanisms of apoptosis induced by Rm-DF, we investigated the effects of Rm-DF on the expression of apoptosis related proteins by Western blot. We examined whether Rm-DF induces the activation of pro-caspase-3 in SiHa and HeLa cells. Our data showed that Rm-DF significantly increased the cleavage of pro-caspase-3 to the active form in a time-dependent manner (Fig.4). Subsequently, the presence of activated caspase-3 is further confirmed by detecting the cleavage of PARP. In Rm-DF-treated cells, the cleavage of PARP (116KDa) into 85KDa fragment occurred in a time-dependent manner. Moreover, as can be seen in Fig.4 a decrease in Bcl-2 expression occurred in Rm-DF-treated cells, in a time-dependent manner.

Table 1. MTT assay: IC_{50} values of extracts and fractions of *Retama monosperma* against SiHa and HeLa cervical cancer cells.

Substances	**IC_{50} (µg/ml)**	
	SiHa	HeLa
Retama monosperma L. extracts		
Hexane extract (Rm-HE)	› 80	› 80
Methanolic extract (Rm-ME)	› 80	› 80
Dichloromethane fraction (Rm-DF)	14.57±4.15	21.33±7.88
Ethyl Acetate fraction (Rm-AF)	27.54±5.64	77.47±2.25
Vinblastin	10.88±0.78	6.28±0,35

Cells were exposed to different concentrations of *Retama monosperma* L. extracts (Hexane and methanol extracts) and fractions (dichloromethane and ethyl acetate fractions) for 72h. Data are expressed as IC_{50} values are means ± SD of three independent experiments. Vinblastin was used as a positive control.

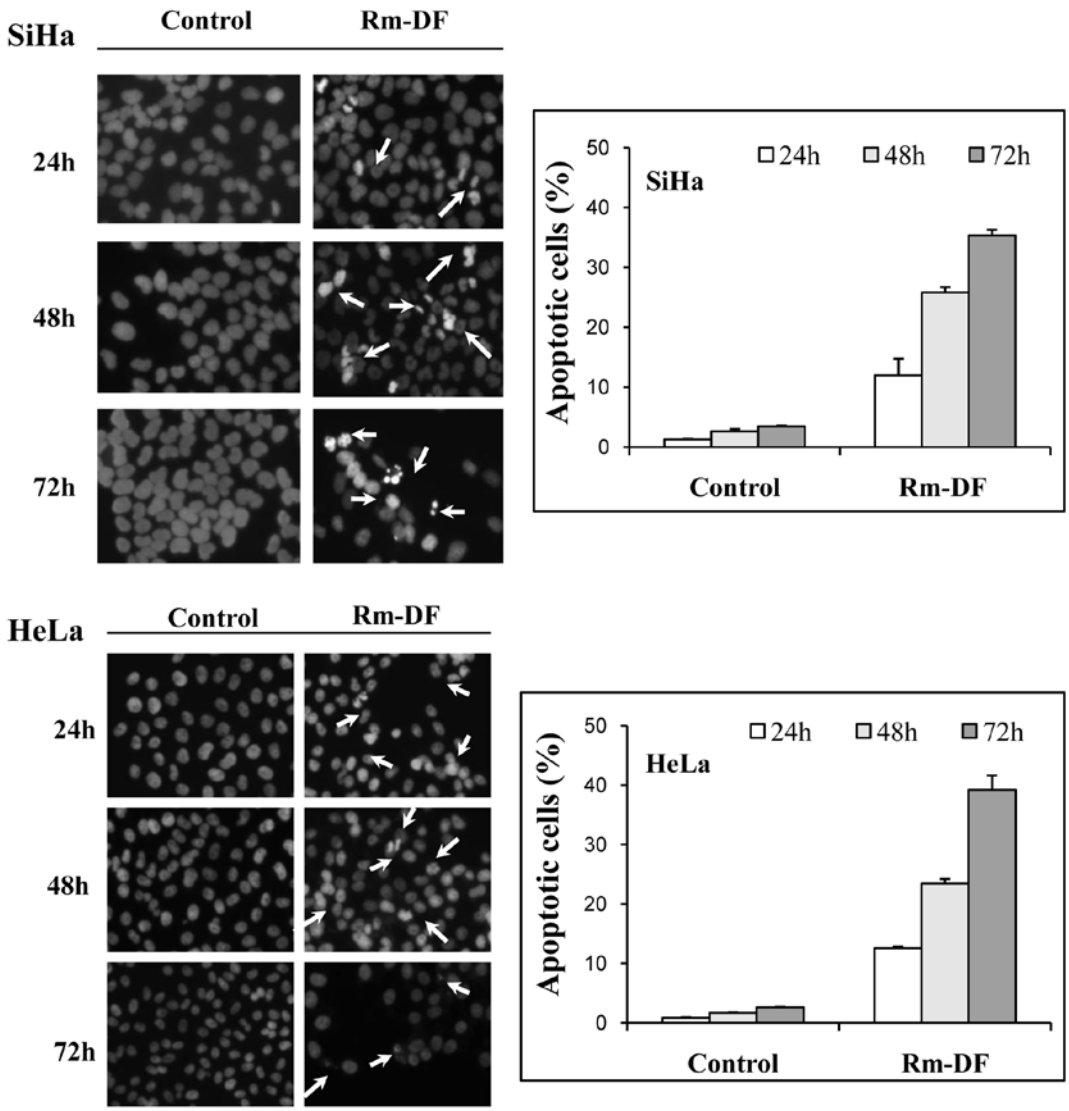

Figure 2. Detection of apoptotic cells in SiHa and HeLa cells treated with Rm-DF by fluorescence microscopy and Hoechst 33342 staining. Cells were treated with 20µg/ml of Rm-DF for 24, 48 and 72h. The stained nuclei were visualized and photographed with an inverted fluorescence microscope (Axiovert 200M Zeiss). The percentage of apoptotic cells, indicated by arrowheads, was calculated relatively to total cell number. Data represent at least two independent experiments (Magnification: 40X).

Depolarization of mitochondrial membrane potential ($\Delta\Psi_m$) in Rm-DF-treated cells

In order to characterize the effect of Rm-DF on the mitochondrial apoptotic pathway, we measured the mitochondrial membrane potential ($\Delta\Psi_m$) in SiHa and HeLa cells after treatment with Rm-DF (20µg/ml) for 24h. As shown in Fig.5, Rm-DF significantly induced a decrease in mitochondrial membrane potential in a dose-dependent manner in SiHa and HeLa cells.

Production of reactive oxygen species (ROS) induced by Rm-DF

In order to investigate whether the intracellular reactive oxygen species ROS are involved in the signal transduction pathways of apoptosis, we measured the production of ROS after cells were treated with Rm-DF (20µg/ml) for 24h, using C2938, an oxydation-sensitive fluorescent probe. Data were normalized relative to the untreated cells. As shown in Fig.6, a significant increase in ROS level was observed in Rm-DF-treated SiHa and HeLa cells after 24h exposure, when compared with control cells.

Characterization of compoundsin the Rm-DF

CG/MS analysis was carried out on the dichloromethane fraction of *Retama monosperma* L. (Rm-DF) that exerted the significant growth inhibitory effect. CG Chromatogram of Rm-DF is presented in figure 7 and analysis of its MS spectral data revealed a total of 11 compounds

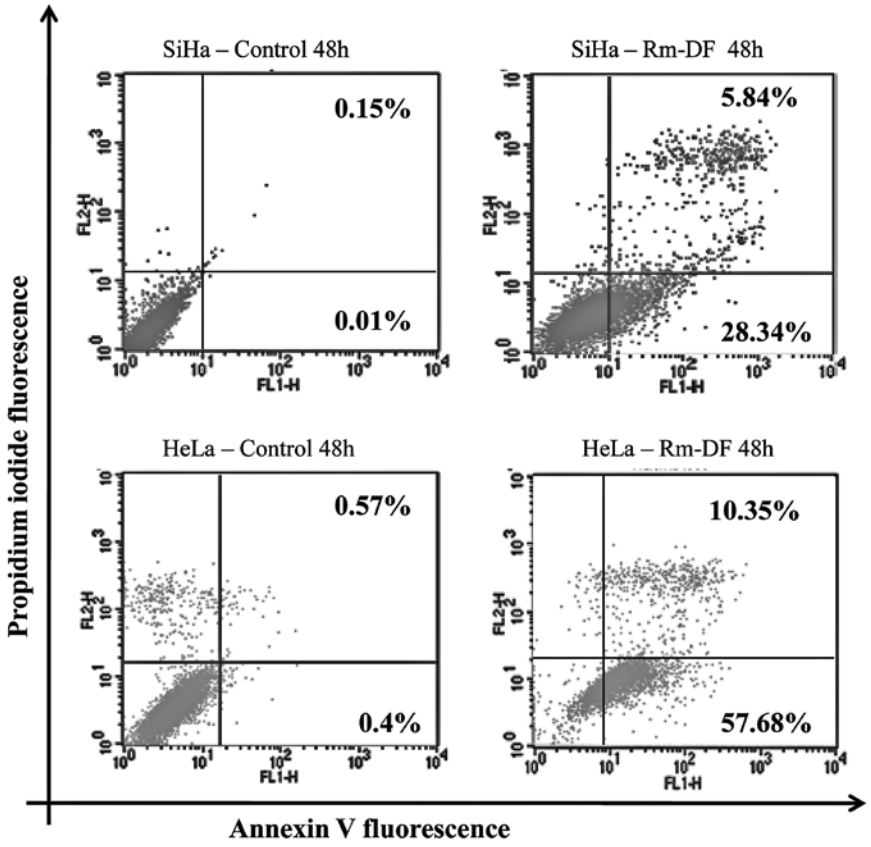

Figure 3. Annexin V and propidium iodide staining of SiHa and HeLa cells exposed to Rm-DF were analysed by flow cytomety . Cells were treated with Rm-DF (20µg/ml) for 48h. The *x*-axis shows Annexin V-FITC binding and the *y*-axis staining of propidium iodide (PI) labeled population. Lower left quadrant are viable cells: AnnexinV negative and PI negative; lower right, early apoptotic cells: AnnexinV positive and PI negative; upper right, necrotic cells or late apoptotic cells: AnnexinV positive and PI positive.

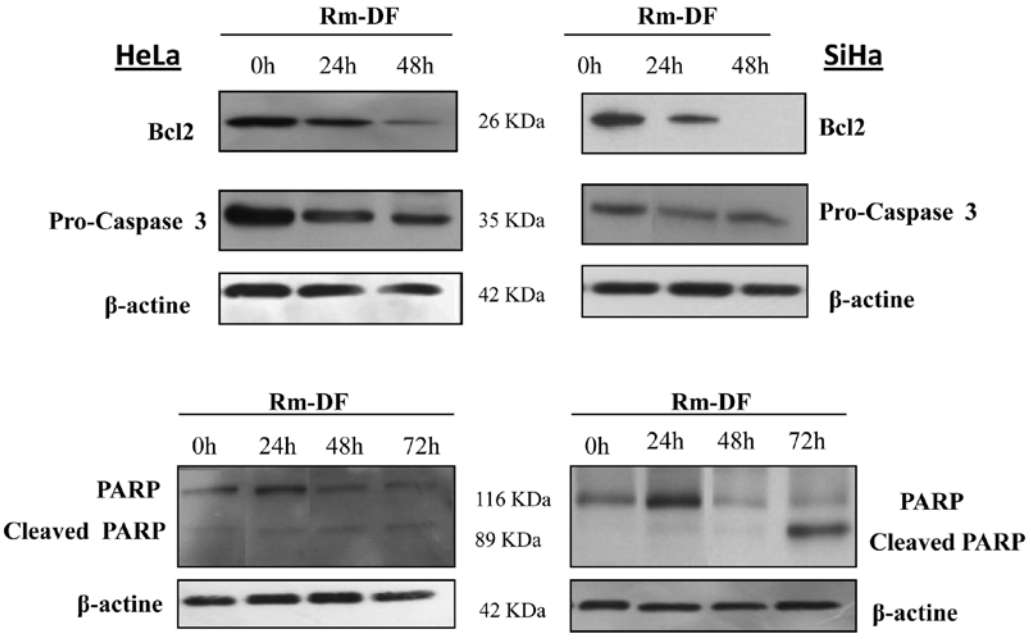

Figure 4. Western blot analysis of Pro-caspase 3 , Bcl-2 and PARP protein expression, performed in HeLa and SiHa cells after treatment with 20µg/ml Rm-DF during 24h , 48h and 72h. Cell lysates were prepared and the proteins were separated on SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. The membranes were probed with the indicated antibodies. β-actin was used as a control for protein loading. The results shown here were from two or three independent experiments.

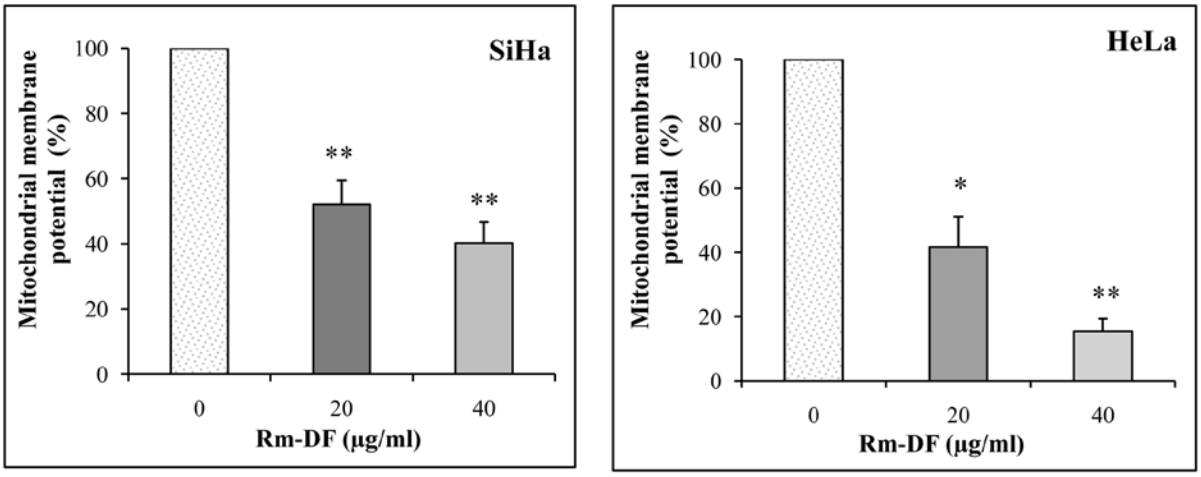

Figure 5. Induction of apoptosis in SiHa and HeLa cells by Rm-DF. Mitochondrial membrane potential was measured by spectrofluorimetry and JC-1 probe in SiHa (A) and HeLa (B). Cells were treated with 20 and 40µg/ml Rm-DF for 24h as described in Materials and Methods. The results are presented as the mean ± SD of three independent experiments.

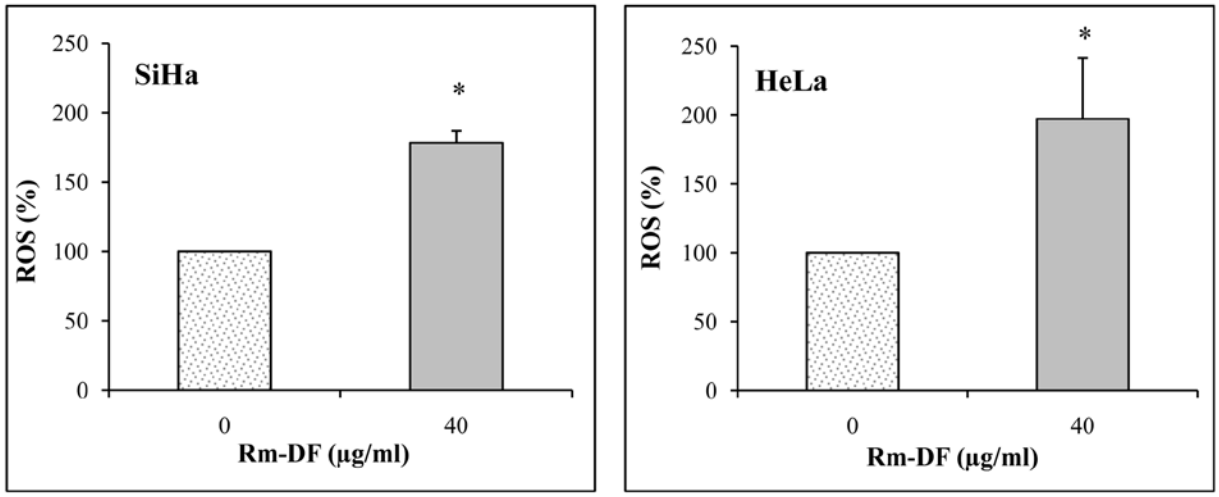

Figure 6. Effects of *Retama monosperma* extract on ROS production in SiHa (A) and HeLa (B). The level of ROS production was measured via oxydation of the carboxydichlorofluorescein analog probe C2938 after treatment with Rm-DF. Data represent the means ± standard deviations for three independent experiments.

which are summarized in Table.2. CG/MS analysis identified compounds including five known quinolizidine alkaloids: Sparteine (10.97%), L-methyl-cytisine (9.11%), 17-oxosparteine (3.49%), Lupanine (0.93%) and anagyrine (39.63%).

DISCUSSION

A large number of novel anticancer drugs have been discovered from natural products and new ones are still under development. The induction of apoptosis in cancer cells is among one of the useful strategies for anticancer drug development. Currently, the plants of the Retama genus have attracted an increasing interest due to their wide range of pharmacological effects, including antihyperglycemic activity (2), cytotoxic effect (6,12,25), antihypertensive activity (12) and anti-inflammatory activity (4), antibacterial, antifungal and antioxidant (11) activities. It has been reported that Retama species contain quinolizidine alkaloids (1,35) and flavonoids (18,24,25) as bioactive constituents.

However, to our knowledge, the anti-proliferative and apoptotic effect of *Retama monosperma* L. (Boiss.) has not yet been explored. The aim of this study was to investigate antiproliferative activity of *Retama monosperma* L. extracts against SiHa and HeLa cervical cancer cell lines. To identify whether the induction of apoptosis was a mechanism underlying the growth inhibition of cancer cells, several assays was performed. We found that the dichloromethane fraction of *Retama monosperma*

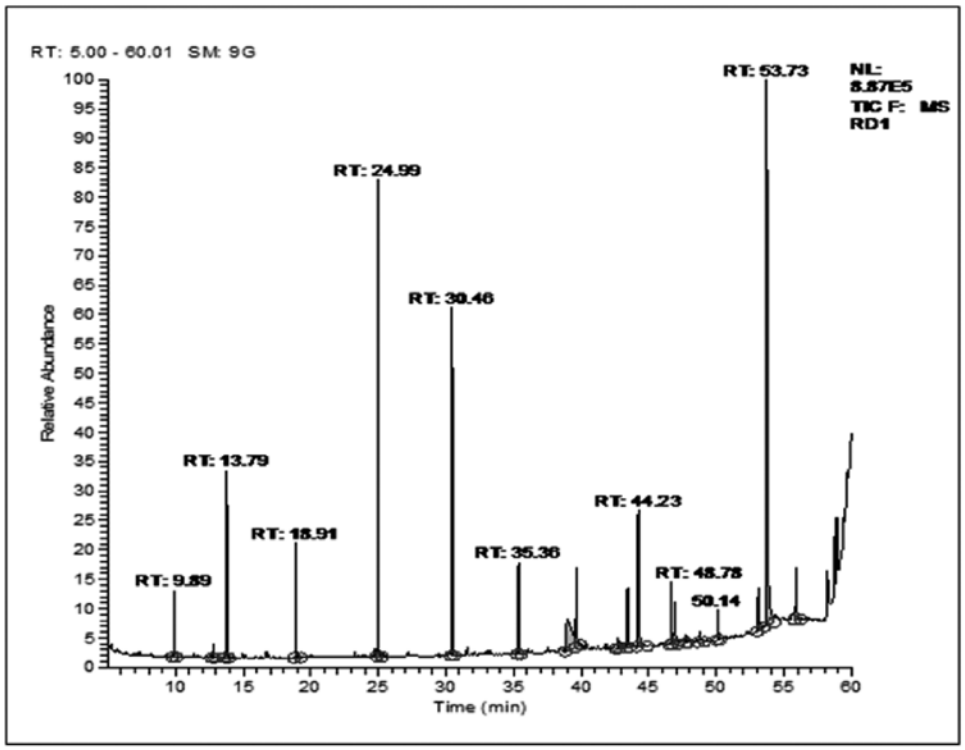

Figure 7. Chromatogram of the *Retama monosperma* dichloromethane fraction (Rm-DF) obtained by CG. Compounds were identified by computer searches in the reference libraries of NIST and Wiley7, and fragmentation patterns were compared with literature data. Rm-DF constituent are presented in Table.2.

Table 2. Compounds present in Dichloromethane fraction of *Retama monosperma* identified by CG/MS.

RT	Identified compounds	CAS	Area (%)
9,89	α-Pinene	7785-70-8	2.73
13,79	1,8-Cineole	470-82-6	8.03
18.91	Benzeneacetic acid, α,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	56114-69-3	4.71
24.99	9H-pyrrolo[3',4':3,4]pyrrolo[2,1-a]phthalazine-9, 11(10H)-dione,10-ethyl-8-phenyl	95647-39-5	19.05
38,98	Sparteine	90-39-1	10.97
42,71	Hexadecanoic acid	57-10-3	0.86
44,23	L methyl cytisine	486-86-2	9.11
46,67	17- oxosparteine	489-79-5	3.49
47.78	4-(N-(3-trifluoromethylphenyl)-amino)-5,6 dimethyl-7H-pyrro[2.3-d]pyrimidine	100633-91-8	0.50
48,78	Lupanine	550-90-3	0.93
53 ,73	Anagyrine	486-89-5	39.63

RT : Retention time (min). - CAS number: Chemical Abstracts Service.
Area (%): area percentage (peak area relative to the total peak area).

(Rm-DF) exhibited the most significant antiproliferative effect in a dose-dependent manner. Rm-DF IC_{50} values were 14 and 22µg/ml in SiHa and HeLa cell lines respectively. This is in agreement with the National Cancer Institute guidelines. In fact, the criteria of cytotoxic activity for the crude extracts is an IC_{50} value < 30µg/ml in preliminary assays (34). Many reports have also shown that other plants of the Retama genus exhibit a cytotoxic effect against various cancer cells. The methanol extracts of Retama raetam subsp. gussonei leaves exhibited a significant cytotoxic activity against COR-L23 cell line with an IC_{50} of 40µg/ml (6). Extracts and flavonoids obtained from aerial parts of *Retama sphaerocarpa* Boiss. exerted a dose-dependent cell growth inhibition against three human cancer cell lines TK-10, MCF-7 and UACC-62 (25).

Apoptosis is a major mode of cell death in response to drug treatment and possesses typical morphological features including cytoplasmic blebbing, chromatin condensation, nuclear fragmentation, cell rounding and cell shrinkage (19,32). The morphological assessment of SiHa and HeLa cells treated with Rm-DF showed significant features associated with apoptosis in a time-dependent manner. We also confirmed that Rm-DF possesses the ability to induce apoptosis via the Annexin V/PI redistribution in plasma membrane. Annexin V positive cells reflect the externalization of phosphatidylserine residues in membrane bilayers in early apoptotic events. We found that after 48h of exposure to Rm-DF, the number of SiHa and HeLa viable cells decreased with a concomitant increase in both early apoptotic and late stage apoptotic cell populations.

Two pathways are known to mediate anti-proliferative drug-induced apoptosis, death receptor-dependent (extrinsic) and mitochondrial-dependent (intrinsic) pathways. In order to elucidate the mechanisms of apoptosis, we investigated the effect of Rm-DF on the expression of specific proteins involved in apoptosis. Caspases 3, 6, 7 have been implicated in the execution phase of apoptosis and their activation and subsequent cleavage of a set of important cellular proteins lead to the appearance of apoptotic morphology (20,32). Our data show that Rm-DF significantly increased the cleavage of pro-caspase-3 to the active form Fig.4. Subsequently, the presence of activated caspase-3 is further confirmed by detecting the cleavage of PARP (116KDa) into 85KDa fragment in Rm-DF-treated cells. PARP is one of the potential target molecules of caspase 3 and PARP cleavage has been widely used as a hallmark of cell apoptosis (37).

One of the most important regulators of the intrinsic pathway is the Bcl-2 family of proteins. Increased expression of the anti-apoptotic protein Bcl-2 causes resistance to chemotherapeutic drugs, while a decrease in Bcl-2 expression may promote apoptotic response to anticancer drugs (16). We demonstrate here that Bcl-2 expression was markedly decreased in Rm-DF-treated cells. Our data clearly show that the molecular mechanisms involved in Rm-DF-induced apoptosis of SiHa and HeLa cells, seemed to be via the intrinsic pathway.

The loss in mitochondrial membrane potential, an early event in apoptosis, represents mitochondrial dysfunction which is an irreversible checkpoint in apoptosis (7,21). Thus, the effect of Rm-DF on the mitochondrial transmembrane potential $(\Delta\Psi_m)$ has been evaluated after 24h, using the lipophilic cationic probe, JC-1. A significant decrease in mitochondrial membrane potentials $(\Delta\Psi_m)$ was observed in Rm-DF-treated SiHa and HeLa cells after 24h, the effect is in dose-dependent. Mitochondrial damage caused by an increase in ROS results in the loss of the membrane potential and cytochrome c release, inducing the execution of apoptosis (31). Intracellular production of ROS active oxygen species such as -OH, O^{2-} and H_2O_2 is associated with the arrest of cell proliferation. Generation of oxidative stress in response to various external stimuli has been implicated in the activation of transcription factors and to the triggering of apoptosis (28). Our data indicate that a significant ROS production occured in Rm-DF treated SiHa and HeLa cells after 24h. These findings confirm that apoptosis induced by Rm-DF could be associated with a caspase-dependent cascade that involves the activation of the mitochondrial pathway.

Data obtained from CG/MS analysis showed the presence of quinolizidine alkaloids as major compounds, particularly the presence of sparteine, L methyl-cytisine , 17- oxosparteine , Lupanine and anagyrine. Our results were in agreement with previous reports (13,35). Considering that quinolizidine alkaloids have been found to elicit a range of biological activities, including antiviral (8,9), antihypoglycemic (5,15,30) anti-tumoral (38) activities, the marked antiproliferative activity of *Retama monosperma* extract could be attributed to the quinolizidine alkaloids, major compounds contained in dichloromethane fraction. Previous

reports have shown that oxymatrine, a natural quinolizidine alkaloid, inhibits proliferation and induces apoptosis of human hepatoma cells. This effect was mediated by a cell cycle arrest in G2/M and S phase, down-regulation of Bcl-2 and up-regulation of p53 (33). Recently, it has been reported that six quinolizidine alkaloids isolated from Sophora *Flavescens Ait*. including, sophoridine, aloperine, sophocarpine, matrine, oxymatrine and cytisine, aloperine exerted the most potent *in vitro* cytotoxic activity against the human cancer cell lines (23).

This study provides evidence that *Retama monosperma* extracts exert antiproliferative effects in cervical cancer cells via induction of mitochondria-dependent apoptosis pathway. Thus, this plant could be a potential source for new lead structures in drug design of quinolizidine alkaloids derivatives against cervical cancer. Further investigations to characterize the mechanistic action of the individual bioactive compounds in the dichloromethane fraction of this plant are required.

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