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16

# Antiproliferative Effect and Induction of Apoptosis by *Inula viscosa* L. and *Retama monosperma* L. Extracts in Human Cervical Cancer Cells

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## 1. Introduction

Worldwide cervical cancer is the second most common malignancy in women with nearly a half million new cases diagnosed and 250,000 deaths each year Almost 80% of cases occur in low-income countries, where cervical cancer is the most common cancer in women (WHO, 2009). In spite of recent advances in the development of new anticancer agents, cancer continues to be one of the major causes of death worldwide. Resistance to chemotherapeutic agents remains a principal obstacle in the successful treatment of cancer. Therefore, development and search of novel and effective anticancer agents to overcome resistance have become very important issues.

As other cancers, radiotherapy and chemotherapy are the conventional cancer treatment used nowadays and remain the routine method for the treatment of cervical cancer. These approaches present sole limits related to the cost, problems of unstable efficiency and severe side effects whose reduce the quality of life and discourage patients to observe medication protocols which then lead to the progression of cancer and associated complications. In addition, many of these treatments present limited anti-cancer activities

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(Mans, 2000). Therefore, development and search of novel and effective anticancer agents to overcome resistance and without severe side effects have become very important issues.

During last decades, natural products have been an important source of chemotherapeutics, more than half of effective cancer drugs can be traced to natural origins (Ma, 2009). Approximately 60% of drugs currently used for cancer treatment have been isolated from natural products (Gordaliza, 2007; Newman, 2007). Currently, medicinal plants constitute a common alternative for cancer treatment in many countries around the world (Gerson-Cwilich, 2006; Tascilar, 2006).

Many candidate compounds that are able to arrest proliferation and induce apoptosis in neoplastic cells have been discovered. These include *Vinca* Alkaloids; *Taxus* diterpenes; *Camptotheca* Alkaloids; and *Podophyllum* lignans. Currently, there are 16 new plant-derived compounds being tested in clinical trials and of these 13 are being tested in phase I or II, and 3 are in phase III. Among these compounds, flavopiridol, isolated from the Indian tree *Dysoxylum binectariferum* and mesoindigo, isolated from the Chinese plant *Indigofera tinctoria*, have been shown to exhibit anti-cancer effects with lesser toxicity than conventional drugs (Saklani A, 2008).

In Morocco, medicinal plants have always been associated with cultural behaviour and traditional knowledge. Herbal remedies are frequently used to treat a large variety of ailments and symptoms, like a fever, inflammation, and pain (Gonzalez-Tejero, 2008). However, there is little information about their anti-cancer properties.

Drug discovery from natural sources involves a multidisciplinary approach combining ethnobotanical, phytochemical and biological techniques to provide new chemical compounds for the development of new drugs against various pharmacological targets, including cancer and related complications. Cytotoxic screening models provide important preliminary data to select plant extracts with potential antineoplastic properties. The initial screenings are cell-based assays using established cell lines, in which the toxic effects of plant extracts or isolated compounds can be measured. Most of the clinically used antitumor agents possess significant cytotoxic activity in cell culture systems (Cardellina, 1999).

In the course to contribute to development of new anticancer drugs against cervical cancer, the human cervical carcinoma SiHa and HeLa cell lines, has been used as a model system in this study for screening promising plant materials from folk Moroccan medicine possessing anticancer effect. Thus, seven medicinal plants: *Inula viscosa* L. (Ait.), *Retama monosperma* L. (Boiss.), *Ormenis mixta* L. (Dumont.), *Ormenis eriolepis* Coss., *Rhamnus lycioides* L., *Berberis hispanica* Bois. and *Urginea maritima* L. (Baker.) were collected and evaluated for the *in vitro* cytotoxic effect against SiHa and HeLa cell lines. The selection was made on the basis of their reputation as folk medicines and ethnobotanic informations to treat different illnesses and diseases. The selected plants have been described to exhibit several biological activities. However, the antiproliferative and apoptotic effects of these plants against cervical cancer cells have not yet been explored. The second part of this study, the most active plants were then selected and were evaluated for their potential antiproliferative effects against SiHa and HeLa cells. Furthers assays were used to elucidate its cytotoxic mechanism.

## 2. Materials and methods

### 2.1 Plant species

Seven plant species were collected from different regions of Morocco and were identified by Dr. M. Fennane from the Scientific Institute of Rabat. Voucher specimens are kept in the herbarium of institute. Table 1 shows the ethnobotanical data of the investigated plant species, including botanical names, local names, ethnomedical uses, as well as the plant parts employed in this study.

| Plants species (Family) | Place of collection | Part plant collected | Traditional use | pharmacological activities |
|---|---|---|---|---|
| *Inula viscosa* L. Ait (Asteraceae) | Ain atik Temara | Leaves | Skin diseases, treats cutaneous abcesses, wound healing, Tuberculosis, bronchial infections (Bellakhdar, 1997) | Anti-inflammatory effects (Hernandez, 2007; Máñez, 2007) Antimicrobial activity (Maoz., 1998) Antifungal activity (Cafarchia, 2002) |
| *Retama monosperma* L. Bois (Fabaceae) | Sidi-Boughaba Mahdia | Leaves | Purgative, vermifuge, antihelmintic, abortive and disinfectant (Benrahmoune, 2003) | No information available |
| *Berberis hispanica* Bois and Reut. (Berberidaceae) | Tamahdit | Bark roots | Blood pressure, digestive, disorders, anorexia, urinary system, nephritic, liver and astrointestinal disorders, ocular affections, febrifuge, antileishmania, antitumoral (Bellakhdar, 1997) | Antimicrobial activity (Ai-Rong, 2007) Antitumor activities (Fukuda, 1999; Meenakshi, 2007) |
| *Ormenis eriolepis* Coss. (Asteraceae) | Ouarzazat | Aerial part | Stomachic, anthelmintic and antidiabetic (Bellakhdar, 1997) | Antibactrial activities (El Hanbali, 2004) Antileishmania activities (El Hanbali, 2005) Antifungic activity (Amani, 2008) |
| *Ormenis mixta* (Asteraceae) | Sidi-Boughaba Mahdia | Aerial part | Drain the buttons, healing wounds (Haddad, 2003) | Antimicrobial activity (Satrani, 2007) |
| *Rhamnus lycioides* ssp. *Oleoides* (Rhamnaceae) | Sidi-Boughaba Mahdia | Leaves | Laxative, diuretic and hepatic affections (Hmamouchi, 2001) | Hypotensive activity (Terencio, 1990) |
| *Urginea maritima* L. *Baker* (Lemnaceae) | Sidi-Boughaba Mahdia | Bulbs | Cardiac failures, whooping-cough, pneumonia, abortive, vipers bites, aphrodisiac, cough, bronchitis and the jaundice, diuretic and internal tumours (Bellakhdar, 1997) | Cytotoxic and antimalarial activities (Sathiyamoorthy, 1999) |

Table 1. Ethnobotanical data and some reported pharmacological activities of plants species used in this study.

### 2.2 Plant extracts preparation

The seven Plants were dried and ground finely. 20g of each powdered plant were extracted by absolute methanol (100 ml, three times) for 72 h at room temperature. The extracts were evaporated to dryness under reduced pressure at 40°C. A total of 40 mg of obtained extract were dissolved in dimethyl sulfoxide (DMSO) to give a solution stock to 40 mg/ml and conserved at -20°C until use.

In second part of the study, the most actives plants were submitted to extraction with solvents with different polarities. *Inula viscosa* L. and *Retama monosperma* L. were extracted successively in a Soxhlet with *n*-hexane and methanol. The resulting extracts were then evaporated by Rotavapor to give dried extracts. The methanol concentrated extract was dissolved in distilled water and was successively extracted with dichloromethane and ethyl acetate. The solvent was evaporated to obtain the crudes extracts, and kept in the dark at +4 °C until tested.

### 2.3 Cell lines

Human cervical cancer SiHa and HeLa cell lines were used in this study. Cells were grown as monolayers in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated fetal calf serum and 1% Penicillin-Spreptomycin mixture. Cultures were maintained at 37°C in 5% CO2. SiHa and HeLa cell lines were kindly provided by Dr. P. Coursaget, INSERM U618, University François Rabelais, Tours, France.

### 2.4 Cytotoxicity assay

Cytotoxicity of the plant extracts was determined using the MTT Assay as described previously (Mosmann, 1983). Cells were seeded in 96-well microplates. After 24 h of culture, the cells were treated with different concentrations ranging from 15.6 to 500 µg/ml, in quadruplicate for 48h or 72h incubation. 10$\mu$L MTT (5mg/mL) was added to each well. After 4 hours incubation, 150$\mu$L DMSO were added to dissolve purple formazan crystals, and absorbance was then determined using a spectrophotometer at 590nm. Mitomycin C and vinblastin (~ 95 % HPLC, sigma-Aldrich) were used as a positive control.

### 2.5 Detection of the morphological changes associated with apoptosis

SiHa and HeLa cells were cultured on glass chamber slides in 2 well plates and were treated with the IV-HE, IV-DF and Rm-DF for 24h , 48h and 72h at a concentration of 20µg/ml. After incubation, cells were washed with PBS twice and fixed with (4% paraformaldehyde and 0.1% Triton X-100) for 5 min. The cells were then washed with PBS and incubated with Hoecsht 33342 (10µg/ml) (Sigma) at 37 °C for 30min. The cells were visualized through fluorescence inverted microscope (Axiovert 200M Zeiss, Germany) equipped with an LD achroplan 40X objective. The images were collected with a CCD cooled camera (Coolsnap HQ, Ropper Scientific).

### 2.6 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 530 nm (green) after

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

### 2.7 Reactive oxygene species (ROS) production

Production of ROS (reactive oxygen species) was monitored via oxidation of the carboxydichlorofluorescein analog probe, C2938. SiHa and HeLa cells (2 x$10^5$) were seeded into a 6-well plate and treated with the appropriate concentration of the extract for 24 h. Control and treated cells were washed and stained with 10 µM 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).

### 2.8 Western blot analysis

Cells were treated with 20 µg/ml of extracts for (24h, 48h and 72h), scrapped, washed with PBS and lysed in ice-cold lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM $Na_3VO_4$, 1mM dithiothreitol, 10µg/ml Leupeptin , 10µg/ml aprotinin, 10% glycerol, 1%Brij (v/v)) , placed on ice for 20 and centrifuged at 14,000g for 15 min at 4 °C. The amount of protein was determined using the Bio-Rad protein quantification kit. Equal amounts of proteins (25-30µg/ml) was subjected to electrophorese 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.

### 2.9 Gas chromatography/mass spectrometry (GC/MS) analysis

The identification of the compounds from *I. viscosa* hexanic fraction (IV-HE) and *R. monosperma* dichloromethane fraction (Rm-DF) was performed by (GC/MS) analysis using a Hewlett Packard 5890 II Gaz Chromatograph, equipped with a HP 5972 Mass selective detector and a VB5 ( 5% phenyl ; 95% methylpolisyloxane) capillary column (30 m, 0.25 mm, film thickness 0.25 µm). Injection volume was 1 µl with a splitless; the injector and detector temperatures was held constant at 250. For GC/MS detection an electron ionization system with ionization energy of 70 eV was used. Helium was used as the carrier gas with an inlet pressure of 10.48 psi, corresponding to a flow rate of 1.0 ml/min. The analytical conditions worked the following programme: oven temperature from 60 to 280°C at rate of 16°C $min^{-1}$,

the final temperature of 300°C was held for 10 min. Tentative 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.

### 2.10 Statistical analysis

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

## 3. Results and discussion

### 3.1 Cytotoxic effect of the medicinal plants extracts from Morocco

Crude extracts of selected plants were made by exhaustive methanol extraction. These plants extracts were tested for their potential cytotoxic effects, SiHa and HeLa cells were treated with plants extracts at different concentrations ranging from 15 to 500 µg/ml for 48h. The cells viability were determined by MTT assay. Among the 7 medicinal plant extracts, methanolic extract from *Inula viscosa* L. and *Retama monosperma* L. have been found to exhibit marked cytotoxic effect on both SiHa and HeLa cell lines. Their $IC_{50}$ values were 54±12 and 99±1 µg/ml in SiHa cells and 60±8 and 112±4 µg/ml in HeLa cells, respectively. The methanolic extract of *Ormenis eriolepis* Coss., *Ormenis mixta* L. and *Berberis hispanica* Boiss. have lower cytotoxic effect on the cancer cell lines tested (Table 2). However, *Urginea maritime* L. and *Rhamnus lycioides* L. had insignificant or no cytotoxic effects at tested concentration with $IC_{50}$ >500µ/ml.

| Methanolic extracts | $IC_{50}$ ± SD (µg/ml) | |
|---|---|---|
| | ***SiHa*** | ***HeLa*** |
| *Inula viscosa* L. | 54 ± 12 | 60 ± 8 |
| *Ormenis eriolepis* Coss. | 94 ± 4 | 112 ± 4 |
| *Ormenis mixta* L. | 383 ± 26 | 311± 14 |
| *Berberis hispanica* Boiss. | 178 ± 5 | 224 ± 10 |
| *Retama Monosperma* L. | 99 ± 1 | 96 ± 4 |
| *Urginea maritime* L. | › 500 | › 500 |
| *Rhamnus lycioides* L. | › 500 | › 500 |
| Mitomycin C | 6 ± 1 | 1 ± 0.30 |

Table 2. Cytotoxic activity of methanolic extracts of some medicinal plants from Morocco on SiHa and HeLa cervical cancer cell lines.

*Inula viscosa* L. (Ait.) and *Retama monosperma* L. methanolic extracts showed the highest cytotoxic activity with lowest $IC_{50}$ values. Previous studies have reported interesting biological activities with potential therapeutic applications of these plants. *Inula viscosa* L. is used in Moroccan folk medicine as antihelmintic, diuretic, anemia and as cataplasm for rheumatic pain (Hmamouchi, 2001), tuberculosis, expectorant and treatment of bronchitis

(Bellakhdar, 1997). The aerial part of this plant is used as decoction in the treatment of diabetes, hypertension and renal diseases (Eddouks, 2002). This plant has been described to exhibit several biological activities such as anti-inflammatory (Hernández, 2007), antimicrobial (Maoz, 1998) and antifungal effects (Cafarchia, 2002). *Retama monosperma* L. is used in the traditional medicine of many countries, as a purgative, vermifuge, antihelmintic and abortive (Bellakhdar, 1997). Moreover, it has been reported that R*etama Genus* for a various pharmacological effects, including an hypoglycemic and diuretic (Maghrani, 2005a; Maghrani, 2005b), cytotoxic (Conforti, 2004; Hayet, 2007; López-Lázaro, 2000), antioxidant and antiviral (Edziri, 2010) and antihypertensive (Eddouks, 2007).

Cells were exposed to different concentrations of extracts for 48h. Data are expressed as $IC_{50}$ values (µg/ml) and are means ± SD of three experiments. Mitomycine was used as positive control.

**The cytotoxic effect of extracts from *Inula viscosa* L. and *Retama monosperma* L.**

As evidenced by MTT assays, we found that hexanic (IV-HE) and dichloromethane (IV-DF) extracts from *Inula viscosa* were able to inhibit cell growth in dose-dependent manner after 72h of treatment, in both cell lines. The $IC_{50}$ values for IV-HE on SiHa and HeLa were 9.56±1.68 and 13.17±0.79 µg/ml, respectively. However, for IV-DF, the $IC_{50}$ values on SiHa and HeLa were respectively 6.54±1.46 and 22.04±3.31 µg/ml. *Retama monosperma* dichloromethane fraction (Rm-DF) was the most active extract, exhibiting also cytotoxic activity against both cells lines in dose-dependent manner. Values of $IC_{50}$ obtained were 14± and 21±µg/ml, in SiHa and HeLa cell lines respectively (Table 3). The American National Cancer Institute assigns a significant cytotoxic effect of promising anticancer product for future bio-guided studies if it exerts an $IC_{50}$ value <30 µg/ml (Suffnes, 1990). The obtained results indicate that IV-HE and IV-DF and Rm-DF were shown to induce significant and dose-dependent inhibitory activities against human cervical cancer cell lines SiHa and HeLa.

| Extracts | $IC_{50}$ (µg/ml) | |
|---|---|---|
| | SiHa | HeLa |
| *Retama monosperma* L. extracts | | |
| Hexane extract (Rm-HE) | › 80 | › 80 |
| Methanol extract (Rm-ME) | › 80 | › 80 |
| Dichloromethane fraction (Rm-DF) | 14.57±4.15 | 21.33±7.88 |
| Acetate ethyle fraction (Rm-AF) | 27.54±5.64 | 77.47±2.25 |
| Inula viscosa L. extracts | | |
| Hexane extract (IV-HE) | 9.56 ± 1.68 | 13.17±0.79 |
| Methanol extract (IV-ME) | 52.83±3.28 | › 80 |
| Dichloromethane fraction (IV-DF) | 6.54±1.46 | 22.04±3.31 |
| Ethyl acetate fraction (IV-AF) | 63.62±10.55 | › 80 |
| Vinblastin | 10.88±0.78 | 6.28±0,35 |

Table 3. Cytotoxic effect of extracts and fractions of *Retama monosperma* L. and *Inula viscosa* L. extracts against SiHa and HeLa cervical cancer cells.

Cells were exposed to different concentrations of extracts for 72h. As determined by MTT assay. Data are expressed as $IC_{50}$ values (µg/ml) and are means ± SD of three experiments. Vinblastin was used as a positive control.

### 3.2 Chemical identification of plants extracts

Analyses of the most active extracts by gas chromatography (GC) coupled with and GC-mass spectrometry (MS) revealed the presence of a sesquiterpene acid: isocostic acid (46.05%) and two sesquiterpenes lactones: tomentosin (33.27%) and inuviscolide (13.04%), as major compounds in IV-HE extract (Table 4). In the fact, *Inula viscosa* L. is source of a number of bioactives compounds as well as flavonoids (Hernandez, 2007) and sesquiterpene derivatives (Fontana, 2007).

| Extracts | Compounds | RT | Area (%) |
|---|---|---|---|
| IV-HE | 1-Amino-1-ortho-chlorophenyl-2-(2-quinoxalinyl)ethene | 12.79 | 0.21 |
| | 3-(4'-Methoxyphenyl)-1-acetyl-2-phenylindolizine | 24.99 | 1.68 |
| | Isocostic acid | 40.56 | 46.05 |
| | Isoaromadendrene epoxide | 41.38 | 1.44 |
| | Phenanthrene, 7-ethenyl-1,2,3,4,4α,4β,5,6,7,8,10,10α-dodecahydro-4α,7-dimethyl-1-methylene-, [4αS-(4αα',4βα',7α',10αα')]- | 42.74 | 0.69 |
| | Iso-velleral | 46.26 | 1.87 |
| | 6,9,12,15-Docosatetraenoic acid, methyl ester | 46.82 | 0.37 |
| | Quercetin 7,3',4'-trimethoxy | 46.93 | 0.22 |
| | Tomentosin | 47.27 | 33.27 |
| | Inuviscolide | 47.39 | 13.04 |
| | Tetracosane | 54.32 | 0.77 |
| | 6-Imino-8-(3',5'-dichlorolphenyl)-3,4-dihydro-2H, 6H-pyrimido[2,1-β][1,3]thiazine-7-carbonitrile | 57.78 | 0.39 |
| IV-DF | Benzeneacetic acid, α',4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester | 18 .91 | 10.44 |
| | 9H-pyrrolo[3',4':3,4]pyrrolo[2,1-a]phthalazine-9,11(10H)-dione,10-ethyl-8-phenyl | 24.99 | 47.85 |
| | Isocostic acid | 40.56 | 2.29 |
| | 1 ,2-longidione | 44.54 | 5.03 |
| | 10 hydroxy-1 ,4,5,8-Tetramethyl anthrone | 44.84 | 2.54 |
| | Chiapin B | 46.27 | 1.55 |
| | 2,4,7 Trimethyl-5 ,6-diphenyl-1H-isoindol-1 ,3(2H)-dione | 47.01 | 9.12 |
| | Tomentosin | 47.26 | 13.93 |
| | Methyl 2,3-Dideoxy-4-O-propargyl-6-O-(tert-butyldimethylsilyl)-α-D-erythro-hex-2-enopyranoside | 55.92 | 7.24 |

Area (%):(%): area percentage (peak area relative to the total peak area percentage).
RT : Retention time (min).

Table 4. Compounds present within *Inula viscosa* L. extracts identified by CG/MS.

However, CG/MS analysis of Rm-DF (Table 5) 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%). The Retama species have been reported to contain alkaloids (Abdel Halim, 1997) and flavonoids (Kassem, 2000). Fifteen quinolizidine and 3 dipiperidine alkaloids were isolated from the leaves of flowering plants of *R. monosperma* collected from Morocco (Touati, 1996).

| Extracts | Compounds | RT | Area(%) |
|---|---|---|---|
| **Rm-DF** | α-Pinene | 9,89 | 2.73 |
| | 1,8-Cineole | 13,79 | 8.03 |
| | Benzeneacetic acid, α,4-bis[(trimethylsilyl)oxy]-, | 18.91 | 4.71 |
| | trimethylsilyl ester | | |
| | 9H-pyrrolo[3',4':3,4]pyrrolo[2,1-a]phthalazine-9, | 24.99 | 19.05 |
| | 11(10H)-dione,10-ethyl-8-phenyl | | |
| | Sparteine | 38,98 | 10.97 |
| | Hexadecanoic acid | 42,71 | 0.86 |
| | L methyl cytisine | 44,23 | 9.11 |
| | 17- oxosparteine | 46,67 | 3.49 |
| | 4-(N-(3-trifluoromethylphenyl)-amino)-5,6-dimethyl-7H-pyrro[2.3-d]pyrimidine | 47.78 | 0.50 |
| | Lupanine | 48,78 | 0.93 |
| | Anagyrine | 53 ,73 | 39.63 |

Area (%):(%): area percentage (peak area relative to the total peak area percentage).
RT : Retention time (min).

Table 5. Compounds present within *R. monosperma* L. extracts identified by CG/MS.

### 3.3 Molecular mechanisms of apoptosis signalling pathways

Induction of apoptosis constitute an important mechanism for anticancer effects of many naturally occurring and synthetic agents. Activation of apoptotic pathways seems to be an effective strategy against tumor progression (Brown, 2005). The caspase pathway plays a pivotal role in the induction, transduction and amplification of intracellular apoptotic signals. Among the caspase family proteins, capase-3 is responsible for the proteolytic cleavage of many key proteins such as PARP, which is considered as a marker of apoptosis (Kothakota, 1997; Wang, 2005).

### 3.4 IV-HE, IV-DF and Rm-DF induced apoptosis in SiHa and HeLa cells

In order to determine whether plant extracts induced cell death was due to apoptosis, we analyzed chromatin condensation and nuclear fragmentation by Hoechst 33342 staining and fluorescence microscopy (Kerr, 1994). SiHa and HeLa cells were treated with IV-HE, IV-DF and Rm-DF for 24h, 48h and 72h. As shown in Figure 1, the rate of apoptotic cells was increased significantly in a time-dependant manner after treatment with IV-HE, IV-DF and Rm-DF (Figure.1).

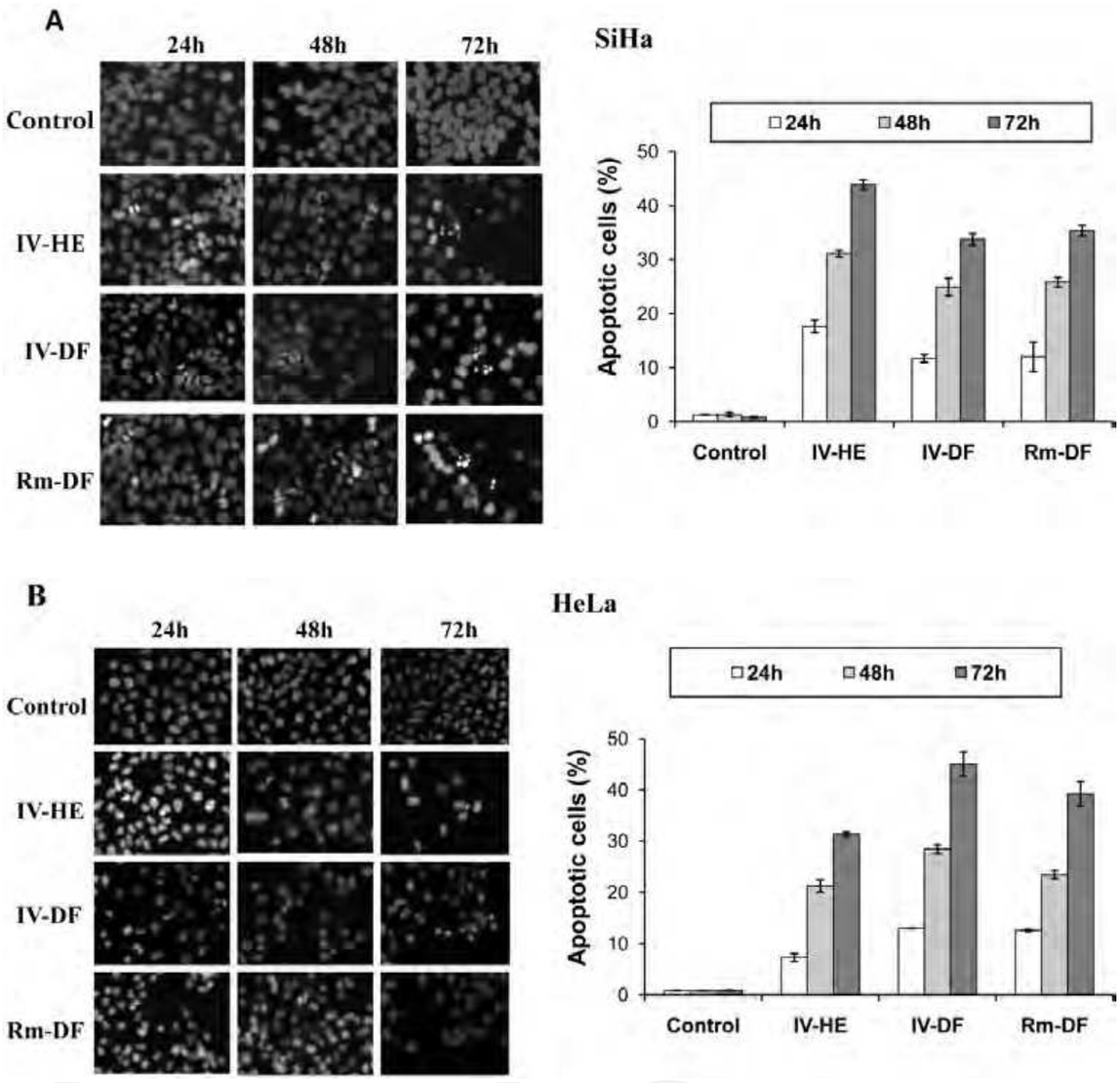


Fig. 1. IV-HE, IV-DF and Rm-DF induce apoptosis of cervical cancer cells. SiHa (A) and HeLa (B) cells were treated IV-HE, IV-DF and Rm-DF and stained with Hoechst 33342. Condensed, fragmented nuclei and apoptotic bodies were seen in the treated cells. The stained nuclei were visualized and photographed with an inverted fluorescence microscope (Axiovert 200M Zeiss). Data represent at least two experiments (Magnification: x 400).

### 3.5 Expression of Pro-caspase, Bcl2 and PARP cleavage

*Inula viscosa* and *Retama monosperma* extracts were able to induce apoptosis in HeLa and SiHa cells as evidenced by western blot analysis. Activation of caspase-3 causes the cleavage of poly-(ADP-ribose)-polymerase (PARP), a hallmark of apoptosis, to produce an 85 kDa fragment during apoptosis (Tewari, 1995). After treatment of cells, a procaspase-3 cleavage and cleavage of poly (ADP-ribose) polymerase (PARP) were observed in time- and dose-dependent manner. IV-HE, IV-DF and Rm-DF caused the proteolytic cleavage of PARP with accumulation of the 85 kDa fragment in SiHa and HeLa cells (Figure 2.A; 2.B). This

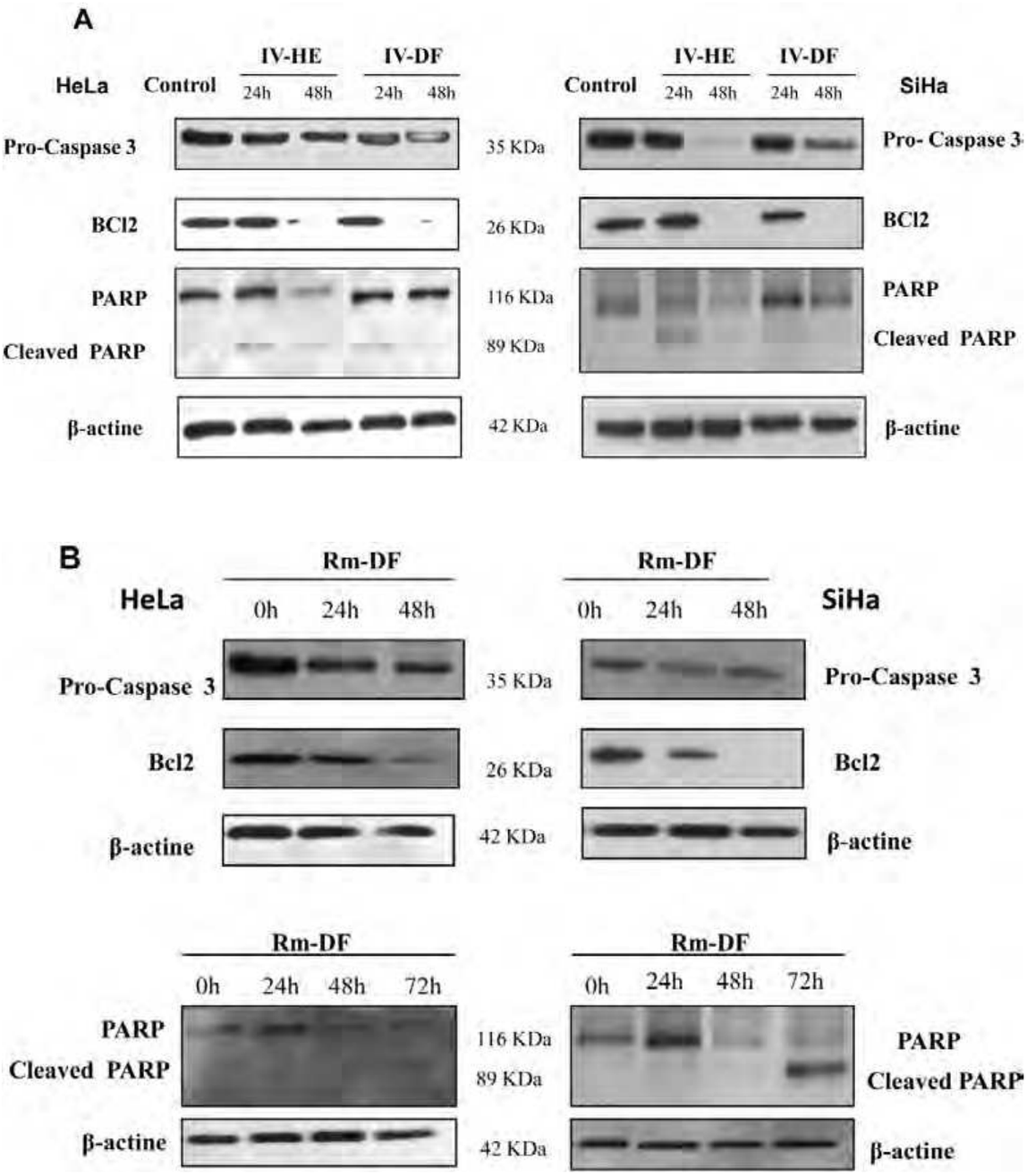


Fig. 2. Expression of Pro-caspase 3 , Bcl-2 and PARP proteins in HeLa and SiHa treated-cells analysed by Western blot. After treatment with 20µg/ml of IV-HE, IV-DF (A) and Rm-DF (B) during 24h and 48 h or 72h, cell lysates were prepared and the proteins were separated on SDS-polyacrylamide gel and transferred into 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 representative experiments.

suggests that apoptosis induced by IV-HE, IV-DF and Rm-DF could be associated with a caspase-dependent pathway.

The activation and function of caspases are regulated by various key of molecules, such as inhibitors of apoptosis protein, Bcl-2 protein family. Increased expression of the anti-apoptotic protein Bcl-2 causes resistance to chemotherapeutic drugs, while decreasing Bcl-2 expression may promote apoptotic responses to anticancer drugs (Reed J.C., 1994). Our investigations showed a significant decrease in Bcl-2 expression after 24h treatment with IV-HE, IV-DF and Rm-DF (Figure 2A, 2B).

### 3.6 Statut of mitochondrial membrane potential

Mitochondrial dysfunction has been shown to participate in the induction of apoptosis. Indeed, opening of the mitochondrial permeability transition pore has been demonstrated to induce depolarization of the transmembrane potential ($\Delta\Psi m$), release of apoptogenic factors and loss of oxidative phosphorylation (Zimmermann, 2001). To characterize the effect of IV-HE, IV-DF and Rm-DF on the mitochondrial apoptotic pathway, we measured the mitochondrial membrane potential ($\Delta\Psi_m$) in SiHa and HeLa cells after treatment for 24h. As shown in (Figure.3), IV-HE, IV-DF and Rm-DF, induced a significant decrease in mitochondrial membrane potential ($\Delta\Psi m$), in both SiHa and HeLa cells.

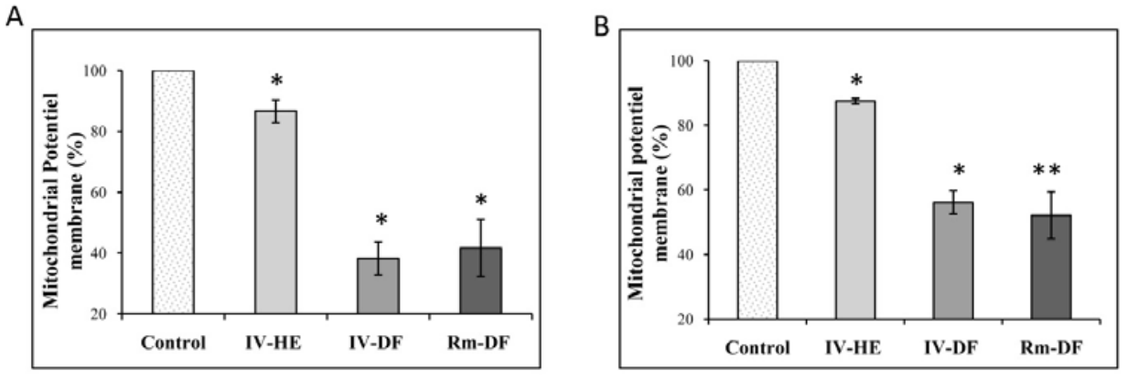


Fig. 3. Mitochondrial membrane potential state in treated cells with IV-HE, IV-DF and Rm-DF, measured by spectrofluorometry and JC-1 probe in SiHa (A) and HeLa (B). Cells are treated with 20µg/ml of extracts for 24h as described in Materials and Methods. The results are presented as the mean ± SD of three independent experiments.

### 3.7 Measurement of ROS production

Mitochondria are a source of ROS during apoptosis and reduced mitochondria membrane potential leads to increased generation of ROS and apoptosis (Zamzami, 1995). We investigate whether the intracellular ROS are involved in the signal transduction pathways of apoptosis. ROS generation was measured after cells treatment with IV-HE, IV-DF and Rm-DF (20µg/ml) for 24h, using a ROS-sensitive fluorescent C2938 probe.

Tested extracts showed a dose-dependent increase in the intracellular ROS production when compared to the control (Figure.4). This indicate that ROS generation induced by IV-HE, IV-DF and Rm-DF in SiHa and HeLa cells can contribute to apoptosis via the mitochondrial pathway.

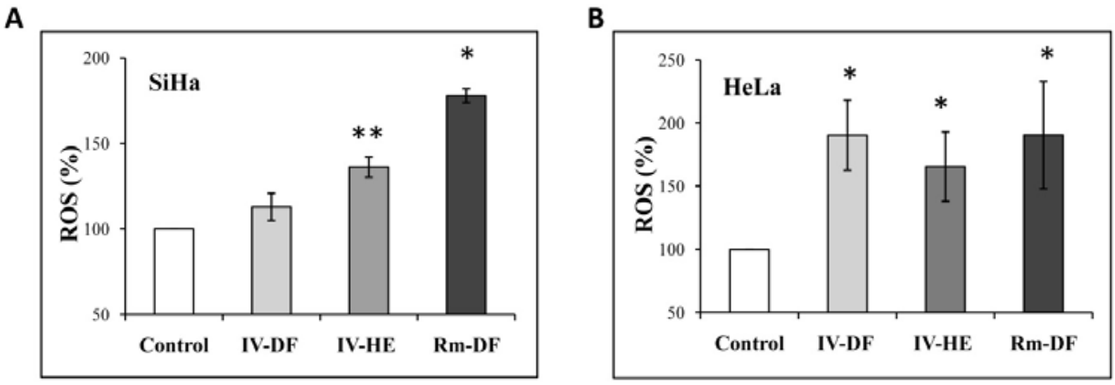


Fig. 4. ROS production in treated cells with IV-HE, IV-DF and Rm-DF, using an oxydation-sensitive fluorescent C2938 probe in SiHa (A) and HeLa (B). Cells are treated with 20µg/ml of extracts for 24h as described in Materials and Methods. The results are presented as the mean ± SD of three independent experiments.

Taken together, these results show clearly that the hexanic extract of *Inula viscosa* and dichloromethane fractions of both *Inula viscosa* and *Retama monosperma* have cytotoxic effects against cervical cancer cell lines SiHa and Hela by inducing apoptotic process. Previous studies have showed that some plant extracts with pronounced cytotoxic in vitro had marked effects in vivo and showed promising potential to be used as an anticancer drugs. Indeed, sesquiterpene lactones, isolated from *Carpesium rosulatum*, have recently been largely studied for their pharmacological proprieties as anti-neoplastic agents (Ma G, 2009; Moon, 2011; Robinson, 2008; Taylor, 2008). Sesquiterpenes lactones, artemisinin, thapsigargin and parthenolide and many of their synthetic derivatives, are in advanced stage for clinical trials (Ghantous, 2010).

Phytochemicals contained in *Inula viscosa* L. extracts including like tomentosin and inuviscolide, as evidenced by CG/MS analysis, have been shown recently to possess an antiproliferative and apoptotic effects on human melanoma cell lines (Rozenblat, 2008).

Quinolizidine alkaloids are known to present in *Retama monosperma* as main active constituents. Quinolizidine alkaloids contained in the dichloromethane fraction of *Retama monosperma* L. extract, may act as potential *in vitro* cytotoxic agents against human cervical cancer cells through the induction of apoptosis. In fact, previous reports have shown that quinolizidine alkaloids have been found to elicit a range of biological activities, including antiviral (Ding, 2006), antihypoglycemic (Brukwicki, 2009) and anti-tumoral (Zhang, 2010) activities.

## 4. Conclusion

The hexanic extract of *Inula viscosa* and dichloromethane fractions of both *Inula viscosa* and *Retama monosperma* showed pronounced cytotoxic effects against cervical cancer cell lines through the inhibition of proliferation and induction of apoptosis caspase-dependent and involving a mitochondria-mediated signaling pathway. Our findings suggest that these extracts might provide compounds which could be potential sources of anticancer drug leads. Further investigation into the isolation, characterization and mechanism of cytotoxic compounds from the selected plants extracts and in vivo are necessary. Moreover it will be interesting to use some *in vivo* models to evaluate the anti-tumor activity of these plant extracts.

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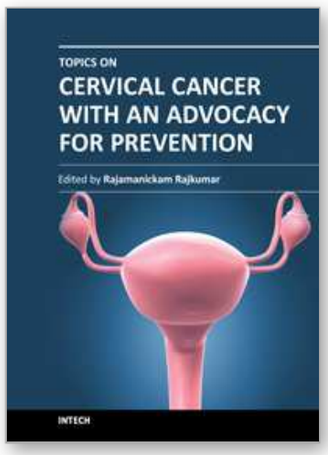


**Topics on Cervical Cancer With an Advocacy for Prevention**
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Cervical Cancer is one of the leading cancers among women, especially in developing countries. Prevention and control are the most important public health strategies. Empowerment of women, education, "earlier" screening by affordable technologies like visual inspection, and treatment of precancers by cryotherapy/ LEEP are the most promising interventions to reduce the burden of cervical cancer.Dr Rajamanickam Rajkumar had the privilege of establishing a rural population based cancer registry in South India in 1996, as well as planning and implementing a large scale screening program for cervical cancer in 2000. The program was able to show a reduction in the incidence rate of cervical cancer by 25%, and reduction in mortality rate by 35%. This was the greatest inspiration for him to work on cerrvical cancer prevention, and he edited this book to inspire others to initiate such programs in developing countries. InTech - Open Access Publisher plays a major role in this crusade against cancer, and the authors have contributed to it very well.



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