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Screening of North African Medicinal Plant Extracts for Cytotoxic Activity Against Tumor Cell Lines

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Authors' contributions

This work was carried out in collaboration between all authors. Author LB performed all experiments and participated in the experimental design, analysis of the data and redaction of the manuscript. Author CAL participated in the experimental design, analysis of the data and preparation of the manuscript. The plants harvest, identification and extraction were done by authors NM and YB. Author SA participated in the analysis of the data and the preparation of the manuscript. Author SFM participated in the experimental design and the analysis of the data. Author PV directed the research and supervised the preparation of the manuscript. All authors have contributed and approved the manuscript.

Research Article

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ABSTRACT

Aims: The aim of this study was to evaluate the in vitro cytotoxic activity and cellular effects of organic extracts and fractions of four plants; Inula viscosa, Ormenis eiriolepis (Asteraceae), *Retama monosperma* (Fabaceae) and *Marrubium vulgare* (Lamiaceae), all of them used in Moroccan traditional medicine.
Methodology: The four plants were extracted using organic solvents and screened on a panel of human cancer cell lines including cell types from both solid and haematological cancer origin as well as non-transformed murine fibroblasts. Cell viability assays were

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performed with sixteen plant extracts. Sensitive cell lines were then exposed to increasing concentrations of the most efficient extracts in order to calculate IC_{50} values. Microscopy, flow cytometry and caspase activity assays were then performed in LN229, SW620 and PC-3 cell lines upon treatment to investigate the cell morphology, cell cycle distribution and cell death.

Results: cell viability assays reveals that at least one extract from each plant was able to exert cytotoxic activity against the majority of cell lines tested, the IC_{50} values of the active extracts were in most cases ≤ 30 µg/ml. the study of the cellular effects of the most active extracts on LN229, SW620 and PC-3 cell lines shows their ability to promote cell cycle arrest and cell death. The data obtained herein support strongly the use of these plants by traditional healers for the treatment of cancer patients and could have some scientific support indicating the presence of bioactive compounds.

Conclusion: The reported biological activity of these four medicinal plants used in traditional Moroccan medicine provides a starting point for forthcoming studies to determine the molecular basis of their activity and to identify the chemical compounds within the most active extracts responsible for their antitumoral effects.

Keywords: Inula viscose; Retama monosperma; Ormenis eriolepis; Marrubium vulgare; Moroccan pharmacopeia; anticancer agents; cell proliferation; cell death.

1. INTRODUCTION

Numerous plant extracts have been used by several civilizations for their scent and medicinal properties. Active compounds within those extracts constitute the basis of traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies [1]. According to the World Health Organization (WHO) about 65–80% of the world's population in developing countries depends essentially on plants for their primary health care due to poverty and lack of access to modern medicine [2]. Medicinal plants have thus a long history of use for almost every group of human diseases including cancer, one of the major causes of mortality throughout the world. Statistics have estimated that cancer will cause 83.2 million deaths between 2005 and 2015 [3]. Drug discovery from medicinal plants plays an important role in the treatment of cancer and, indeed, most new clinical applications of plant secondary metabolites and their actives compounds derivatives over the last half century have been applied against cancer [4]. Of all available anticancer drugs between 1940 and 2002, 40% were natural products or natural product-derived mimics, including Vinca alkaloids, Taxusditerpenes, Camptotheca alkaloids, and Podophyllumlignans [5]. Currently, there are 16 new plant derived compounds being tested in clinical trials and 13 of these compounds 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 Indigo feratinctoria, have been shown to exhibit anti-cancer effects with lesser toxicity than conventional chemotherapeutic drugs [6]. Ethnomedical knowledge is one of the main strategies to select plant species in cancer drug discovery [7]. In Morocco, traditional medicine belongs to one of the world's oldest pharmacopoeia; in fact it disposes of a wide arsenal of plants remedies because of the natural diversity of the environment and flora [8]. The aim of this study was to evaluate the in vitro cytotoxic activity and cellular effects of organic extracts and fractions of four plants; Inula viscosa, Ormenis eiriolepis (Asteraceae), *Retama monosperma* (Fabaceae) and *Marrubium vulgare* (Lamiaceae), all of them used in Moroccan traditional medicine. The plant selection was based on their reputation as folk

medicines in the treatment of tumors [9]; and related diseases and also on their previously reported phytochemical and biological properties (Table 1).

2. MATERIALS AND METHODS

2.1 Plant Material

The selected plants were collected in different areas of Morocco during the period 2007-2011 and were identified by Dr. M. Fennane from the Scientific Institute of Rabat (Table 1).

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

Plants species (family)	Trivial name	Place of collection	Part plant collected	Traditional use	Pharmacological activities and references
***Inula viscosa* L. Ait. (Asteraceae)**	Magramane-Terhala	Ain atik Temara	Leaves	Skin diseases, treat cutaneous abscesses, wound healing, Tuberculosis, bronchial infections [9]	Antimicrobial activity [11]. Antifungal activity [12] Anti inflammatory effects [13]. Antitumoral activity [14], [15], [16].
***Retama monosperma* L. Bois (Fabaceae)**	R'tm	Sidi-Boughaba Mahdia	Leaves	Purgative, vermifuge, antihelmintic, abortive and disinfectant [10]	Antitumoral activity [15], [16]
Ormenis eriolepis Coss. (Asteraceae)	Hellala	Ouarzazat	Aerial part	Stomachic, anthelmintic and antidiabetic [9]	Antibacterial activities [17] Antileishmania activities [18] Antifungic activity [19]
***Marrubium vulgare* L. (Lamiaceae)**	Merriwut Merriwa	Temara	Aerial part	Antidiabetic, antidiarrheal, febrifuge, expectorant, antiicteric, treatophthalmia, otitis, abscesses feversandboils [9]	Antispasmodic [20] Antinociceptive [21] Analgesic [22] Antioxidant, Anti cholesterol [23] Antioeudematogenic [24] Antidiabetic [25], [26], [27], [28].

2.2 Experimental Design

Plant extracts were screened across a panel of human cancer and a non-tumoral mouse fibroblast cell lines from different tumor origin (Table 2) to analyze the cytotoxic activity and determines the IC_{50} of the most active extracts. In order to examine the cellular effects of the

most active extracts, we analyzed cell morphology, cell cycle distribution, cell death induction and caspase activity in LN229, SW620 and PC-3 cell lines.

Table 2. Cell lines used in the study related to the cells type and diseases

	Cell lines	Cell type	Disease
Human	LN-229	epithelial	Glioblastoma
	T98G	fibroblast	Glioblastomamultiforme
	U-87 MG	epithelial	Glioblastoma; Astrocytoma grade IV
	Jurkat	T lymphocyte	Acute T cell Leukemia
	JeKo-1	lymphoblast	non-Hodgkin lymphomas
	SW620	epithelial	Colorectal adenocarcinoma
	SW480	epithelial	Colorectal adenocarcinoma
	PC-3	epithelial	Prostate adenocarcinoma
	U2OS	epithelial	Osteosarcoma
Mice	NIH/3T3	Embryonic fibroblast	Normal

2.3 Preparation of Organic Extract

The collected parts of the selected plants were air-dried and mechanically ground to produce a fine powder. 200g of each plant powder was successively extracted using a Soxhlet apparatus with *n*-hexane (1.3L) and methanol (1.3L) to obtain hexanic extract and methanolic extract 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 (1.3L) and ethyl acetate (1.3L) to obtain dichloromethane fraction and ethyl acetate fraction. All extracts were evaporated by a Rotavapor and kept at -20ºC until use.

2.4 Cell Culture

Jurkat and Jeko-1 cells were maintained in RPMI1640 with L-Glutamine and HEPES (Invitrogen, Carlsbad, CA). LN229, T98G, U87MG, SW620, SW480, U2OS, PC-3 and NIH3T3 cells were maintained in DMEM High Glucose (4.5 g/l) with L-glutamine (Invitrogen, Carlsbad, CA). All cells were grown in a humidified incubator at 37ºC with 5% CO2. RPMI and DMEM were supplemented with 10% heat inactivated foetal bovine serum and 100 units/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA). All cell lines were subconfluently grown and passaged, routinely tested for mycoplasma contamination and subjected to frequent morphological tests and growth curve analysis as quality-control assessments. All cell lines were treated at a prophylactic concentration of 5 µg/ml with Plasmocin™ (InvivoGen, San Diego, CA).

2.5 Cell Viability Assays

The number of viable cells in culture was determined based on quantification of ATP, which signals the presence of metabolically active cells, using the Cell Titer-Glo Luminiscent assay kit (Promega, Madison, WI, USA) following the manufacturer's instructions. Briefly, cells were plated in 96-well plates, treated 24h later with 50 µg/ml of extracts dissolved in DMSO

for 48h followed by addition of Cell Titer-Glo reagent. Luminiscence was detected using a multi-well Synergy Mx scanning spectrophotometer (Biotek, Winooski, VT).

2.6 Cellular Morphology Analysis

Cells were plated in 6-well plates and treated with 50 µg/ml of the indicated extract for 24h and 48h. Representative images were collected under a light optic microscope (Leica Microsystems, Wetzlar, Germany).

2.7 Cell Cycle Analysis

Cell cycle analysis was performed following propidium iodide staining. Briefly, trypsinized cells were washed in phosphate-buffered saline (PBS) and fixed in 70% ethanol. Fixed cells were then washed twice in PBS and DNA was stained with propidium iodide (50 µg/ml) in the presence of 50 µg/ml RNase A (Sigma-Aldrich, Saint Louis, MO), then analysed by flow cytometry using a FACS can (Coulter Epics XL-MSL; Beckman Coulter, Fullerton, CA, USA) and winMDI software.

2.8 Annexin V-FITC/Propidium Iodide FLow Cytometric Analysis

Analysis of phosphatidylserine externalization in apoptotic cells was determined by Annexin-V-FITC (Invitrogen, Carlsbad, CA) staining, according to the manufacturer's instructions. Briefly, $2x10^5$ cells were seeded in 6-well plates and treated with 50 µg/ml of the extracts for 48h. They were then collected and suspended in 100µl of Annexin Vbinding buffer. Following incubation with Annexin-V-FITC and propidium iodide for 15 min at room temperature in the dark, flow cytometry analysis was carried out using a FACScan (Coulter Epics XL-MSL; Beckman Coulter, Fullerton, CA, USA) and analyzed with the winMDI software.

2.9 Caspase Activity Assays

Caspase activity was determined by measurement of caspases 3 and 7 activity in control and treated cells with 50 µg/ml of indicated extracts plated in 96-well plates for 48h using the luminometric Caspase-Glo 3/7 assay (Promega) according to the manufacturer's protocol, using a Synergy HT multidetection microplate reader (Bio- Tek, Winooski, VT).

2.10 Statistical Analysis

Data are presented as means ± SD of at least three different assays performed in triplicate. IC_{50} values and the statistical significance of differences by Student's t test were assessed using GraphPad Prism (GraphPad Software Inc. La Jolla, CA). Statistically significant differences are indicated by ****P,0.001*, ***P,0.01* and **P,0.05.*

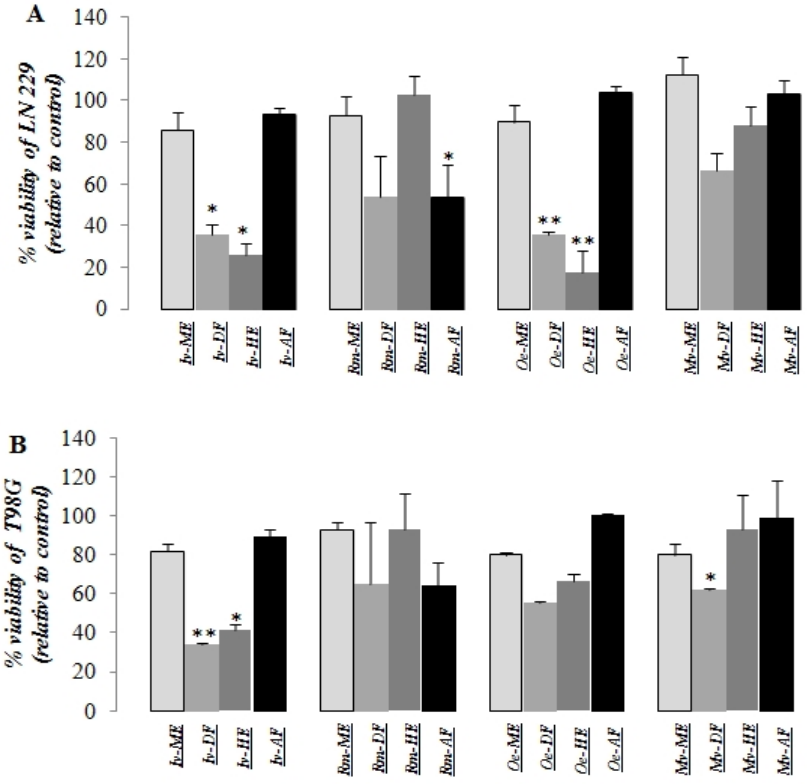
3. RESULTS AND DISCUSSION

For this study, four medicinal plants used for various diseases were harvested in different areas of Morocco. Four organic extracts of *Inula viscosa, Ormenis eiriolepis* (Asteraceae), *Retama monosperma* (Fabaceae) *and Marrubium vulgare* (Lamiaceae) were prepared. Table 1 summarizes the ethnobotanical data including botanical and local names, ethnomedical uses and the relevant plant parts employed together with their reported

pharmacological activity. The cytotoxic screening was performed on a panel of established human cancer cell lines including Glioblastoma Multiforme, Acute T cell Leukemia, Mantle Cell Lymphoma, Colon cancer, Prostate Cancer and Osteosarcoma cells. The NIH3T3 nontransformed mouse fibroblast cell line was employed as a control cell line to explore the cytotoxic effects of the extracts on nontumoral cells. Table 2 provides an overview of the characteristics of the cell lines used. In our screening program, all cell lines were initially treated with all sixteen extracts at a starting concentration of 50µg/ml to determine the selective activity of the extracts.

3.1 Selected Plant Extracts Show Cytotoxicity against Human Glioblastoma Cell Lines

The human glioblastoma cell lines LN229, T98G and U87MG were treated with the complete selection of plants extracts and fractions at 50µg/ml for 48h. Fig. 1 shows the percentage of cell viability compared to vehicle-treated cells. The results revealed that cell lines responded differently and each one was selectively sensitive to specific extracts. A significant reduction in cell viability was particularly observed in LN229 cells which were sensitive to *Iv-DF* (37.72%)*, Iv-HE* (25.93%)*, Oe-DF* (35.87%) and *Oe-HE* (17.81%) extracts (Fig. 1A), while T98G (Fig. 1B) and U87MG cell lines (Fig. 1c) were sensitive to *Iv-DF* (34.01%).and *Rm-DF* (34.01%).



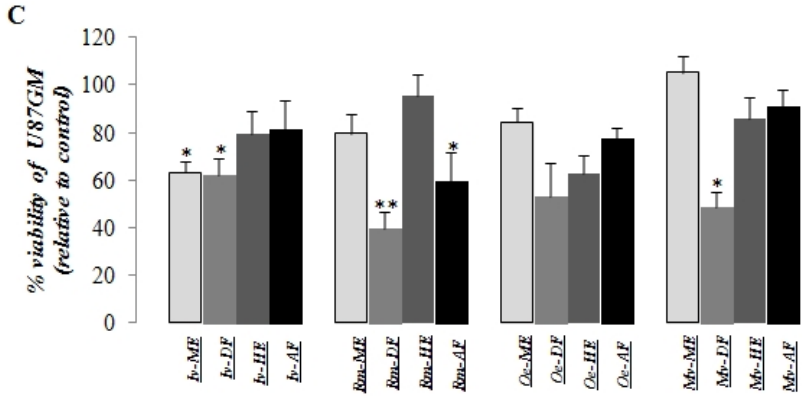


Fig. 1. Cell viability analysis of Glioblastoma cell lines

*LN229 (A), T98G (B) and U87MG (C) cell lines were treated for 48 h with extracts from Inula viscosa (Iv), Retama monosperma (Rm), Ormenis eiriolepis (Oe) and Marrubium vulgare (Mv) with 50µg/ml of methanol (ME) and hexane extracts (HE) and dichloromethane (DF) and ethyl acetate (AF) fractions Results represent the mean ± SD of at least 3 independent experiments indicating the percentage of viable cells relative to vehicle-treated (control) cells. The differences between control and the indicated plants extracts treatement are statistically significant (Student t-test *P<0.05; **P<0.01 and ***P<0.001 respectively).*

In Glioblastoma cells several key regulatory elements of cell homeostasis and apoptosis are altered at different levels, such as the status of the tumor suppressor p53 [29]. Previous characterization of p53 genotype and drug sensitivity of human cancer cell lines has revealed that cells with mutant or absent p53 are less sensitive than cells with wild-type p53 to the majority of clinically-used anticancer agents [30]. Interestingly, our results show that p53-mutant LN229 cells are the most sensitive glioblastoma cell line to the tested plant extracts, in comparison with U87MG cells, expressing wild-type p53.

3.2 Acute T-cell Leukemia and Mantle Cell Lymphoma Cell Lines Show Increased Sensitivity to the Tested Extracts

Jurkat (Acute T-cell leukemia) and Jeko-1 (Mantle Cell Lymphoma) cells were next evaluated with the panel of extracts. Both cell lines were very sensitive to at least one extract of each of the four medicinal plants selected. Fig. 2 reveals that, particularly, the dichloromethane fraction and hexanic extract of each plant are very effective on Jurkat (Fig. 2A) and Jeko-1 cells (Fig. 2B) with a percentage of viability lower than 6%.

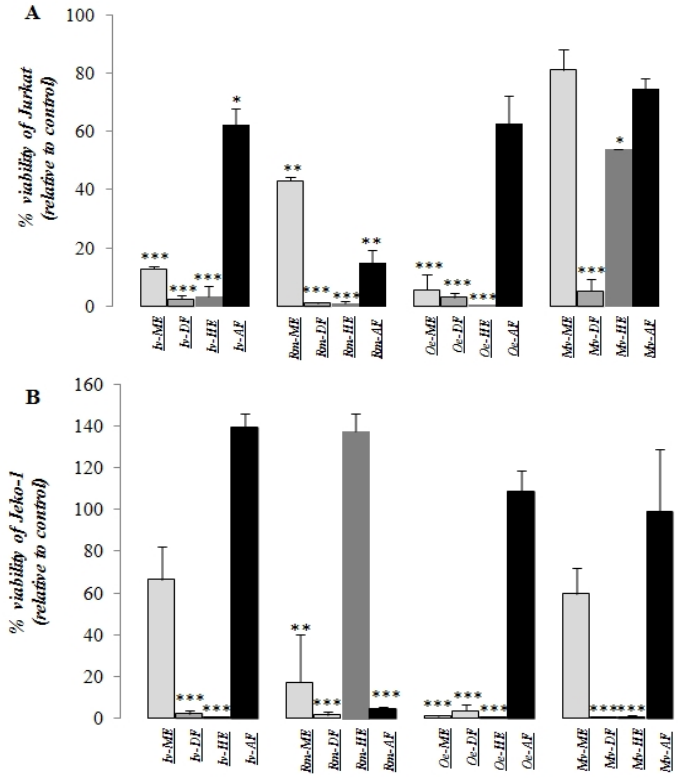


Fig. 2. Cell viability analysis of acute T-cell leukemia and mantle cell lymphoma cell lines

Jurkat (A) and Jeko-1 (B) cells were treated and the data obtained represented as in Fig. 1. Results represent the mean ± SD of at least 3 independent experiments indicating the percentage of viable cells relative to vehicle-treated (control) cells. The differences between control and the indicated plants extracts treatement are statistically significant (Student t-test *$P<0.05$; **$P<0.01$ *and* ***$P<0.001$ respectively).

Both hematological malignancies are characterized by a deregulated expression of apoptosis-related molecules. Overexpression of anti-apoptotic oncogenes is one of the mechanisms that decrease the efficacy of therapeutic agents [31]. Our results indicate that both oncohematological cell lines tested were very sensitive to several extracts. More studies are required to elucidate which survival pathways are affected.

3.3 Effects on Colorectal Cancer Cell Lines

The cell viability analysis of colorectal cancer cell lines (Fig. 3) indicates that three extracts induced clear significant effects on SW620 cells. *Iv-DF* (28, 35%), *Iv-HE* (35, 38%) and *Mv-DF* (33, 11%) (Fig. 3A), whereas *Iv-HE* (22, 63%) and *Oe-HE* (35, 38%) were active against SW480 cells (Fig. 3B). Colon cancer becomes resistant to apoptosis as it acquires metastatic potential [32]. SW480 and SW620 colon cancer cells were established from the same patient at different stages of tumor progression [32]. Metastatic SW620 colon cancer cells have undergone multiple gene product modifications resulting in resistance to cell death [32]. Interestingly, our data indicate that SW620 cells were more sensitive, suggesting

that our extracts may contain molecules with high therapeutic potential for multidrug resistant cell lines.

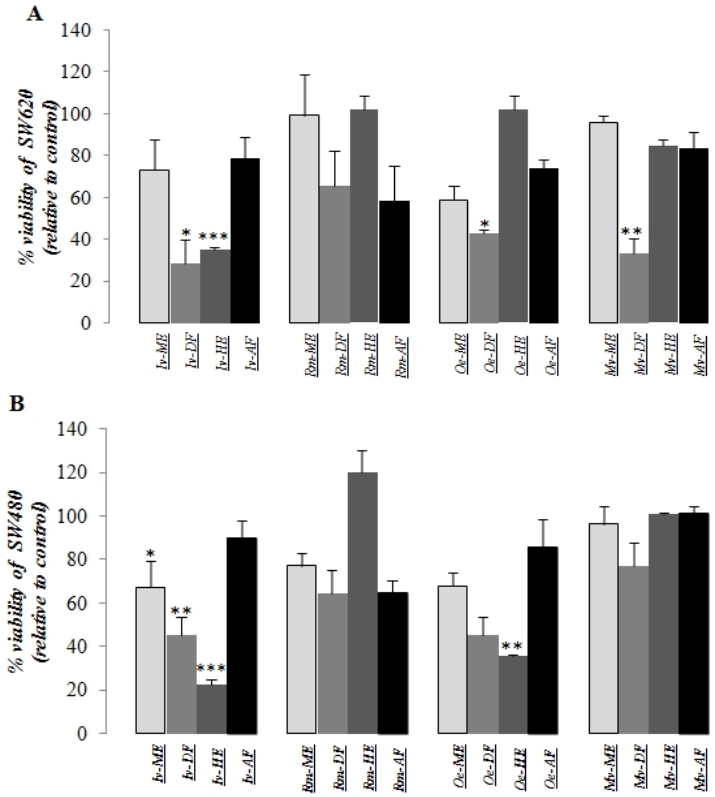


Fig. 3. Cell viability analysis of colon cancer cell lines

*SW620 and SW480 colorectal cancer cells were were treated and the data obtained represented as in Fig. 1. Results represent the mean ± SD of at least 3 independent experiments indicating the percentage of viable cells relative to vehicle-treated (control) cells. The differences between control and the indicated plants extracts treatement are statistically significant (Student t-test *P<0.05; **P<0.01 and ***P<0.001 respectively).*

3.4 Analysis of Extract Activity against Prostate Cancer and Osteosarcoma Cells

The effect of the different extracts was also tested in the PC-3 Prostate cancer cell line and in U2OS Osteosarcoma cells, which were exposed to the same conditions described above (Fig. 4). Fig. 4A illustrates that only *Iv-HE* (1,2%) and *Oe-HE* (8,2%) were active against PC-3 cells, although they both induced a dramatic effect on these cells. Unfortunately, conventional chemotherapeutic drugs have a discreet effect on prostate cancers and do not provide a marked survival advantage for patients [33]. In contrast, in the Osteosarcoma cell line U2OS (Fig. 4B) almost none of the extracts exerted a significant activity at the tested concentration, except *Rm-AF* (42, 3%). This could be explained by the fact that U2OS cell line is chromosomally highly altered.

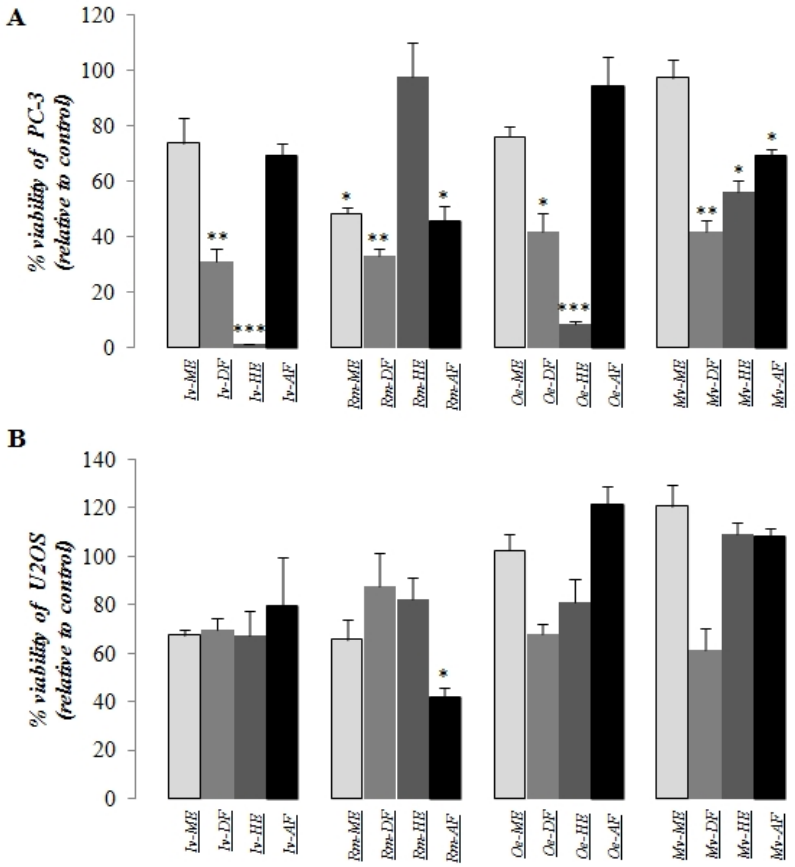


Fig. 4. **Cell viability analysis of prostate cancer and osteosarcoma cell lines**
*Prostate cancer PC-3 (A) and osteosarcoma U2OS (B) cells were treated and the data obtained represented as in Fig. 1. Results represent the mean ± SD of at least 3 independent experiments indicating the percentage of viable cells relative to vehicle-treated (control) cells. The differences between control and the indicated plants extracts treatment are statistically significant (Student t-test *P<0.05; **P<0.01 and ***P<0.001 respectively).*

3.5 Effects on Non-Transformed Cells

The murine fibroblast cell line NIH3T3 was chosen to test the toxicity of all the extracts. Interestingly, all the extracts with the exception of *Iv-AF* (26,32%), *Oe-AF* (52,59%) and *Mv-HE* (65,78%) were non-toxic for these cells at the concentration of 50µg/ml (Fig. 5; note the different scale of the *y*-axis). Taken together, the low cytotoxic profile exerted in both NIH3T3 and U2OS cells indicates that these extracts do not act as indiscriminate cellular poisons, rather reflecting a specific cell type-based.

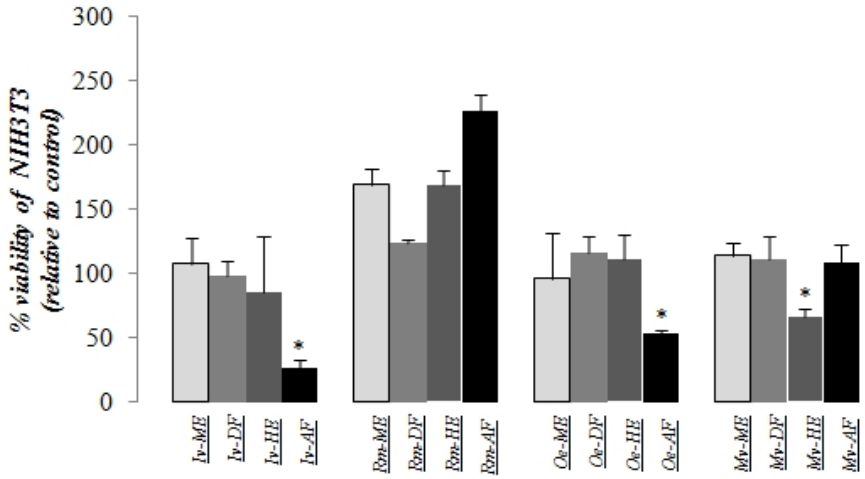


Fig. 5. Cell viability analysis of mouse embryonic fibroblast cell line

*NIH3T3 mouse fibroblasts were treated and the data obtained represented as in Fig. 1. Results represent the mean ± SD of at least 3 independent experiments indicating the percentage of viable cells relative to vehicle-treated (control) cells. The differences between control and the indicated plants extracts treatment are statistically significant (Student t-test *P<0.05).*

3.6 Analysis of the IC_{50} of the Most Active Extracts in Sensitive Cell Lines

According to NCI (National Cancer Institute, U.S.A.) recommendations to consider a crude extract promising for further purification, IC_{50} values need to be lower than 30μg/mL in order to discover and develop potential anticancer natural compounds [35,36]. For this purpose increasing concentrations ranging from 1μg/ml to 50μg/ml of selected extracts were then tested and the IC_{50} values were calculated. All tested extracts were able to reduce cell viability in a dose-response manner after treatment. All determined IC_{50} values for the most active extracts in sensitive cell lines are included in Table 3. In most cases the IC_{50} values were under 30μg/ml, and specifically *Iv-DF, Iv- HE, Rm-DF; Rm-HE; Oe-DF; Oe-HE* and *Mv-DF* showed the most promising activities in this assay.

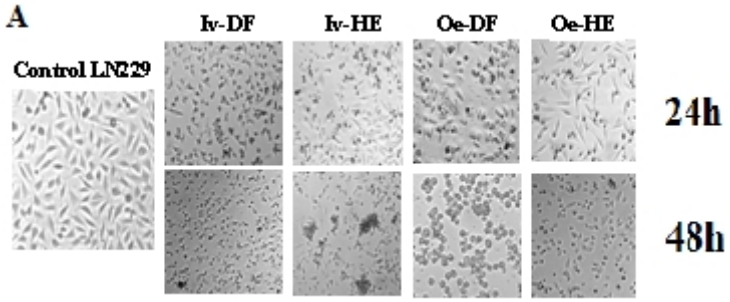
Table 3. IC_{50} values of extracts *(ME, HE)* and fractions *(DF, AF)* of *Inulaviscosa (Iv), Retamamonosperma (Rm), Ormeniseiriolepis (Oe) and Marrubiumvulgare (Mv)* against selected cell lines. Jurkat, Jeko-1, LN229, T98G, U87MG, SW620, SW480, U2OS and PC-3 were exposed to increasing concentration fromdifferent extracts range 1µg/mlto 50 µg/ml for 48h. The IC_{50} values were determined only for the most active and non toxic extracts exhibiting a significant cell death effect usingGraphPad Prism 5 software and Data are expressed as IC_{50} ± SD of three independent experiments

Organic Extracts	IC $_{50}$(µg/ml) ±SD								
	Jurkat	**Jeko-1**	**LN229**	**T98**	**U87**	**SW620**	**SW480**	**U2OS**	**PC-3**
***Inula viscose* L. Ait.**									
Iv-ME	25,81±2,92	>50	NA	NA	>50	NA	>50	>50	NA
Iv-DF	11,16±6,95	8,68±3,1	17,66±5,4	20,50±2,21	>50	23,30±6,33	>50	>50	20,15±9,09
Iv-HE	7,52±4,57	<1	5,9±3,57	>50	NA	8,40±3,31	10,36±6,59	>50	6,53±1,42
Iv-AF	NA	NA	NA	NA	NA	NA	NA	NA	NA
***Retama monosperma* L.Bois.**									
Rm-ME	>50	21,47±3,05	NA	NA	NA	NA	NA	>50	>50
Rm- DF	9,12±4,20	24,77±4,85	>50	>50	47,85±4,08	NA	>50	NA	45,41±4,6
Rm- HE	34,44±3,38	NA	NA	NA	NA	NA	NA	NA	NA
Rm- AF	20,22±10,51	12,01±5,14	>50	>50	>50	>50	>50	NA	>50
Ormenis eriolepis Coss.									
Oe-ME	27,45±4,03	<1	NA	NA	NA	>50	>50	NA	NA
Oe- DF	11, 33±5,37	13,63±1,67	21,70±2,37	>50	>50	>50	>50	>50	>50
Oe- HE	9,81±8,44	<1	41,67±1,98	>50	>50	NA	19,31±4,88	NA	12,59±4,69
Oe- AF	NA	NA	NA	NA	NA	NA	NA	NA	NA
***Marrubium vulgare* L.**									
Mv- ME	NA	>50	NA	NA	NA	NA	NA	NA	NA
Mv- DF	11,54±2,83	6,04±2,77	>50	>50	>50	10,58±4,37	NA	>50	>50
Mv- HE	NA	NA	NA	NA	NA	NA	NA	NA	NA
Mv- AF	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA: Not active; >50: above 50µg /ml; <1: below 1µg/ml

3.7 Analysis of the Cellular Effects of the Most Active Extracts in LN229 Glioblastoma Cells

Four extracts have shown a robust growth inhibitory activity against LN229 cells: *Iv- DF, Iv-HE, Oe-DF* and *Oe-HE.* We used these extracts at 50 µg/ml to treat LN229 cells for 24 and 48h and investigate their cellular effects. We observed that all extracts clearly induced cell shrinkage and floating cell formation, which are suggestive of cell death (Fig. 6A). We next performed flow cytometry following propidium iodide staining to quantitate the cell cycle distribution after treatment (Fig. 6B). *Iv-DF* causes a cell cycle arrest in G1 at 24h with a significant increase (46,17%) of the sub- G1 population at 48h. Similarly, *Iv-HE* induced a cell cycle arrest in G1 at 24h and increased the proportion of sub-G1 cells (43,22%) at 48h. Interestingly, *Oe-DF* seems to induce a G2/M arrest at 24h, followed by an increase in sub-G1 cell population (24,2%) at 48h (Fig. 6B), whereas *Oe-HE* mainly exerts a combination of G1 arrest and sub- G1 increase at 24h, with this latter population further increasing at 48h up to 42% (Fig. 6B). We next performed double Annexin V/Propidium Iodide staining following treatment with the selected extracts for 24h and 48h. In agreement with our previous results, cells shifted upon treatment in a time-dependent manner to regions mainly indicative of early apoptosis, late apoptosis and necrosis, showing a total percentage of cell death higher than 50% (Fig. 6C). We next performed caspase activity assays upon treatment of LN229 cells with 50 µg/ml of each extract for 48h. *Oe-DF* seemed to induce the higher increase in caspase activity, although a parallel increase in the proportion of apoptotic, as opposed to necrotic, cells was not detected by flow cytometry. This discrepancy may reflect an underestimation of the percentage of apoptotic cells detected by Annexin V staining. Altogether, the results obtained with LN229 cells are in agreement with our previous data showing that these cells were the most sensitive Glioblastoma cell line, compared with T98G and U87MG cells. Therefore, active compounds from *Inula viscosa* and *Ormenis eiriolepis* extracts may be attractive therapeutic agents; specially in Glioblastoma cells that present multiple alterations at different levels including loss of heterozygoty (LOH), inactivating mutations, methylation or altered expression of cancer-relevant signaling intermediates such as bcl-2 family members, inhibitors of apoptosis (IAPs) or receptor tyrosine kinases like the epidermal growth factor receptor(EGFR) and their down-stream effectors [30].



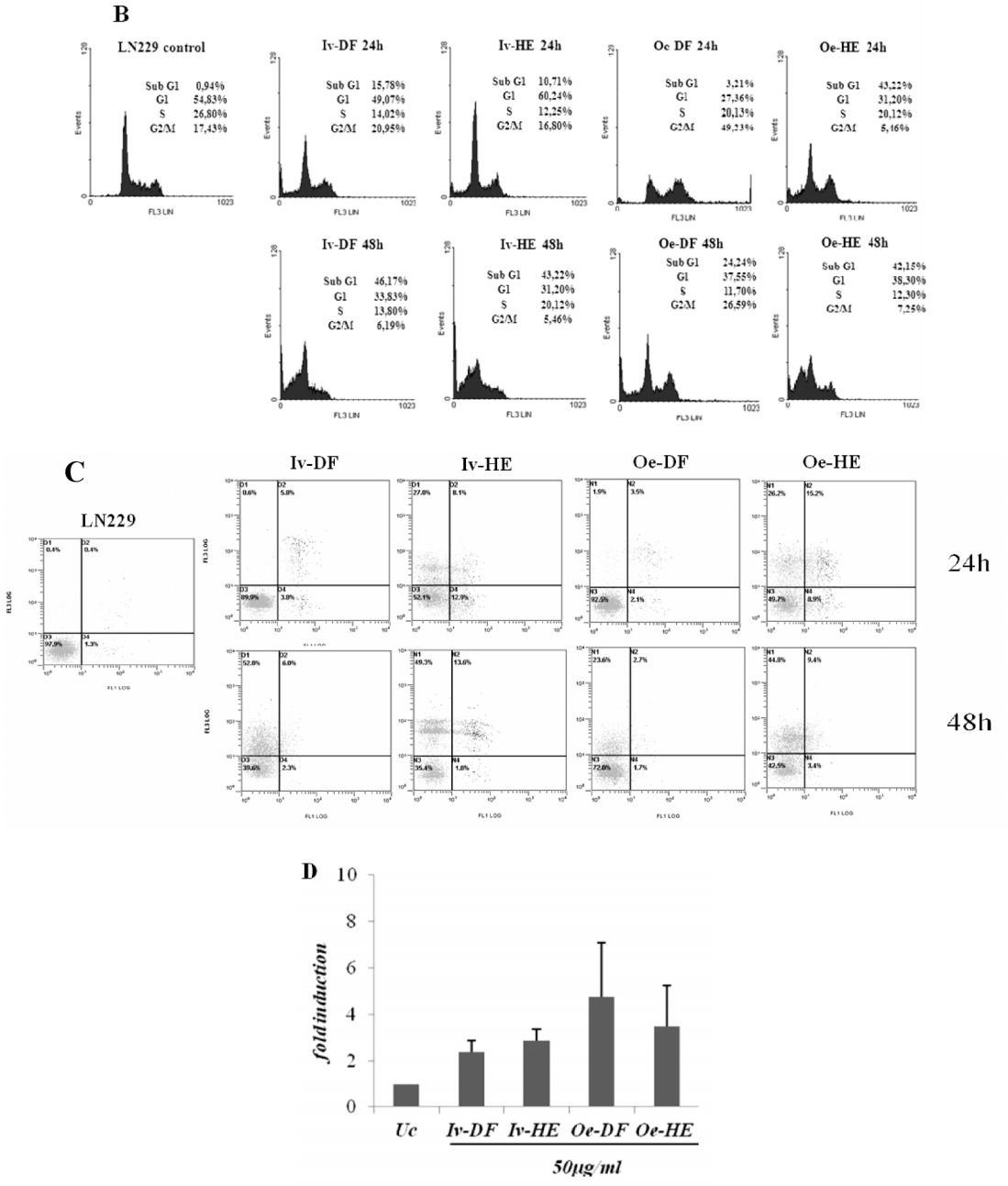
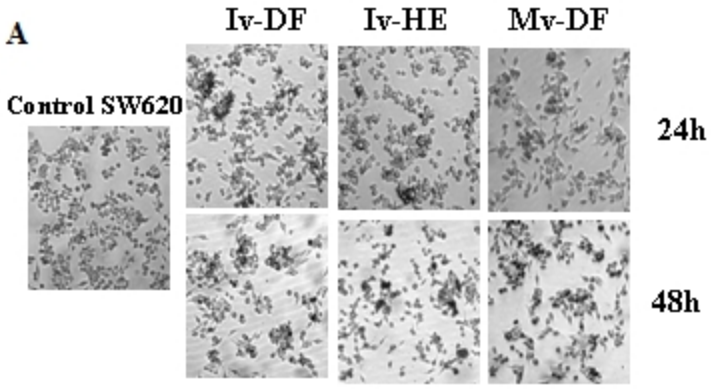


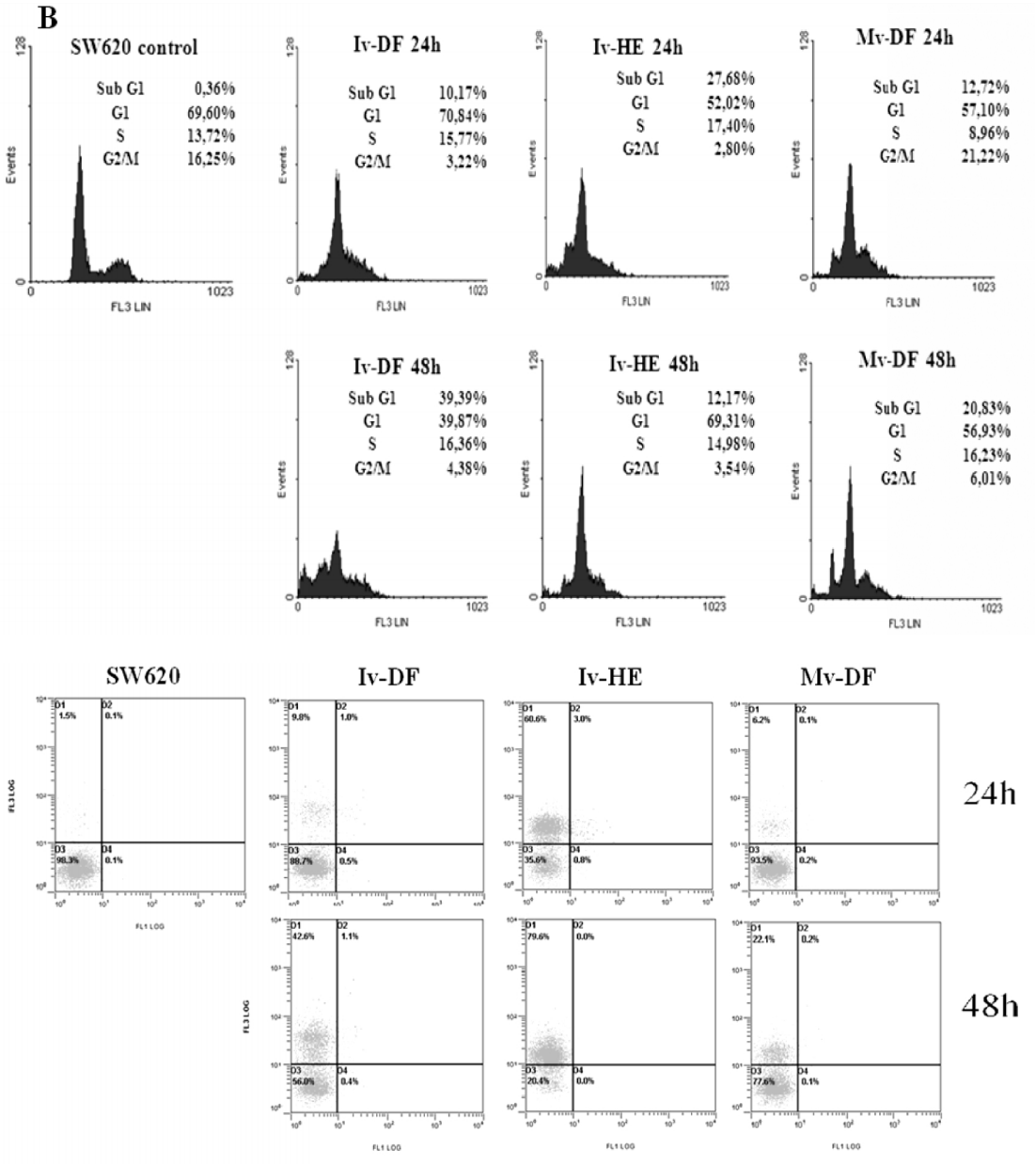
Fig. 6. Cellular effects analysis of the most active extracts on LN229 glioblastoma cell line

(A) images of morphological changes of LN229 cells under treatment with 50µg/ml of Iv-DF, Iv-HE, Oe-DF and Oe-HE for 24h and 48h (magnification 5×) (B) cell cycle analysis of LN229 cells treated same as in (A). (C) cell death analysis of LN229 at 24h and 48h under the same conditions as in (A) and (B).(D) caspase activity analysis of LN229 cells treated with Iv-DF, Iv-HE, Oe-DF and Oe-HE for 48h. Results show fold induction respective to untreated cells. Data is means ± SEM from three independent determinations performed in duplicate.

3.8 Analysis of the Cellular Effects of the Most Active Extracts in SW620 Colon Cancer Cells

Viability assays in colon cancer cells indicated that SW620 cells were the most sensitive to *Iv-DF, Iv-HE* and *Mv-DF*, which all exhibited clear cytotoxic effects (Fig. 3A). We thus used the same experimental approach as with LN229 cells to investigate if those extracts may affect SW620 cell morphology, cell cycle distribution and cellular death. Cell shrinkage and floating cell formation were observed upon treatment suggesting that *Iv-DF, Iv-HE* and *Mv-DF* were inducing cell death (Fig. 7A). Cell cycle analysis upon propidium iodide staining revealed that *Iv-DF, Iv-HE* and *Mv-DF* were able to induce cell cycle arrest in G1 at 24h and a clear increase in the sub-G1 population at 48h, with 39%, 12% and 21% cells in sub-G1, respectively (Fig. 7B). Annexin V/Propidium Iodide staining was then performed to better analyze cellular death. Treatment with all extracts dramatically increased cellular necrosis, without the presence of a detectable apoptotic cell fraction as indicated by the low numbers of Annexin V-positive cells (Fig. 7C). The percentages of necrotic cells at 48h were 42%, 79% and 22% for *Iv-DF, Iv-HE* and *Mv-DF*, respectively. Surprisingly, however, *Iv-HE* specifically induced a dramatic induction of caspase activity (30-fold) which again suggests that the detection of Annexin V-positive cells in these assays may not accurately reflect caspase-dependent apoptosis (Fig. 7D). In comparison with SW480 cells, their non metastatic counterpart, SW620 cells have acquired genetic defects both in the intrinsic and extrinsic pathways of apoptosis and are thus more resistant to apoptosis induced by CH-11, cisplatin, and ionizing radiation, respectively [32]. In our study SW620 cells were sensitive to *Inula viscosa* and *Marrubium vulgare* extracts, suggesting that those plants may lead to new therapeutic molecules that could challenge the acquired mechanisms leading to tumor cell resistance to current cytotoxic drugs in colon cancer.




B
SW620 control
Sub G1 0,36%
G1 69,60%
S 13,72%
G2/M 16,25%
Iv-DF 24h
Sub G1 10,17%
G1 70,84%
S 15,77%
G2/M 3,22%
Iv-HE 24h
Sub G1 27,68%
G1 52,02%
S 17,40%
G2/M 2,80%
Mv-DF 24h
Sub G1 12,72%
G1 57,10%
S 8,96%
G2/M 21,22%
Iv-DF 48h
Sub G1 39,39%
G1 39,87%
S 16,36%
G2/M 4,38%
Iv-HE 48h
Sub G1 12,17%
G1 69,31%
S 14,98%
G2/M 3,54%
Mv-DF 48h
Sub G1 20,83%
G1 56,93%
S 16,23%
G2/M 6,01%
Events
FL3 LIN
SW620
Iv-DF
Iv-HE
Mv-DF
24h
48h
FL3 LOG
FL1 LOG

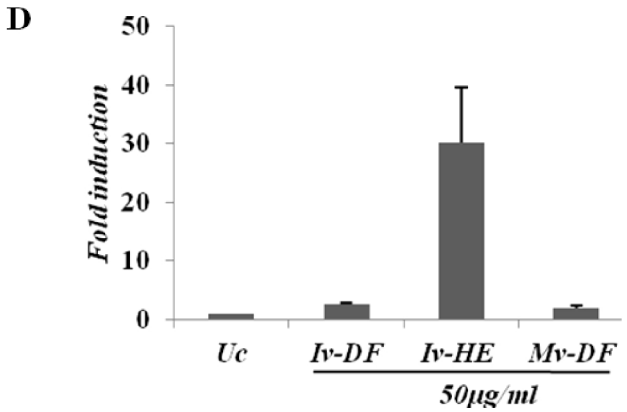
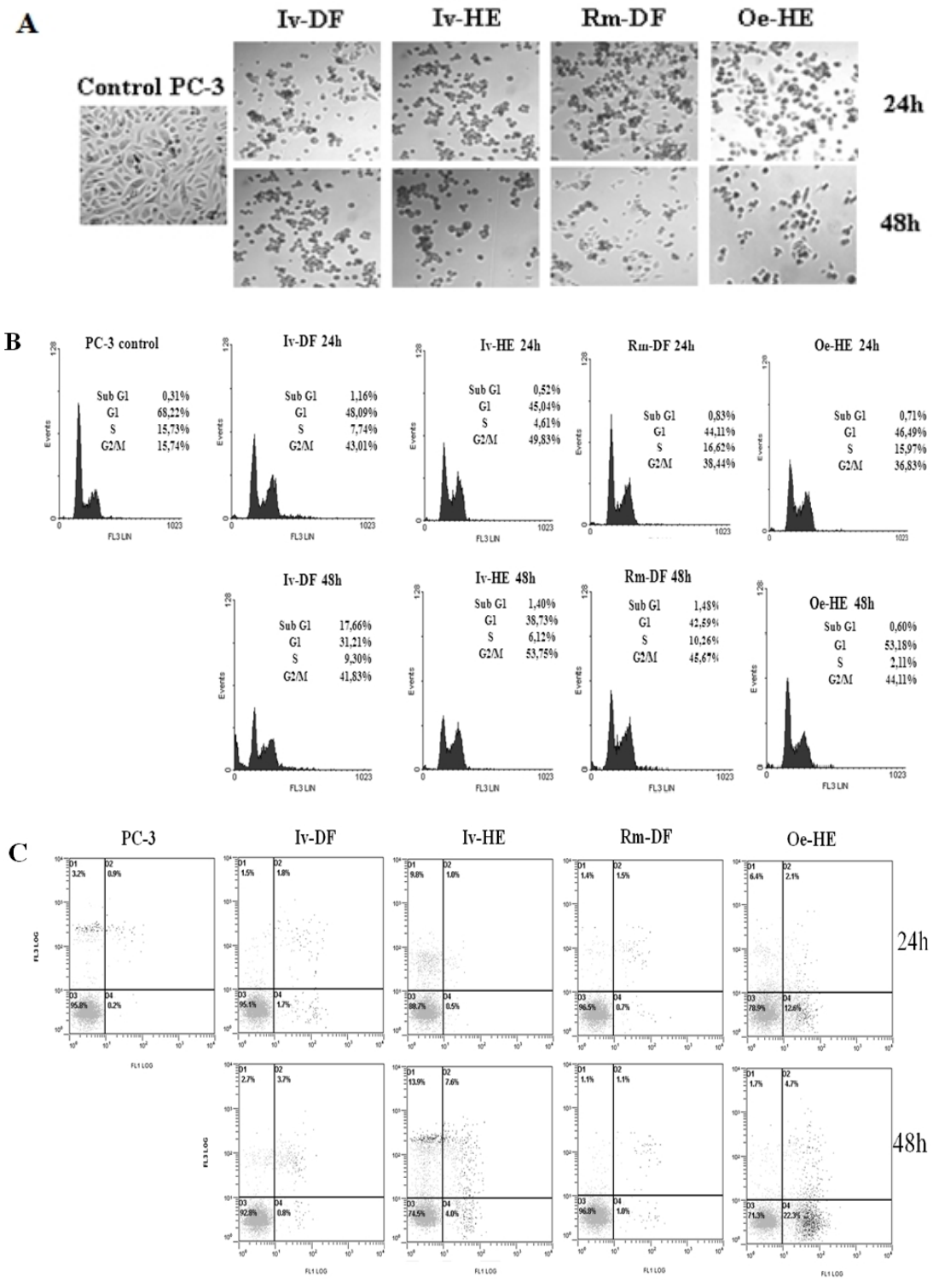


Fig. 7. Cellular effects analysis of the most active extracts on SW620 colon cancer cells

(A) images of morphological changes of SW620 cells under treatment with 50µg/ml of Iv-DF, Iv-HE and Mv-DF for 24h and 48h (magnification 5×) (B) cell cycle analysis of SW620 cells treated same as in (A).(B) cell cycle analysis of SW620 cells treated same as in (A). (C) cell death analysis of SW620 at 24h and 48h under the same conditions as in (A) and (B). (D) caspases activity analysis of SW620 cells treated with Iv-DF, Iv-HE and Mv-DF for 48h. Results show fold induction respective to untreated cells. Data is means ± SEM from three independent determinations performed in duplicate.

3.9 Analysis of the Cellular Effects of the Most Active Extracts in PC-3 Prostate Cancer Cells

The same experiments were next performed in PC-3 prostate cancer cells to figure out if the most active extracts in viability assays could also affect cell morphology, cell cycle progression and cell death. Morphological changes were observed in PC-3 cells upon treatment with *Iv-DF, Iv-HE, Rm-DF* and *Oe-HE* extracts for 24h and 48h. Rounding up and cell shrinking, with a clear reduction of cellular and nuclear volume were observed (Fig. 8A). Flow cytometry analysis of cell cycle distribution and cell death upon *Iv- DF, Iv-HE, Rm-DF* and *Oe-HE* treatment revealed that the four extracts induced a clear G2/M arrest which was already detectable at 24h leading at 48h to a G2/M cell population of 41,83%, 53,75%, 48,67% and 44.11%, respectively (Fig. 8B). The results obtained with Annexin V/Propidiume iodide indicate that only *Iv-HE* and, more robustly, *Oe-HE* were able to induce cell death in PC-3 cells, reaching at 48h a percentage of 25% and 29% of cell death, respectively. Interestingly, in *Oe-HE*-treated cells the results primarily indicate apoptotic cell death. Accordingly, the results of caspase assays showed the highest increase of caspase activity in *Iv-HE* and *Oe-HE* treated cells. PC-3 prostate cancer cells are androgen-insensitive and apoptosis-resistant; they survive and eventually develop into androgen-refractory and metastatic clones [33]. Our data suggest that *Inula viscosa* and *Ormenis eiriolepis* extracts exert growth inhibition, cell cycle arrest and apoptosis induction and could contain active compounds with the potential to effectively target apoptosis extrinsic and intrinsic pathways. Therefore, more studies are clearly needed to clarify the molecular mechanisms involved.


A
Iv-DF
Iv-HE
Rm-DF
Oe-HE
Control PC-3
24h
48h
B
PC-3 control
Sub G1 0,31%
G1 68,22%
S 15,73%
G2/M 15,74%
Iv-DF 24h
Sub G1 1,16%
G1 48,09%
S 7,74%
G2/M 43,01%
Iv-HE 24h
Sub G1 0,52%
G1 45,04%
S 4,61%
G2/M 49,83%
Rm-DF 24h
Sub G1 0,83%
G1 44,11%
S 16,62%
G2/M 38,44%
Oe-HE 24h
Sub G1 0,71%
G1 46,49%
S 15,97%
G2/M 36,83%
Iv-DF 48h
Sub G1 17,66%
G1 31,21%
S 9,30%
G2/M 41,83%
Iv-HE 48h
Sub G1 1,40%
G1 38,73%
S 6,12%
G2/M 53,75%
Rm-DF 48h
Sub G1 1,48%
G1 42,59%
S 10,26%
G2/M 45,67%
Oe-HE 48h
Sub G1 0,60%
G1 53,18%
S 2,11%
G2/M 44,11%
Events
FL3 LIN
C
PC-3
Iv-DF
Iv-HE
Rm-DF
Oe-HE
24h
48h
FL3 LOG
FL1 LOG

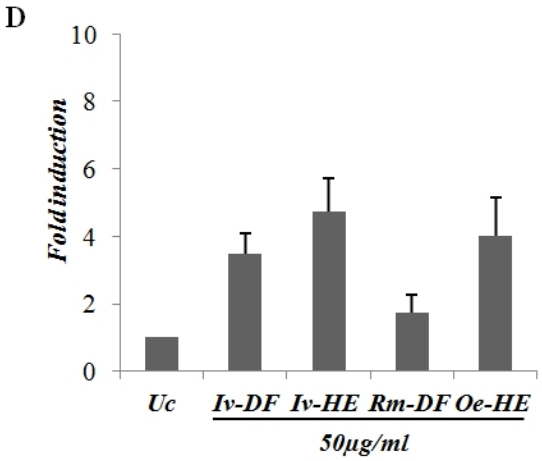


Fig. 8. Cellular effects analysis of the most active extracts on PC-3 prostate cancer cells

(A) images of morphological changes of PC-3 cells under treatment with 50µg/ml of *Iv-DF, Iv-HE* , *Rm-DF* and *Oe-HE* for 24h and 48h (magnification 5×). (B) cell cycle analysis of PC-3 cells treated same as in (A). (C) cell death analysis of PC-3 at 24h and 48h under the same conditions as in (A) and (B). (D) caspases activity analysis of PC-3 cells treated with *Iv-DF, Iv-HE* , *Rm-DF* and *Oe-HE* for 48h. Results show fold induction respective to untreated cells. Data is means ± SEM from three independent determinations performed in duplicate.

4. CONCLUSION

In agreement with our data, the antitumoral activity of the hexanic extract and the dichloromethane fraction of *Inula viscosa* and *Retama monosperma* were also observed in the cervical cancer cell lines HeLa and SiHa, where they exhibited a high level of cytotoxity by inducing apoptosis following pro-caspase activation, Bcl-2 expression and PARP cleavage [16]. The chemical composition of *Inula viscosa* and *Retama monosperma* extracts has already been investigated. Numerous studies have reveale d the presence of a sesquiterpene acid (isocostic acid) and two sesquiterpenes lactones: tomentosin and inuviscolide, as major compounds in *Inula viscosa* extract [16]. Indeed, this plant is a source of a number of bioactive compounds as well as flavonoids [13] and sesquiterpene derivatives [37]. However, the dichloromethane fraction of *Retama monosperma* revealed the presence of five known quinolizidine alkaloids together with sparteine, L-methyl cytisine, 17-oxosparteine, lupanine and anagyrine [16]. The Retama species have been reported to contain alkaloids [38] and flavonoids [39]. Accordingly, 15 quinolizidine and 3 dipiperidine alkaloids were isolated from the leaves of flowering plants of *R. monosperma* collected from Morocco [40]. The compositional analysis of the aqueous infusion of *Marrubium vulgare* has revealed the presence of fifteen metabolites, all belonging to the class of polyphenols. Particularly, seven flavonoids have been detected, together with 5-caffeoylquinic (chlorogenic) acid in small amounts; the extract is dominated by the presence of a series of complex molecules, characterized as verbascoside (acteoside) derivatives [26]. However, the chemical constituents of *Ormenis eiriolepis* still remain to be investigated. The selected species are reported to have both preventive and therapeutic properties. According to our findings, the use of these plants by traditional healers for the treatment of cancer patients

could have some scientific support. Because cancer drug-resistance is a significant health problem, the screening of medicinal plants for new anticancer agents remains a priority in drug discovery programs. The herein reported biological activity of medicinal plants used in traditional Moroccan medicine provides a starting point for forthcoming studies to determine the molecular basis of their activity and to identify the chemical compounds. Within the most active extracts responsible for their antitumoral effects.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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DECLARATION OF COMPETING INTEREST

The authors declare that there are no competing interests.

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