



Cytotoxic Effect and Chemical Composition of *Inula viscosa* from Three Different Regions of Morocco

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Authors' contributions

This work was carried out in collaboration between all authors. Authors NC and MM have participated equitably in the study and consequently shared the first place. Author MEM carried out the toxicity tests, participated in the project design and drafted the manuscript. Author NC participated in the project design, carried out the plant collection and participated in the toxicity tests. Author MEM conceived the study and participated in the design and coordination of the project and drafted the manuscript. Author SG carried out in the chemical analysis of plant extracts. Author SA participated in the conception and design of the study and review of the final manuscript. Author LB participated in the conception and design of the study, and supervised the toxicity tests. Author MEH participated in the design and coordination of the project and review of the final manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Worldwide, growing interest was given to find new cytotoxic agents that could be used in breast cancer treatment. In this context, we have planned to evaluate cytotoxic activity of *Inula viscosa* extracts, on two breast cancer cell lines. The plant was harvested in three different regions of Morocco; Imouzzar, Tounate and Sefrou.

Methodology: The evaluation of cytotoxicity of *Inula viscosa* ethanol and ethyl acetate extracts of the three regions was performed by measuring cell viability using the WST1 test after 72 h of exposure.

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Results: The IC₅₀ of the various tested extracts vary between 100 and 400 µg/ml. Sefrou ethanol and ethyl acetate extracts presented the best activities with IC₅₀ values of 195.42 µg/ml and 112.20 µg/ml, respectively. Chemical analysis of tested extracts showed that the composition of *Inula viscosa* extracts varies by region and impact the corresponding activity. Moreover, the cytotoxic activity of *Inula viscosa* is strongly related to the presence of tomentosine, inuviscolide and isocostic acid.

Conclusion: These preliminary results provide evidence that *Inula viscosa* induces a significant cytotoxic effect on both human cancer cell line, MCF-7 and MDA-MB231. This cytotoxic activity could be attributed to the presence and the concentration of tomentosine, inuviscolide and isocostic acid.

Keywords: Inula viscosa; cytotoxic activity; breast cancer; tomentosine; inuviscolide; isocostic acid.

1. INTRODUCTION

Breast cancer (BC) is a serious public health concern being the second most common of all cancers and by far the most frequent death reason of cancer amongst women [1]. BC incidence is rising rapidly in low and middle income countries (LMC) due to population aging and changes in underlying risk factors, in particular reproductive patterns [2,3].

In Morocco, as other developing countries, BC represents a serious public health problem since it's the first cancer among women and the third one of all registered cancer cases [4,5].

BC is traditionally treated with a combination of chemotherapy and surgery; hormonal therapy could be used depending on the stage and hormones receptors expressions. Although chemotherapies have been found to effectively control the progression of BC, the adverse effects and the development of resistance to anticancer drugs are a common clinical problem in the treatment of breast cancer [6]. Thus, there is a critical and urgent need for developing new therapeutic approaches for this disease.

In the quest for new therapeutics, plants were and are still considered as one of the main sources of biologically active materials. In this field, great interest was given to plants used in the traditional medicine, as a potential source of active compounds. Moreover, products of plants are frequently considered less poisonous and having fewer side effects than synthetic drugs, and consequently still widely used by the population to treat several diseases. In the Moroccan traditional medicine, as in other developing countries, plants are commonly used among people of rural communities, and their use is increasing in urban populations [7].

Inula viscosa L. Ait (Compositae) (common local name: Trehla or Magramane) is a perennial plant distributed in different regions of the Mediterranean Basin [8]. In traditional medicine, *Inula viscosa* L. has many uses, including anti-inflammatory [9]; anthelmintic [10]; antipyretic, antiseptic and antiphlogistic activities [11,12]. Moreover, this plant is also used for treatment of lung and gastroduodenal disorders [10,13]. Crude extracts prepared from different parts of *Inula viscosa* L. (Ait) exhibit antifungal [14], antioxidant [15], antiulcerogenic [16] and anthelmintic [17] properties and have cytotoxic activities on large variety of cancerous cells [18-23]. *Inula viscosa* L. is widely characterized by the presence of sesquiterpene lactones that are involved in many biological effects and associated with the main cellular activities, especially cytotoxic and anti-inflammatory activities [24].

This study was planned to evaluate the cytotoxic activity of different extracts obtained from *Inula viscosa* L on two breast cancer cell lines: MCF7, an estrogen receptor-positive cell line, and MDA-MB-231, an estrogen receptor-negative cell line. Indeed, hormone receptors status is widely used in the classification of breast cancer and is a key marker in breast cancer treatment. On the other hand, an interest was given to compare the cytotoxic activity of *Inula viscosa* extracts obtained from three different regions of Morocco to assess the relationship between site of collection, chemical composition and biological activity of these extracts.

2. MATERIALS AND METHODS

2.1 Plants Materials Extraction and Preparation

Inula viscosa L. was collected from the regions of Imouzzer, Sefrou and Taounate (Fig. 1). Plants

were identified by Professor Amina Bariin, from the Department of Biology, Faculty of Sciences Dhar El Mehraz, Fes – Morocco, where specimen was preserved and deposited under (ADV14101) reference in the University Herbarium. Plants were dried for a week at ambient temperature and then ground finely. Ethanol and Ethyl Acetate extractions were performed at the ratio of 10% (w/v) for 3 h under agitation [25]; the mixture was then filtered and concentrated in vacuum at 45°C to obtain an oily, dry and green paste, then stored at 4°C for further use. The obtained extracts were dissolved in dimethyl sulfoxide (DMSO) to give solution stocks of 40 mg/ml and conserved at -20°C until use.

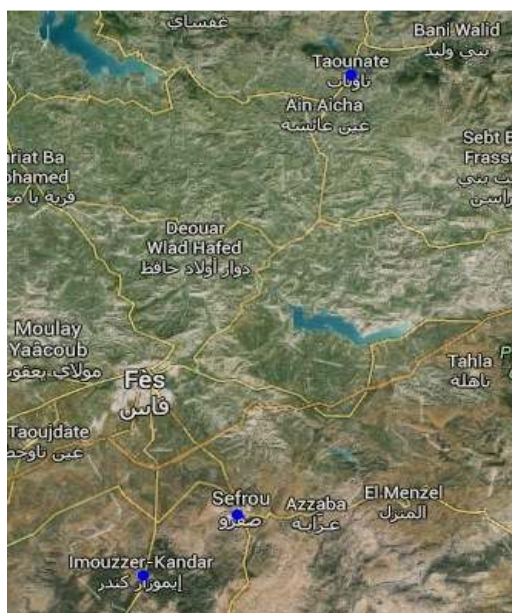


Fig. 1. A map showing the three regions of (Sefrou, Taouinate and Imouzzar) from where *Inula viscosa* L (Ait) was collected

2.2 Cell Viability Assays

Two well-characterized human breast cancer cell lines (MCF-7 and MDA-MB-231) were used in this study. MCF-7 and MDA-MB-231 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotics in tissue culture flasks under a humidifying atmosphere containing 5% CO₂ and 95% air at 37°C. Cytotoxic effect of *Inula viscosa* L. (Ait) Ethanolic and Ethyl Acetate extracts was assessed by WST-1 test [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Roche

Diagnostics GmbH, Mannheim, Germany), a colorimetric assay that quantifies mitochondrial activity and reflects cell viability. Exponentially growing MCF7 and MDA-MB-231 cells were washed and seeded at 10.000 cells/well, in 100 µl of growth medium, in 96 well microplates (Nunc, Denmark). After, 24 h of incubation, 100 µl of the medium containing the extracts (initially dissolved in DMSO) were added in each well, with different concentrations ranging from 15.6 to 500 µg/ml, in duplicate, and re-incubated for 72 h. Then, 100 µl of the medium were aspirated and 10 µL of the WST1 were added in each well. Cell viability was assessed by absorbance reading of each well at 450 nm using a Wallac Victor2 multiplate reader. For every test, DMSO and mitomycin C (~ 95% HPLC, sigma-Aldrich) were tested, representing respectively negative and positive controls.

Data are expressed as percentages of absorbance between treated and control wells. The IC₅₀ values, representing concentrations which reduce the absorbance of treated cells by 50%, were obtained from non-linear dose-response curves, obtained by plotting the percentages of inhibition versus the concentrations of tested extracts (µg/ml), using Origin 7.0 software.

2.3 Gas Chromatography / Mass Spectrometry (GC / MS) Analysis

The identification of compounds of *Inula viscosa* L. (Ait) from the three different regions of Morocco 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% methylpolysiloxane) capillary column (30 m, 0.25 mm, film thickness 0.25 µm). Injection volume was 1 µl with a splitless; the injector and detector temperature was held constant at 250°C. 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 program: 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.

3. RESULTS

Cytotoxic activities of *Inula viscosa* Ethanolic and Ethyl Acetate extracts from the three regions were evaluated on two human breast cancer cell lines, MCF-7 and MDA-MB231. The cytotoxic effect of all extracts was reported in Fig. 2, and the corresponding IC₅₀ are summarized in Table 1.

Overall, ethyl acetate and ethanolic extracts were able to inhibit cell growth in dose dependent manner after 72 h of treatment, in both cell lines. However, ethyl acetate extracts showed higher cytotoxic activities in both MCF-7 and MDA-MB231 cells as compared to the ethanolic extracts. In MDA-MB-231 cell line, ethyl acetate extract from *Inula viscosa* L. (Ait) collected in Sefrou region is the most active extract, showing a significant growth inhibitory effect, the corresponding IC₅₀ was 112.2 µg/ml. Ethyl acetate extracts from *Inula viscosa*, collected in Imouzzer and Taounate showed moderate cytotoxicity, the corresponding IC₅₀ are 199.73 and 316.22 µg/ml, respectively.

Similar results were also obtained on MCF-7 cell line. Indeed, ethyl acetate extract of *Inula viscosa* from Sefrou was the most cytotoxic compound with an IC₅₀ of 186.2 µg/ml. ethyl acetate extracts from plants collected in Imouzzer and Taounate

exhibited moderate cytotoxicity with higher IC₅₀ values.

Comparison between the cytotoxicity of the extracts on both breast cancer cell lines MCF-7 and MDA-MB-231, clearly showed that ethyl acetate and ethanolic extract of *Inula viscosa* from the three regions are more active on MDA-MB-231 than on MCF-7. For all extracts, IC₅₀ obtained on MCF-7 cell line are significantly higher than the corresponding IC₅₀ obtained on MDA-MB-231 cell line (Table 1).

Chemical composition analysis of Ethyl Acetate and Ethanolic extracts of *Inula viscosa* L. (Ait) from the three different regions has revealed the presence of isocostic acid, a sesquiterpene acid, and two sesquiterpenes lactones: tomentosin and inuviscolide, as major compounds (Table 2).

Great difference was observed between ethyl acetate extracts from the three regions. The tomentosin is present at high concentrations in the three extracts, ranging from 22% in the extract from Sefrou to 64% in the extract from Taounate, whereas the inuviscolide is absent in the extract from Taounate. The isocostic acid, present at relatively high concentration in the extract from Taounate, exhibited very low concentrations in the extracts from Imouzzer and Sefrou.

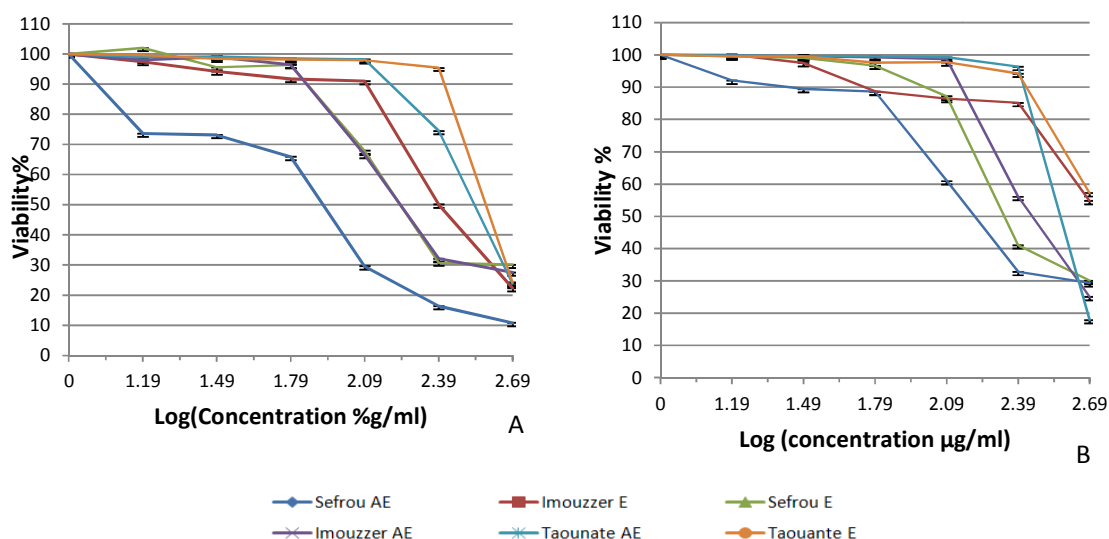


Fig. 2. Cytotoxic effect of Ethyl Acetate (AE) and Ethanolic (E) extracts of *Inula viscosa* L. (Ait) collected from three different regions of Morocco: Sefrou, Imouzzer and Taounate, against MDA-MB-231 (A) and MCF7 cell lines (B)

Cells were incubated for 72 h with different concentrations of the plant extracts (ranged from 15.62 to 500 µg/ml). The test was performed in duplicates

Table 1. IC₅₀ values of Ethyl Acetate and Ethanolic extracts of *Inula viscosa* L (Ait) collected from Sefrou, Imouzzer and Taounate regions, in both MDA-MB-231 and MCF-7 cell lines

<i>Inula viscosa</i>		MDA-MB231	MCF-7
Region of collect	Solvent for extraction		
Sefrou	AE	112.20 ± 1.28	186.20 ± 2.57
	E	195.42 ± 1.81	234.22 ± 1.23
Imouzzer	AE	199.73 ± 1.23	285.42 ± 1.51
	E	251.52 ± 1.99	>500
Taounate	AE	316.22 ± 1.20	351.03 ± 2.04
	E	392.12 ± 2.56	>500
Mitomycin C		6.1 ± 0.2	5.45 ± 1.1

Table 2. Chemical composition of Ethyl Acetate and Ethanolic extracts of *Inula viscosa*. (L) from Taounate, Imouzzer and Sefrou regions

Compounds		RT	Taounate		Imouzzer		Sefrou	
			E	AE	E	AE	E	AE
Major	Isocosticacid	41.11	19.7%	21.9%	0.18%	0.22%	2.78%	2.23%
	Tomentosin	47.62	29%	64%	4.98%	39%	3%	22%
	Inuviscolide	47.83	30%	-	57%	42.23%	46%	58%
Minor	1,2-longidinone	48.18	12.9%	-	4.98%	3.2%	7%	18%
	Iso-velleral	-	-	1.08%	0.2%	0.98%	-	1.79%
	6,9,12,15Docosatetraenoicacid, methyl ester	-	-	0.38%	-	0.25%	0.07%	1.22%
	Quercetin 7,3',4'-trimethoxy	-	-	1.25%	-	1.37%	-	2.05%
	Chiapin B	45.98	1.09%	-	4.18%	2.19%	3.77%	-
	1-Amino-1-ortho-chlorophenyl-2-(2-quinoxaliny) ethane	12.78	-	0.25%	-	0.78%	-	-
	3-(4'-Methoxyphenyl)-1-acetyl-2-phenylindolizine	25.08	0.52%	3.57%	-	2.76%	0.12%	3.08%
	Methyl 2,3-Dideoxy-4-O-propargyl-6-O-(tert-butyl)dimethylsilyl)-α-D-erythro-hex-2-enopyranoside	54.78	4.89%	0.34%	5.90%	1.78%	6.88%	1.56%
	Benzeneacetic acid, 4[(bis(trimethylsilyl)oxy)-, trimethylsilyl Ester	19.09	2.67%	0.78%	1.22%	0.09%	1.55%	-
	1-Tetralone	-	-	-	-	0.48%	-	-
	Tetracosane	53.87	0.09%	2.07%	0.11%	2.35%	-	0.12%
	Isoaromadendreneepoxide	42.23	-	2.53	-	2.53	-	-

In the ethanolic extracts, the isocostic acid is present at the same concentration as in the ethyl acetate extracts, whereas the inuviscolide is present in the three extracts and at high rates. However, the concentration of tomentosin in the extract from Taounate is less than 50% of the concentration obtained in the ethyl acetate extract and is very low in the extracts from Sefrou and Imouzzer, not exceeding 5%.

4. DISCUSSION

Recently, screening plants and their products for their cytotoxic and antiproliferative effects have become the major strategy in the search for new anticancer agents. In this optic, we have

conducted this study to evaluate the cytotoxic activity of ethanolic and ethyl acetate extracts of *Inula viscosa*, collected from three different regions in Morocco, on 2 breast cancer cell lines. Our previous study have reported an interesting cytotoxic effect of extracts from *Inula viscosa* L. collected against cervical cancer cell lines, SiHa and Hela, by inducing apoptotic process [26,27]. This activity was mainly attributed to the presence of tomentosin, a sesquiterpene lactone, widely reported as an active compound against cancer cells [27,28].

Obtained results clearly showed that both ethanolic and ethyl acetate extracts exhibit cytotoxic activities on both breast cancer cell

lines after 72 h of treatment, but with different IC_{50} . Extracts from Sefrou region, especially the ethyl acetate extract, seem to be the most active. Several studies have already reported that plants of the genus *Inula* exhibit *in vitro* cytotoxic effects on various human cancer cell lines [18-23]. The *Inula helenium* extract revealed a highly selective cytotoxicity toward four different tumor cell lines (HT-29, MCF-7, Capan-2 and G1).

In this study, even there's a cytotoxic activity with a dose dependent manner, the IC_{50} values are high. According to the National Cancer Institute, the criteria of cytotoxicity activity for the crude extracts is an IC_{50} values $<30 \mu\text{g/ml}$ [29]. It's widely accepted that the activity of plant extract is mainly due to chemical composition, especially on major compounds, this activity is due to the combination of multiple products, with protagonist/antagonist effects. In this field, it seems that the cytotoxic activity of *Inula viscosa* extracts is mainly due to the presence of tomentosin. This effect could be potentiated by the presence of the inulinolide and decreased in the presence of isocostic acid. Ethyl acetate extract from Sefrou, with high rates of tomentosin and inulinolide, and low rate of isocostic acid, exhibit the lower IC_{50} on both MCF-7 and MDA-MB-231 breast cancer cell lines. Interestingly, higher IC_{50} were obtained with the ethanolic extract of *Inula viscosa* from Taounate, having a high rate of isocostic acid, and the ethanolic extract from Imouzzer, showing a low rate of tomentosin. In these extracts, the concentration of chemical compounds is variable and depends on the solvent used and also according to the environmental conditions in which the plants were developed. Indeed, there's evidence that environmental conditions, including climate, type and composition of the soil, affect the chemical composition of the plants, and consequently affect the biological activities.

Many studies have highlighted the biological activity of tomentosin. Indeed, we have already reported that tomentosin is able to inhibit significantly the growth of SiHa and HeLa cervical cancer cell lines in dose and time-dependent manner and induces caspase-dependent apoptosis in cervical cancer cells [30]. Furthermore, studies of Rozenblat et al. [28] have shown that tomentosin and inulinolide inhibit the growth of three human melanoma cell lines. Sesquiterpenes lactones, including tomentosin, are widely reported to inhibit tumour growth by selective alkylation of growth-regulatory biological macromolecules, such as

key enzymes, which regulates cell division, thereby inhibiting a variety of cellular functions, which induce the cells to undergo apoptosis [31].

The presence of inulinolide is usually associated with the presence of tomentosin, and together forms the major compounds of *Inula viscosa*. Muhammad et al. have already reported a great cytotoxic effect on different human cancer cell lines, highlighting an interest of this molecule as a potent anticancer product [32].

To our knowledge, this is the first time that isocostic acid is investigated in the cytotoxic activity on cancer cell lines. Previous studies have studied biological activities of essential oils containing both costic and isocostic acids, and showed interesting antibacterial and antifungal effects [33,34]. Other studies have showed that costic acid and isocostic acid from *Inula viscosa* leaf extracts were found to be nematocidal phytochemicals [35].

In this study, *Inula viscosa* was harvested from three different regions; Taouante, Sefrou and Imouzzer. Corresponding ethyl acetate and ethanol extracts exhibited different chemical composition on both major and minor compounds, and are quite different from those reported for *Inula viscosa* growing over the world [36-38]. Our results are in agreement with previously reported data showing that *Inula viscosa* composition is strongly associated with the soil and environmental conditions [34]. Moreover, ethanol and ethyl acetate extracts of *Inula viscosa* from Taounate, Imouzzer and Sefrou have different rates of polyphenols associated with different antioxidant activities [39].

Of particular interest, a difference in the susceptibility of the two breast cancer cell lines to the effect of *Inula viscosa* extracts have been reported. Indeed, obtained results clearly showed that both ethyl acetate and ethanolic extracts are less active on the MCF-7 cell line as compared to the MDA-MB-231 cell line. This differential susceptibility could be explained by the fact that the cytotoxic effect of these extracts depends on the signalization pathway of the two cell lines. In fact, the breast cancer cell line MCF7 is estrogen receptor-positive whereas the MDA-MB 231 is estrogen receptor-negative [40,41].

5. CONCLUSION

In conclusion, this study revealed that *Inula viscosa* extracts have a moderate cytotoxic effect

on the breast cancer cell lines MCF-7 and MDA-MB 231. Moreover, obtained results highlighted that this cytotoxic activity is an integral effect of the combination of three major compounds, tomentosin, inuviscolide and isocostic acid. Further studies are required to confirm these results with purified products, to evaluate chemical interactions and to study the molecular mechanisms generating the cytotoxic activity on breast cancer cell lines.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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