Cytotoxic effect of some Moroccan medicinal plant extracts on human cervical cell lines

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Organic extracts of 7 selected plant species, used by Moroccan traditional healers to treat cancer or non-cancer diseases, were tested for their anti-cancerous activities. The plant selection was based on ethnobotanic information and interviews with local healers. Extracts from \textit{Inula viscosa} (L.) Ait., \textit{Retama monosperma} (L.) Bois., \textit{Ormenis mixta} (L.) Dumont., \textit{Ormenis eriolepis} Coss., \textit{Rhamnus lycioides} (L.), \textit{Berberis hispanica} Bois and Reut. and \textit{Urginea maritima} (L.) Baker. were tested for their potential cytotoxic effects on the human cervical cancer cell lines SiHa and HeLa, harbouring HPV16 and HPV 18 respectively. MTT (Tetrazolium blue) colorimetric assay was used to evaluate the viability of cell cultures in the presence of the extracts. The extract from \textit{Inula viscosa} (L.) Ait., \textit{Retama monosperma} (L.) Bois., \textit{Ormenis eriolepis} Coss. exhibited marked cytotoxic effect on the two cell lines. These species could be considered as potential sources of anticancer compounds. Further studies are necessary for chemical characterization of the active principles and more extensive biological evaluations.

\textbf{Key words:} Moroccan medicinal plants, cervical cancer cells, cytotoxic activity.

\section*{INTRODUCTION}

Cervical cancer is a major cause of death. It is the second most frequent cancer in women worldwide, accounting for 15% of all cancer related deaths in women (Boyle and Ferlay, 2005). Each year 470,000 women are diagnosed with invasive cervical cancer worldwide, 230,000 women die of this disease and 80% of these occur in developing countries (Bosh and de Sanjosé, 2003). In Morocco, cervical cancer is the second most frequent female cancer after breast cancer and represents a major public health problem. The diagnosis is usually made in advanced stages and mortality is high (Amrani et al., 2003).

Human Papillomavirus (HPV) is considered as the etiologic agent of cervical cancer. Epidemiological and biological studies have shown close relationship between HPV infection and cervical cancer development. High risk HPV, such as HPV16 and HPV18, has been detected in 94 - 100% of cervical precancerous lesions and cancer (Castellsagué et al., 2006).

Though the cervical cancer therapy is in advance, side effects due to the non-specific cytotoxicity of drugs and resistance to treatment represent a great problem in the cervical cancer management. Therefore, development and search of novel and effective anticancer agents, which in addition should overcome resistance, have become very important issues (Cameron and Bell, 2004).

Natural compounds have provided many effective anticancer agents in current use. Currently, over 50% of drugs used in clinical trials for anticancer activity were isolated from natural sources or are related to them (Newman and Gragg, 2007). The use of plants or plant products, traditionally, as antiviral agents is relatively wider than their use in modern medicine. Some antiviral substances have so far been isolated from higher plants, algae and lichens (Abonyi et al., 2009).

To our knowledge, few studies described the use of

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medicinal plants in treatment of infections by Human Papilloma Virus, HPV. Craigo et al. (2000) have shown that, some methylated derivatives of plant lignan, nord-hydroguaiaretic acid, which were found to be a potent antiviral agent against the Human Immunodeficiency Virus (HIV) and Herpes Simplex Virus (HSV), inhibit the gene expression from the early promoter P97 of HPV 16 and can be used in the therapy of papillomavirus infections. Likewise, Deng et al. (2004) have also found that water extracts from Asarum heterotropoides are effective on anti-human papillomavirus.

There are two main strategies for the selection of plants species in anticancer drug discovery: random screening and ethnomedical knowledge. The second approach includes plants used in organize traditional medical systems like herbalism and folklore (Pieters and Vlietn, 2005).

Uncontrolled proliferation is a universal property of tumour cells. Investigation of the cellular growth control mechanisms has contributed to the understanding of carcinogenesis and identification of compounds with specific antitumoral activities. Thus, cytotoxicity screening models provide important preliminary data to help select plant extracts with potential antitumoral properties for future studies (Cardellina et al., 1999).

In Morocco, the use of traditional medicine is widespread practice. The ethnombotanical and ethnopharmacological surveys conducted in different areas allowed the compilation of an inventory of 360 species and more than 500 prescriptions are recorded (Bellakhda, 1997). In the Moroccan traditional medicine, the use of plants in the form of infusions or decoctions is a common practice among people of rural communities and their use is increasing in urban populations. Moroccan medicinal plants were already studied for their use in different human diseases (Gonzalez-Tejero et al., 2008).

In the course of our screening strategy for the anticancer compounds from plants, we undertook the present study to evaluate the in vitro cytotoxic activity of seven plants used in the Moroccan traditional medicine for various diseases such as cancer, inflammation or infectious diseases. The selection of plants was made on the basis of their reputation as folk medicines in the treatment of tumours and related diseases. Table 1 shows the ethnombotanical data of the investigated plant species, including botanical names, local names, ethnomedical uses, as well as the plant parts employed in this study. The cytotoxic activity of selected plants was studied on the human cervical carcinoma cells lines, SiHa and HeLa, harbouring HPV16 and HPV18 respectively.

MATERIALS AND METHODS

Plant material

The selected plants were collected in different areas of Morocco in May, 2007 and were identified by Dr. M. Fennane from the Scientific Institute of Rabat. Voucher specimens are kept in the “Herbarium” of the Institute.

Preparation of extract

Plant materials were dried at room temperature in the dark and ground finely using blender. Exactly 20 g of each powdered sample (aerial plants, leaves, root bark or bulbs) were extracted by absolute methanol (100 ml, three times) for 72 h at room temperature with constant shaking. The extracts were pooled and 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. Extracts were sterilized by filtration using sterile 0.22 µm pore size filters.

In vitro cytotoxic activity assay

Cell lines and culture medium

Cancer cell lines were kindly provided by Dr. P. Coursaget, INSERM U618, University François Rabelais, Tours, France. Two human cervical cancer cell lines were used in this study, SiHa harbouring the HPV16 and HeLa harbouring the HPV18. 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 and 100% relative humidity atmosphere.

Cytotoxicity assay

Cytotoxicity of sample on tumor cells was measured by microculture tetrazolium (MTT) assay (Mosmann, 1983). For the assays, 96-well microplates were seeded with 100 µl medium containing 10,000 cells in suspension. After 24 h incubation and attachment, the cells were treated with 6 fourfold dilution of crude extracts. Exactly from the stock solution (40 mg/ml), each extract sample was applied in a series of 6 dilutions (final concentrations ranging from 15.6 to 500 µg/ml) with a final DMSO concentration of 0.1% and was tested in quadruplicate.

After 48 h incubation, cell viability was determined by adding (Sigma) tetrazolium salt as cytotoxicity indicator and by reading absorbance at 590 nm with a scanning multiwell spectrophotometer. Tetrazolium salts are cleaved to formazan dye by cellular enzymes (only in the viable cells). The level of absorbance directly correlates to the metabolically active cells. Mitomycin C (~ 95 % HPLC, sigma-Aldrich) was used as a positive control.

RESULTS AND DISCUSSION

Using the ethnomedical data approach, some Moroccan plants that are used in the Moroccan traditional medicine for various diseases, including cancer, were collected and evaluated for their cytotoxic activities. The search for new anti-cancer drugs is one of the most prominent research areas of natural products. To investigate the cytotoxic potential of 7 extracts from Morrocan plants used in traditional medicine for the treatment of various diseases such as cancer, inflammation or infectious diseases. We collected a selection of seven plants in order to screen them for possible cytotoxic activity against cervical cancer cell lines.

The cytotoxic activity was evaluated on two human cer-
Table 1. Ethnobotanical data and some reported pharmacological activities of plants species used in this study.

<table>
<thead>
<tr>
<th>Plants species (Family)</th>
<th>Trivial name</th>
<th>Place of collection</th>
<th>Part plant collected</th>
<th>Traditional use</th>
<th>Pharmacological activities</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Inula viscosa</em> L. Ait (Asteraceae)</td>
<td>Magramane-Terhala</td>
<td>Ain atik Temara</td>
<td>Leaves</td>
<td>Skin diseases, treats cutaneous abscesses, wound healing, Tuberculosis, bronchial infections (Bellakhdar, 1997)</td>
<td>Inflammatory effects (Hernandez et al., 2007) Antimicrobial activity (Maoz and Neeman, 1998) Antifungal activity (Cafarchia et al., 2002) Antitumoral activity (Rozenblat et al., 2008)</td>
</tr>
<tr>
<td><em>Retama monosperma</em> L. Bois (Fabaceae)</td>
<td>R'tm</td>
<td>Sidi-Boughaba Mahdia</td>
<td>Leaves</td>
<td>Purgative, vermifuge, antihelmintic, abortive and disinfectant (Benrahmoune, 2003)</td>
<td>No information available.</td>
</tr>
<tr>
<td><em>Berberis hispanica</em> Bois and Reut. (Berberidaceae)</td>
<td>Argis - Azargnat</td>
<td>Tamahdit</td>
<td>Bark roots</td>
<td>Blood pressure, digestive disorders, anorexia, urinary system, nephritic, liver and gastrointestinal disorders, ocular affections, febrifuge. Antileishmania, antitumoral. (Bellakhdar, 1997)</td>
<td>Antimicrobial activity (Li et al., 2007; Singh et al., 2007) Antilurum activities (Fukuda et al., 1999)</td>
</tr>
<tr>
<td><em>Ormenis eriolepis</em> Coss. (Asteraceae)</td>
<td>Helala</td>
<td>Ouarzazat</td>
<td>Aerial part</td>
<td>Stomachic, antihelmintic and anti diabetic (Bellakhdar, 1997)</td>
<td>Antibacterial activities Antileishmania activities (Antifungic activity)</td>
</tr>
<tr>
<td><em>Ormenis mixta</em> (Asteraceae)</td>
<td>Helala</td>
<td>Sidi-Boughaba Mahdia</td>
<td>Aerial part</td>
<td>Antioxidant, nervous breakdown, hepatic and gastric insufficiencies (Haddad et al., 2003)</td>
<td>Antimicrobial activity</td>
</tr>
<tr>
<td><em>Rhamnus lycioides</em> ssp. <em>Oleoides</em> (Rhamnaceae)</td>
<td>S’afira El- harcha</td>
<td>Sidi-Boughaba Mahdia</td>
<td>Leaves</td>
<td>Laxative and diuretic</td>
<td>Hypotensive activity (Terencio et al., 1990)</td>
</tr>
<tr>
<td><em>Urginea maritima</em> L. Baker (Lemnaceae)</td>
<td>Ansal-Baslet el dib</td>
<td>Sidi-Boughaba Mahdia</td>
<td>Bulbs</td>
<td>Cardiac failures, whooping-cough, pneumonia, abortive, vipers bites, and aphrodisiac Cough, bronchitis, the jaundice, diuretic, and internal tumours (Bellakhdar, 1997)</td>
<td>Cytotoxic and antimalarial activities (Sathiayamoorthy et al., 1999) Anti-insect activity (Pascual-Villalobos and Fernandez, 1999)</td>
</tr>
</tbody>
</table>
Figure 1. Cytotoxic activity of methanolic extracts from 7 medicinal plants against SiHa cells (A) and Hela cells (B).

Cells were incubated with different concentrations of the plant extracts (ranged from 15.6 to 500 µg/ml) for 48 h. Cell viability was determined by the MTT assay (n=4). Viability curves: Percentage viability = absorbance of test wells/absorbance of control wells) × 100) plotted against the concentration of extract.

Among the 7 medicinal plants tested methanolic extracts from *Inula viscosa* (L.) Ait., *Retama monosperma* (L.) Bois. and *Ormenis eriolepis* Coss. showed significant growth inhibitory effects in both SiHa and HeLa cells compared to the control. Their IC$_{50}$ were 54, 99 and 94 µg/ml in SiHa cells respectively. In HeLa cells, the IC$_{50}$ of the same plant extracts were 60, 112 and 96 µg/ml respectively. To be a good drug candidate, the IC$_{50}$ value

vical cancer cell lines, SiHa and HeLa, harbouring respectively HPV 16 and 18, the high oncogenic human papillomavirus (Pater and Pater, 1985).

The cytotoxic effect of 7 methanolic plant extracts on SiHa and HeLa cell lines was determined using the MTT assay. The MTT assays data are presented respectively in Figures 1(A and B) and the corresponding IC$_{50}$ are summarized in Table 2.
of such agent should be sufficiently low to avoid any possible unspecific effects. The American National Cancer Institute assigns a significant cytotoxic effect of promising anticancer product for future bioguided studies if it exerts an IC\textsubscript{50} value \( \leq 30 \) \( \mu \text{g/ml} \) (Suffness and Pezzuto, 1999). In this preliminary study, we have focused our interest on crude plant extracts, the cytotoxic activity could be due to the presence in the methanolic extracts of active products that could probably have highly anti-growth effects.

\textit{I. viscosa} (L.) Ait. extract had the greatest activity with lowest IC\textsubscript{50} values. Several studies have been reported on the phytochemical and other biological properties of \textit{Inula viscosa} (L.) Ait. It has been described to exhibit several biological activities such as anti-inflammatory (Hernandez et al., 2007), antimicrobial (Maz and Neeman, 1998) and antifungal effects (Cafarchia et al., 2002). This plant is a source of a number of bioactive compounds as well as flavonoids (Hernandez et al., 2007) and sesquiterpene derivatives (Fontana et al., 2007). Recently, Rozenblat et al. (2008) reported that tomentosin and inuvicolide, a sesquiterpene lactones isolated from \textit{I. viscosa} (L.) Ait. were able to inhibit cell growth of three different human melanoma cell lines.

\textit{R. monosperma} (L.) Boiss. and \textit{O. eriolepis} Coss. extracts showed also a significant growth inhibitory effects in both SiHa and HeLa cells. Theses effects are less marked than that obtained with \textit{I. viscosa} (L.) Ait. extract. These two plants were also reported in previous studies. The Retama species have been reported to contain alkaloids (Abdelhalim et al., 1997) and flavonoids (Kassem et al., 2000). Fifteen quinolizidine and 3 dipiperidine alkaloids were isolated from the leaves of flowering plants of \textit{R. monosperma} subsp. \textit{Eumonosperma} collected from Morocco (Touati et al., 1996). On the other hand, Retama genus has been described to contain flavonoids, alkaloids and tannins. In addition, a variety of plant flavonoids and alkaloids have also been shown to be anti-carcinogenic in several animal models (Cassady et al., 1990).

\textit{O. eriolepis} Coss. has been described to have an antibacterial, antileishmania and antifungic effects. To our knowledge, no data relative to the chemical constituents of \textit{O. eriolepis} Coss. has been proposed. This plant has not yet been assessed for \textit{in vitro} cytotoxicity against cancer cells.

The results obtained in this preliminary study indicate that the methanolic extracts of the plants \textit{I. viscosa} (L.) Ait. \textit{R. monosperma} (L.) Boiss and \textit{O. eriolepis} Coss. were shown to induce significant and dose-dependent inhibitory activities against human cervical cancer cell lines SiHa and HeLa. There remains interesting to evaluate the cytotoxic activity of selected plants \textit{in vitro} on other cancer cell lines.

This study provides an important basis for further investigation into the isolation, characterization and mechanism of cytotoxic compounds from the screened medicinal plants. We also plan to carry more biological activities, including the \textit{in vivo} studies and the statute of inhibition of HPV. Thus, these plants could be as a source for new lead structures in drug design to combat cancer.

Experiments were performed in quadruplicate (\( n = 4 \)) and data were expressed as means \( \pm \) SDs. IC\textsubscript{50} (inhibitory concentration 50\%) and SD (standard deviation for 95\% confidence) were determined by interpolation from the viability curves of SiHa and HeLa cells versus methanolic plant extract concentrations. Mitomycin C was used as a positive control.

Cells were incubated with different concentrations of the plant extracts (ranged from 15.6 to 500 \( \mu \text{g/ml} \)) for 48 h. Cell viability was determined by the MTT assay (\( n = 4 \)). Viability curves: Percentage viability = absorbance of test wells/absorbance of control wells) \( \times 100 \) plotted against the concentration of extract.

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