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Antiproliferative activity of selected medicinal plants of Jordan against a breast adenocarcinoma cell line (MCF7)

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Abstract

76 ethanolic extracts of medicinal herbs from the Jordanian flora, belonging to 67 species and 34 families, were evaluated for their antiproliferative activity on a breast cancer cell line (MCF7). The cells were cultured in RPMI 1640 medium and incubated with the extracts for 72 hours. Sulphorhodamine B (SRB) assay was used to test cytotoxicity.

From the tested crude extracts, *Inula graveolens, Salvia dominica*, *Conyza canadiensis* and *Achillea santolina* showed potent antiproliferative activity and the activity resided in the chloroform/ethanolic extracts. The most active plant was *I. graveolens* with an IC_{50} of 3.83 µg/ml. Phytochemical screening indicated the presence of flavonoids, terpenoids, and phenolics in all active extracts. These results indicate the possible potential use of medicinal plants from the Jordanian flora as antineoplastic agents.

Key words

Antiproliferative activity, SRB, Medicinal plants, Jordan, MCF7

Introduction

Since medieval times, plants have been the source of medicines for the treatment of diseases. Regardless of the availability of a wealth of synthetic drugs, plants remain – even in the 21st century – an integral part of the health care in different countries, especially the developing ones. In the late 90's, the WHO stated that a big percentage of the world's population depends on plant based therapies to cover the needs of the primary health care (WHO 1999) [1]. Moreover, towards the end of the 20th century, plant based OTC products, nutroceuticals and food supplements comprising the complementary and alternative therapies have gained a big share in the drug market in the developed countries.

Medicinal plants –either through systematic screening programs or by serendipity - possess an important position in the drug discovery and many modern drugs have their origin in traditional medicine of different cultures. Hence, despite the advantages of the synthetic and combinatorial chemistry as well as molecular modeling, medicinal plants remain an important source of new drugs, new drug leads and new chemical entities [2, 3]. The latter study reported that of the 877 small molecule new chemical entities (NCEs) introduced between 1981 and 2002 nearly the half (49%) were natural products, semi-synthetic natural products, semi-synthetic natural products analogues or synthetic compounds based on natural products.

The areas of cancer and infectious diseases have a leading position in utilization of medicinal plants as a source of drug discovery. Among FDA approved anticancer and anti-infectious preparations drugs of natural origin have a share of 60% and 75% respectively [3]. It is worthy to mention the vivid current interest in discovery of natural drugs for cancer treatment and chemoprevention [4, 5]. Huge number of plant species is screened and bioassayed for this purpose worldwide [6-17].

In many countries, cancer is the second leading cause of death after heart diseases [15, 18]. The estimated worldwide incidence of different carcinomas is about

10 million; half of these are in developed countries [13]. Among the cancer patients in the USA, the use of complementary and alternative medicine, represented mainly by plants, ranges between 30-75% [6]. This in turn justifies the interest in search of possible anticancer agents from the flora of different countries.

In accordance with this worldwide trend, the current study was undertaken to screen the ethanolic extracts of 67 plant species found in the Jordanian flora or sold by the local herbalist shops. Among the screened plants there are only few plants recommended by the traditional healers for the treatment of cancer (i.e *Arum palestinum*) while some tested plants are belonging to the genera with reported anticancer activities (i.e. *Salvia dominica*).

Experimental

Plant extracts' preparation

Plant samples collected during spring/summer 2005 or purchased in the same period were dried at room temperature and finely ground with a hammer mill. Each 2.5 g powdered plant material was extracted by refluxing with 25 ml ethanol for 30 min and kept overnight at room temperature before filtration. After filtration, ethanol was evaporated until dryness and the crude extracts were weighed. 0.1 g of the crude extract was dissolved in dimethyl sulphoxide (DMSO) to a final stock concentration of 10 mg/ml. All extracts were kept at −20 °C until cytotoxicity tests were carried out.

Phytochemical screening

Phytochemical screening using thin layer chromatography (TLC) was carried out only for plant species (*Achillea santolina, Conyza canadiensis, Inula graveolens* and *Salvia dominica*) indicating promising anticancer activity using MCF7 cell line. The ethanolic extracts were subjected to TLC examination for group determination of the

secondary metabolites. Modified Dragendroff's reagent for alkaloids, ferric chloride reagent for phenolics, Naturstoff reagent for flavonoids, ethanolic KOH for coumarins and vanilline/sulfuric acid reagent for terpenoids were used. Solvent systems for the development of ready coated analytical TLC plates (Merck) were selected according to Wagner and Bladt [19].

In vitro assay for cytotoxic activity

Cell culture

The cell line under investigation was human breast adenocarcinoma (MCF7). It was purchased from the European Collection of Animal Cell Culture (ECACC No. 86012803). The cells were cultured in RPMI 1640 medium supplement with 10% heated foetal bovine serum, 1% of 2 mM l-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin.

According to the cells growth profile, cells were seeded with a density of 5000 cell/well. This number was sufficient to give a reliable reading with the SRB assay, which corresponded well with the cell number and was the one that gave exponential growth throughout the incubation period with the plant extracts.

Cytotoxicity assay

For the assay, cells were washed three times with phosphate buffer saline (PBS). PBS was decanted and cells detached with 0.025% trypsin-EDTA (Sigma). RPMI 1640 was added to a volume of 10 ml. The cell suspension was centrifuged at 1000 X g for 10 minutes and the pellet was resuspended in 10 ml of medium to make a single cell suspension. Viability of the cells was determined by trypan blue exclusion and it exceeded 90% as counted in a haemocytometer. The cell suspension was diluted afterwards to give the optimal seeding density and 100 µl of the cell suspension was plated in a 96 well plate and incubated at 37 °C in a humidified atmosphere containing 5% CO2. After 24 hours the cells were treated with the extracts or pure compounds.

Each extract (initially dissolved in DMSO), was diluted with the medium and passed through a 0.2 µm filter. 50 µg/ml of each extract was tested initially, and, from the results, the active extracts were considered to be those which gave less than 50% survival at exposure time 72 hours. The active extracts were further diluted in medium to produce eight concentrations (0.1, 0.5, 1, 5, 10, 25, 50, 100 µg/ml) of each extract. 100 µl/well of each concentration was added to the plates in six replicates. The final dilution used for treating the cells contained not more than 1% of the initial solvent, this concentration being used in the solvent control wells. The plates were incubated for 72 hours. At the end of the exposure time, cell growth was analyzed using the SRB assay. Two replicate plates were used to determine the cytotoxicity of each extract.

As positive control vincristine sulphate was used (Sigma, Lot No. 34H0447) at the concentrations of 0.05, 0.1, 0.5, 1, 5, 10, 25, 50, 100 nM. For the calculation of IC_{50} WinNonLin Professional Version 5.0.1 was used.

Sulphorhodamine B assay

After incubation for 72 hours, adherent cell cultures were fixed *in situ* by adding 50 µl of cold 40% (w/v) trichloroacetic acid (TCA) and incubated for 60 min at 4 °C. The supernatant was then discarded and the plates were washed five times with demonized water and dried. 50 µl of SRB solution (0.4% w/v in 1% acetic acid) was added to each well and incubated for 30 min at room temperature. Unbound SRB was removed by washing five times with 1% acetic acid. Then, the plates were air-dried and 100 µl of 10 mM Tris base pH 10.5 (Sigma) were added to each well to solubilize the dye. The plates were shaken gently for 20 minutes on a plate shaker and the absorbance (OD) of each well was read on an ELISA reader at 570 nm. Cell survival was measured as the percentage absorbance compared to that of the control (non-treated cells).

Results and Discussion

In the present study, the cytotoxic effect of 76 ethanolic plant extracts that belong to 34 families on MCF7 cells were characterized by conducting cell viability assay stained with sulphorohdamine B. Cultures of MCF7 cells were treated with the extracts first at one concentration of 50 µg/ml and the results are shown in Table 1. Control assays were carried out for samples containing only the appropriate volumes of blank solutions and those showed no effect on cell growth.
For plant extracts that showed less than 50% survival rate, further dilutions were made to calculate the exact IC_{50} values (Figure 1 and Table 2). For the most potent extracts, their antiproliferative activity was studied again using chloforom and water extracts (Table 3).

In the US NCI plant screening program, a crude extract is generally considered to have *in vitro* cytotoxic activity if the IC_{50} value (concentration that causes a 50% cell kill) in carcinoma cells, following incubation between 48 and 72 hours, is less than 20 µg/ml, while it is less than 4 µg/ml for pure compounds [20]. Table 2 shows that four ethanolic plant extracts exhibited high cytotoxicity against MCF7 cells.

Number	Plant Family	Plant Name	Part Used	% survival ± standard deviation
1	Araceae	*Arum palaestinum*	L	99.81 ± 4.25
2	Arecaceae	*Phoenix dactylifera*	L	88.55 ± 9.11
3	Boraginaceae	*Alkanna strigosa*	H	99.93 ± 6.81
4	Boraginaceae	*Asperugo procumbens*	H	92.42 ± 11.52
5	Cactaceae	*Opuntia ficus-indica*	L	78.81 ± 7.69
6	Capparaceae	*Capparis spinosa*	L, St	111.06 ± 8.97
7	Capparaceae	*Capparis spinosa*	Fr	111.32 ± 12.52
8	Capparaceae	*Capparis spinosa*	R	65.2 ± 7.53
9	Capparaceae	*Capparis spinosa*	L	111.44 ± 11.07
10	Capparaceae	*Capparis spinosa*	F	117.22 ± 5.67
11	Caprifoliaceae	*Sambucus nigra*	L	94.89 ± 7.26
12	Caprifoliaceae	*Sambucus nigra*	F	100.56 ± 8.89
13	Caryophyllaceae	*Ankyropetalum gypsophloides*	R	90.93 ± 9.33
14	Caryophyllaceae	*Paronychia argentea*	F	84.11 ± 7.32
15	Chenopodiaceae	*Beta vulgaris*	H	103.1 ± 3.62
16	Cistaceae	*Cistus creticus*	H	72.32 ± 5.38
17	Compositae	*Achillea biberstenii*	L, F	81.64 ± 3.3
18	Compositae	*Achillea santolina*	L, F	46.54 ± 4.00
19	Compositae	*Anthemis palaestinum*	L, F	91.71 ± 4.78
20	Compositae	*Centaurea iberica*	L, F	102.15 ± 8.20
21	Compositae	*Cichorium intybus*	L,F	105.15 ± 5.74

22	Compositae	*Conyza bonariensis*	H	84.89± 17.26
23	Compositae	*Conyza canadiensis*	H	40.28 ± 3.39
24	Compositae	*Inula graveolens*	F	5.29 ± 0.41
25	Compositae	*Varthemia iphionoides*	L,F	84.95 ± 8.91
26	Convolvulaceae	*Convolvulus betonisifolius*	H	95.44 ± 7.38
27	Cruciferae	*Cardaria draba*	H	111.05 ± 6.47
28	Cruciferae	*Eruca sativa*	R	98.75 ± 6.46
29	Cruciferae	*Lepidium sativum**	L	105.23 ± 8.03
30	Cucurbitaceae	*Momordica balsamina*	L, St	100.44 ± 9.83
31	Elaeagnaceae	*Eleagnus angustifolia*	L	77.8 ± 7.29
32	Ericaceae	*Arbutus andrachne*	L	103.48 ± 10.30
33	Ericaceae	*Arbutus andrachne*	St	111.05 ± 8.84
34	Euphorbiaceae	*Euphorbia hierosolyminata*	H	74.68 ± 7.75
35	Euphorbiaceae	*Euphorbia peplus*	H	78.16 ± 4.58
36	Euphorbiaceae	*Mercurialis annua**	L	102.57 ± 9.26
37	Euphorbiaceae	*Mercurialis annua*	L	100.88 ± 8.23
38	Hippocastanaceae	*Aesculus hippocastani*	L	85.29 ± 7.22
39	Hypericaceae	*Hypericum triquetrifolium*	L, St	97.12 ± 9.24
40	Iridaceae	*Iris germanica*	F*	73.85 ± 11.16
41	Iridaceae	*Iris nigricans*	F*	91.07 ± 7.81
42	Labiatae	*Ajuga chia*	H	101.08 ± 1.87
43	Labiatae	*Ballota undulata*	L	94.83 ± 6.07
44	Labiatae	*Marrubium vulgare*	L,F	72.68 ± 6.12

45	Labiatae	*Nepeta curviflora*	L,F	90.93 ± 6.83
46	Labiatae	*Phlomis syriaca*	F	103.86 ± 6.54
47	Labiatae	*Salvia dominica*	L	9.69 ± 0.84
48	Labiatae	*Salvia hierosolymitana*	L	85.92 ± 10.25
49	Labiatae	*Salvia indica*	L	100.56 ± 6.70
50	Labiatae	*Teucrium leucocladum*	F,L	60.25 ± 10.33
51	Labiatae	*Teucrium polium*	F,L	77.85 ± 8.25
52	Labiatae	*Thymus capitatus*	L,F	86.61 ± 5.95
53	Leguminosae	*Alhagi maurorum*	R	69.90 ± 4.14
54	Leguminosae	*Anagyris foetida*	L	106.19 ± 2.51
55	Leguminosae	*Astragalus oocephalus*	L	74.72 ± 5.20
56	Leguminosae	*Melilotus indicus*	L	80.30 ± 6.31
57	Leguminosae	*Ononis natrix*	L, F	90.01 ± 4.68
58	Leguminosae	*Trifolium purpureum*	F, L	95.79 ± 5.76
59	Liliaceae	*Allium neoplitanum*	H	99.86 ± 5.15
60	Ranunculaceae	*Clematus cirrhosa*	H	77.82 ± 9.37
61	Resedaceae	*Reseda lutea*	Fr, L, F	104.69 ± 8.68
62	Rhamnaceae	*Zizyphus jujuba*	L	96.42 ± 9.27
63	Rubiaceae	*Gallium arabicum*	H	100.47 ± 2.67
64	Rubiaceae	*Rubia tinctoria*	L	101.03 ± 6.84
65	Rutaceae	*Ruta chalepensis*	F, L	80.33 ± 4.76
66	Santalaceae	*Ossyris album*	L,S	87 ± 17.23
67	Simarubiaceae	*Ailanthus altissima*	F	106.22 ± 11.37

68	Simarubiaceae	*Ailanthus altissima*	L	109.4 ± 9.87
69	Simarubiaceae	*Ailanthus altissima*	Fr	94.57 ± 5.59
70	Tiliaceae	*Tilia cordata*	L	91.42 ± 9.44
71	Umbelliferae	*Ammi majus*	F, S	87.4 ± 8.97
72	Umbelliferae	*Apium graveolens**	L	101.76 ± 8.88
73	Urticaceae	*Pariteria alsinifolia*	L	66.87 ± 8.31
74	Urticaceae	*Urtica dioica*	L, St	93.12 ± 8.88
75	Urticaceae	*Urtica urens*	L	103.94 ± 5.2
76	Zygophyllaceae	*Peganum harmala*	L	97.94 ± 9.00

Tab. 1. Percentage cell survival of MCF7 cells following 72 hours exposure to 50 µg/ml fractions from ethanolic plant extracts.
L: leaves, S: seeds, St: stems, F: flowers, Fr: fruits, R: Roots, H: herb, *: fresh plant used.

The data presented in Table 1 show that the ethanolic extracts of five plants that belong to the families of Compositae and Labiatae exhibited high cytotoxic activity. Calculation of the IC_{50} values for these extracts confirmed that the most potent plant extract was *I. graveolens (*IC_{50} 3.83 µg/ml) followed by *S. dominica* (IC_{50} 7.28 µg/ml), *C. canadiensis (*IC_{50} 12.76 µg/ml) and *A. santolina* (IC_{50} 24.12 µg/ml). Among these five plant species the least active was *T. leucocladum* (IC_{50} 39.242 µg/ml).

Plant extract	**IC_{50} ± Standard deviation**
Achillea santolina	24.12 ± 1.927
Salvia dominica	7.28 ± 1.150
Inula graveolens	3.833 ± .177
Teucrium leucocladum	39.242 ± 8.727
Conyza canadiensis	12.76 ± 2.475

Tab. 2. In vitro cytotoxic activity (IC 50 µg/ml **±** Standard deviation) of crude extracts tested against MCF7 cell line for exposure of 72 hours.

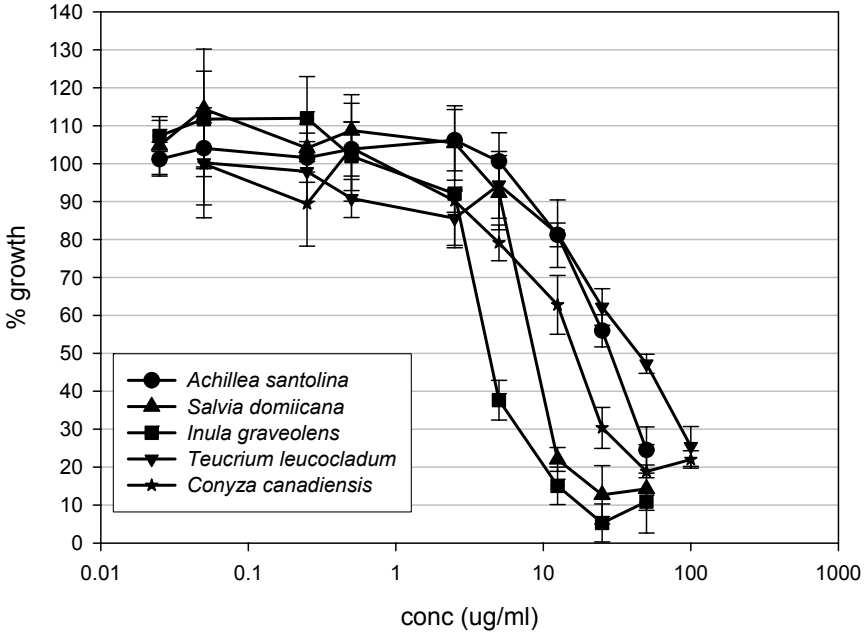

Fig. 1. Growth inhibition of MCF7 cell line by active plant extracts. Results present the average and standard deviation of 6 replicates.

The cytotoxic activity was further studied using chloroform and water extracts of the potent plants and for these, the activity was mainly in the chloroform and/or ethanolic extracts (Table 3).

Plant extract	Chloroform extract	Water extract	Ethanolic extract
Achillea santolina	15.49 ± 1.45	96.79 ± 3.04	46.54 ± 4.0
Salvia dominica	47.43 ± 4.57	100.96 ± 8.30	9.69 ± 0.84
Inula graveolens	6.80 ± 1.73	74.23 ± 8.93	5.288 ± 0.41
Conyza canadiensis	36.32 ± 9.35	105.13 ± 5.56	40.281 ± 3.40

Tab. 3. Survival rate of MCF7 cells treated with different extracts at the concentration of 50 µg/ml at exposure time of 72 h ± S.E.M.

The results of the phytochemical screening of the plant species with potent anticancer activity are given in Table 4. Flavonoids, terpenoids and phenolics were identified in all tested plants while the presence of alkaloids could be weakly detected only in one species (*A. santolina*).

Plant name	alkaloids	flavonoids	terpenoids	phenolic	coumarins
Achillea santolina	–/+	+ +	++	++	+
Salvia dominica	-	+	+	+	+
Inula graveolens	-	++	+	+ +	+
Conyza canadiensis	-	++	++	+	-
Teucrium leucocladum	–	++	++	++	-

Tab. 4. Phytochemical screening of plant extracts with high cytotoxic activity

Cytotoxicity screening models provide important preliminary data to help selecting plant extracts with potential antineoplastic properties for future work [21]. Sulforhodamine B assay is a well-established *in vitro* method for cytotoxicity against cancer cell lines and non-cancer cell lines, and here it was utilized to determine the selective activity of the extracts [15].

Several plant species rich in flavonoids are reported having disease preventive and therapeutic properties. This observation is of particular importance since flavonoids are ingredients of many vegetables and fruits and the association of vegetable and fruit consumption with reduced cancer risk has been reported [22, 23]. Antiproliferative activity recorded in the present study is in accordance with this finding, since the phytochemical evaluation indicated the presence of flavonoids in all of the four plant species with promising activity. Among these active plant species the genus

Salvia has been investigated for cytotoxic activities on cultures of different human tumor cell lines using crude extracts, oily fractions or isolated compounds [24-26]. In a recent study Fiore et al. demonstrated antiproliferative activity for *S. dominica*, collected from Jordan, against colorectal adenocarcinoma, choriocarcinoma, prostate adenocarcinoma, B lymphoblast, glioblastoma and endometrium adenocarcinoma cell lines [27]. For the remaining plants with cytotoxic activities only scarce reports are available [28].

The ethanolic and chloroform extracts of *I graveolens* and *C. canadiensis* showed little difference in their potency, while the chloroform extract of *A. santolina* was found to be more active than the ethanolic extract indicating for non polar active principles responsible for the antiproliferative activity. In *S. dominica*, the ethanolic extract was found to be nearly five times more active than the chloroform extract.

In a parallel study, the 67 plants are under investigation for the antiproliferative activity against other cancer cell lines, namely A549 and HL60. In both cell lines and for the concentration 50 µg/ml, the extracts were not toxic and the proliferation rate of the cells was not significantly different from that of the controls (untreated cells). The toxicity of these extracts on normal tissue culture and the exact determination of IC_{50} value is another point to be further assessed in order to clarify their safety.

Further studies are also in process to evaluate the most potent fraction of the four active plants and to isolate the constituents of these fractions.

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