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Original Article

Antioxidant and Cytotoxic Effect of Aqueous and Hydroalcoholic Extracts of the Achillea Millefolium L. on MCF-7 Breast Cancer Cell Line

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Breast cancer is the second main cause of death among women. The use of medicinal plants has been common in many countries since ancient times. The aim of this study was to evaluate the antioxidant and anti-proliferative potential of Achillea millefolium L. The antioxidant activity of the aqueous and hydroalcoholic extracts of Achillea millefolium L. leaf and flower was measured by DPPH and FRAP method while its anti-proliferative activity on MCF-7 human breast cancer cell line was evaluated using MTT assay. The ethanolic extract of the leaf and the methanolic extract of the flower had the highest radical scavenging and ferric ion reducing activities. Time and dose-dependent cytotoxic effects of different extracts were observed on MCF-7 cells. The difference in cell viability between hydroalcholic (methanol and ethanol) and aqueous extracts of leaf and flower was significant ($P< 0.05$), but there was no significant difference in cell viability between methanolic and ethanolic extracts of leaf ($P< 0.05$). IC50 values varied between 7 and 93 µg/ ml with ethanolic extracts being more cytotoxic and flower extract exhibiting a higher antiproliferative effect than leaf extract. The presence of antioxidant activity as well as high cytotoxic effect of all examined extracts suggest that Achillea millefolium may possess a potential chemotherapeutic activity for breast cancer treatment.

Keywords: *Achillea millefolium*, antioxidant, antiproliferative, MCF7, FRAP assay

Cancer is a condition in which cells grow out of control in the body. It is the leading cause of death worldwide with about 25% of deaths in 2013 in the United States being due to cancer (1). Among all cancers, breast cancer is the second main cause of death among women after lung cancer (2). Different strategies such as surgery, radiotherapy, chemotherapy and hormone therapy are currently used for breast cancer treatment. However, these methods are expensive and have many side effects. Many attempts have been made to use plants for cancer treatment (3-7). The use of medicinal plants

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has been common in many countries since ancient times. Since, pharmaceutical products originate from plants, therefore, finding more effective drugs with fewer side effects is important (8, 9). *Achillea millefolium L. Subsp* (yarrow) is a flowering and aromatic herb which is a member of the Asteraceae family. It is growing wild in the British Isles; is native to Europe, Asia, Australia and North America. *Achillea millefolium L.* extract contains compounds such as isovaleric acid, salicylic acid, asparagin, sterols, flavonoids, tannins, and coumarone. The essential oil of *Achillea millefolium* is commonly used in folk medicine for the treatment of several diseases and properties such as antibacterial, astringent and anti-blood transfusion, diuretic, digestive system stimulator, anti-inflammatory, anticancer and anti-allergic characteristics were assigned to this plant's oils (9, 10). In this study, we investigated the antioxidant and anti-proliferative activity of the aqueous, ethanolic and methanolic extracts of the flowers and leaves of the *Achillea millefolium L.* on breast cancer MCF-7 cell line.

Materials & Methods

Samples preparation

Achillea millefolium L. Subsp leaves and flowers were harvested in June, 2013 from Polour, Mazandaran, Iran. Dried leaf and flowers were powdered and extracted (10 g) with either ethanol 85%(v/v) or methanol 85%(v/v) or distilled water (72 h) in an orbital shaker at room temperature. After filtering through #1 filter paper (Whatman Inc., Hillsboro, OR, USA) followed by centrifugation at 8000 rpm for 15 min, supernatants were again filtered through a 0.2 µm filter under the laminar flow hood. Then, supernatants were evaporated and dissolved in dimethyl sulfoxide (DMSO) to a final stock concentration of 200 mg/ml. All extracts were stored at -20 °C for further use.

Ferric-reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) assay was performed as previously described (11). Briefly, the fresh working solution was prepared by combining acetate buffer (0.3 mM, pH 3.6), 2,4,6-Tripyridyl-s-Triazine (TPTZ) solution (10 mM) in HCl (40 mM), and FeCl3 _ 6H2O solution (20 mM), and then warmed at 37 °C before use. A 50 µl sample was mixed with 1.5 ml of FRAP reagent and the absorbance of the reaction mixture was measured at 593 nm after incubation at 37 °C for 10 min. A calibration curve was prepared, using an aqueous solution of ascorbic acid. All measurements were performed in triplicate and the mean values± standard deviations (SD) are reported.

Table 1. Scavenging activity percentage and IC_{50} values (mg/ ml) of different extracts measured by DPPH

		Leaf			Flower			
	Concentration mg/ml	Aqueous	Methanol	Ethanol	Aqueous	Methanol	Ethanol	Ascorbic acid µM
	3.75	21.49±1.20	74.52±0.63	79.66±1.15	32.13±3.20	81.10±0.40	75.98±1.90	-
	1.88	14.99±2.20	46.73±3.60	75.63±1.60	20..97±2.40	78.54±0.70	44.96±2.50	-
	0.94	6.50±5.40	24.66±3.80	43.38±0.40	6.35±1.30	43.70±1.10	18.60±3.00	-
	0.47	3.03±1.17	13.67±5.60	25.81±2.10	3.04±1.00	19.59±1.80	6.07±0.70	-
	0.23	3.33±2.00	9.47±3.78	13.76±3.00	1.40±0.30	11.64±1.7	3.64±2.20	-
IC_{50} mg/ml		7>	2.31	1.16	7>	1.16	2.31	98 µM

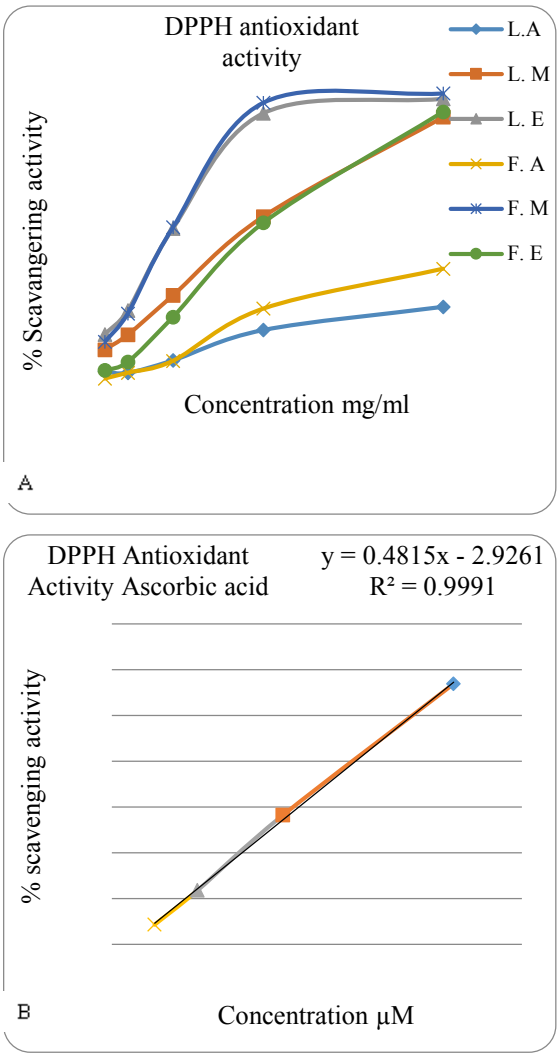

Figure 1. Free radical-scavenging capacities of different extracts of *Achillea millefolium* (A) and ascorbic acid (B). L: leaf; F: flower; A: aqueous; M: methanol and E: ethanol.

DPPH radical scavenging assay

The free radical scavenging activity of the extracts was determined by stable radical 1,1-Diphenyl-2-picrylhydrazyl (DPPH) previously described (12)with slight modifications in a microtiter plate where 25 µl of each extract at various concentrations (3.75, 1.88, 0.94, 0.47, and 0.23 mg/ml) were added to 100 µl of freshly prepared DPPH solution (0.5 mM) in methanol. Ascorbic acid was used as standard control. The reaction was allowed for 30 min and absorbance was measured at 515 nm by using a microplate reader (ELX 800/ Biotek). The percentage of scavenging activity at different concentrations was determined and the IC_{50} value of the extracts was compared with that of ascorbic acid. The inhibition of the DPPH radical by the sample was calculated according to the following formula:

%DPPH scavenging activity= [(Abs. of control –Abs.of sample)/Abs. of control] ×100

Cell culture

MCF-7 cell line was purchased from Pasteur institute, Tehran, Iran. The cell line was cultured in RPMI-1640 medium (PAA, Austria) supplemented

with 10% fetal bovine serum and 1% antibiotics (penicillin/streptomycin) (Invitrogen, USA) in a humidified atmosphere containing 5% CO2 and 95% air, at 37 °C.

Cytotoxicity assay

In vitro cytotoxicity of the *Achillea millefolium* against MCF-7 breast cancer cell line was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, the MCF-7 cells were seed on 96-well microplates at a concentration of 5×10^3 per well. After 24 h, the cells were incubated with different concentrations of two-fold serial dilution (500- 7.8 µg/ml) of plant extracts for 24 and 48 h. DMSO (the vehicle) was used as control. At the end of the exposure time, the cells were washed with phosphate buffer solution (PBS), then MTT stock solution (5 mg/ml) was added to each well and the plates were further incubated for 4 h at 37 °C. Acidic isopropanol was added to the wells and then the absorption at 570 nm of formazan product was

Table 2. Antioxidant activity of different extracts of *Achillea* measured by FRAP assay

Extract	µmol vitamin C/mg sample
Aqueous Leaf	8.47±0.50
Methanol Leaf	30.15±2.47
Ethanol Leaf	49.05±3.74
Aqueous Flower	7.68±1.04
Methanol Flower	54.79±11.75
Ethanol Flower	37.20±7.06

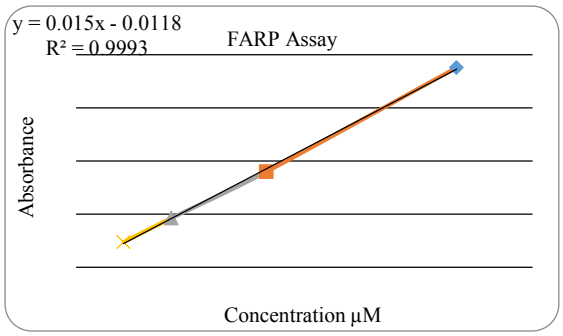

Figure 2. Standard curve for ascorbic acid. Regression factor squared for the linear regression, r2= 0.999.

measured using a microplate reader (ELX 800/ Biotek).

Results

Antioxidant activity

The antioxidant activity was evaluated by DPPH and ferric reducing assays. Ascorbic acid was considered as positive control. Figure 1A and 1B show the dose- response curve for the radical-scavenging activity of different extracts of *Achillea millefolium L.* and ascorbic acid using the DPPH colorimetric method, respectively. The radical-scavenging activity of different extracts of leaf and flower are represented in Table 1. The ethanolic extract of the leaf and the methanolic extract of the flower had the highest radical-scavenging activity at 1.88 mg/ml. The aqueous extract of the leaf and flower showed the lowest antioxidant activity and an IC_{50} value greater than 7 mg/ml. Standard ascorbic acid showed an IC_{50} value of 98 µM. The ability to reduce ferric ions was measured by FRAP method and is summarized in Table 2. As in DPPH assay, the methanolic extract of flower exhibited strong ferric ion reducing activity followed by the ethanolic extract of leaf. Figure 2 shows the dose- response curve for ascorbic acid reducing power by FRAP colorimetric method.

MTT cell proliferation assay

Time- and dose- dependent cytotoxic effects of different extracts on the growth of the human breast cancer MCF-7 cell line are summarized in Figure 3. The difference in cell viability between hydroalcoholic (methanol and ethanol) and aqueous extracts of leaf and flower was significant ($P< 0.05$). But there was no significant difference in cell viability between methanolic and ethanolic extracts of leaf (Figure 3A) ($P< 0.05$). However, there was a significant difference between methanolic and ethanolic extracts of flower (Figure 3B) ($P < 0.05$). As shown in Figure 4A, there was no significant difference in cell viability between the aqueous extracts of flower and leaf for concentrations up to 58.75 µg/ml. But there was a significant difference in the viability between hydroalcoholic extracts of flower and leaf (Figure 4B and 4C). Table 3 presents *in vitro* cytotoxic activities of different extracts of *Achillea millefolium*, which were expressed as IC_{50} values. All extracts exhibited a high antiproliferative effect against MCF-7 cell line, giving IC_{50} values at the level of µg/ml. The IC_{50} values corresponding to flower were highly cytotoxic in comparison to leaf. In addition, the IC_{50} values of ethanolic extracts were higher than other extracts.

Discussion

Concerns about health and nutrition are growing. Emerging medicinal plants as natural products are used not only in traditional medicine but also in a number of food and pharmaceutical products, due to their nutritional properties and bioactivity (13). Among *Achillea* species, the

Table 3. IC_{50} values of different extracts of *Achillea millefolium*

Extract method	Plant part	Time of exposure(h)	IC 50(µg/ml)
Aqueous	Leaf	24	147.49
Aqueous	Leaf	48	97.47
Methanol	Leaf	24	54.91
Methanol	Leaf	48	40.54
Ethanol	Leaf	24	47.05
Ethanol	Leaf	48	37.46
Aqueous	Flower	24	92.04
Aqueous	Flower	48	79.80
Methanol	Flower	24	14.60
Methanol	Flower	48	14.60
Ethanol	Flower	24	11.77
Ethanol	Flower	48	7.30

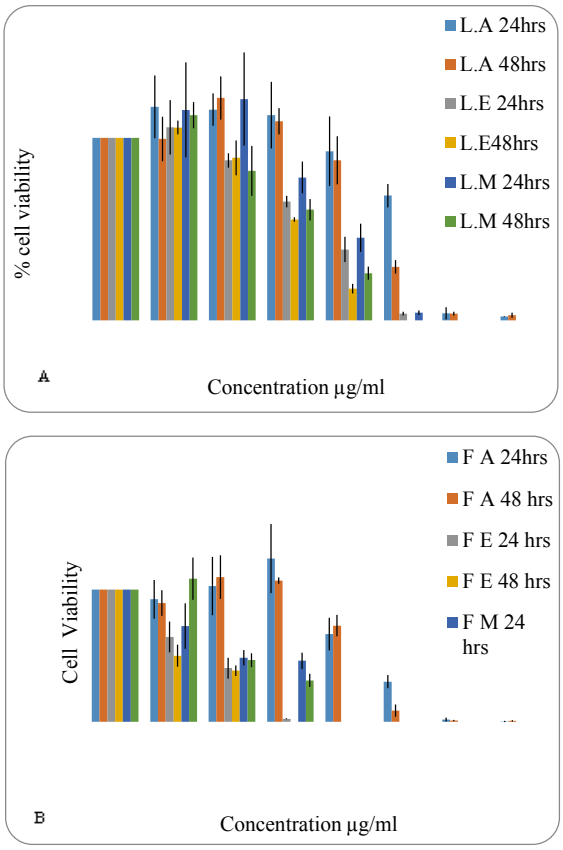

Figure 3. Dose-dependent antiproliferative effects of different extracts of *Achillea millefolium* leaves (A) and flowers (B) against MCF-7 cell line. L: leaf, F: flower, A: aqueous, M: methanol and E: ethanol.

cytotoxic effects of *Achillea alexandri-regis*, *A. lavennae* and *A. millefolium* have been reported against various tumor cell lines. Also, anti tumoral activity of n-hexane, chloroform, aqueous-methanol and aqueous extracts of the aerial parts of the *Achillea millefolium* have been assayed on three human tumor cell lines. The chloroform-soluble extract had high tumor cell proliferation inhibitory activities on HeLa and MCF-7 cells (14). Antioxidant and antibacterial properties of *A. millefolium* have previously been reported in hydroalcoholic, methanol and aqueous extracts as well as in essential oil (15-19). Candan et al. have investigated the antioxidant activity and monoterpens content of methanol extracts and essential oils of *A. millefolium*. They found that DPPH and superoxide radical scavenging activities of methanol extracts were lower than essential oils (19). Keser et al. have studied water and ethanol extracts of flowers, leaves and seeds of *A. millefolium* by DPPH and showed that the flower's ethanol extract has the highest DPPH radical scavenging activity (91.03%), while the seed's ethanol extract has the lowest activity (79.94%) (20). These results correlate with our findings showing that the ethanol extract of flower is more effective than its aqueous counterpart. The DPPH radical scavenging activity of *A. millefolium* was also reported by others (16, 20). Cytotoxicity against human tumor cell lines was only evaluated for the ethanol extract (21). Such activity was related to the presence of sesquiterpene lactones and flavonols (14) . Bhat et al. found that the methanol extract of the aerial part of *Achillea millefolium* exhibited a dose dependent oxidation potential with an IC_{50} around 2.5 µg/ml. Cytotoxic activity evaluation on MCF-7 cells for methanol extract of aerial parts and flowers showed that the flower parts were more effective than aerial parts ((22). The results reported for MCF-7 cell line, mainly in the case of ethanol extract samples, are consistent with those obtained with ethanol extracts of *A. millefolium* from Iran (GI_{50}= 64.08 µg/ml) (21). Correspondingly, *Achillea millefolium* showed promising antioxidant and cytotoxic actitivities against MCF-7 cell line. The ethanol extract of leaf and methanol extract of flower showed the best radical scavenging activities with an IC_{50} of 1.16 µg/ml and the ethanol extract of flower demonstrated an IC_{50} of 7.4 µg/ml, respectively. The results of this study showed that the *Achillea millefolium* possesses antioxidant and antitumoral activities and thus, can be considered as a source of natural antioxidant or a possible supplement in food industry or in pharmaceutical industry. More researches are needed to find the active components present in this plant and their mechanism of action.

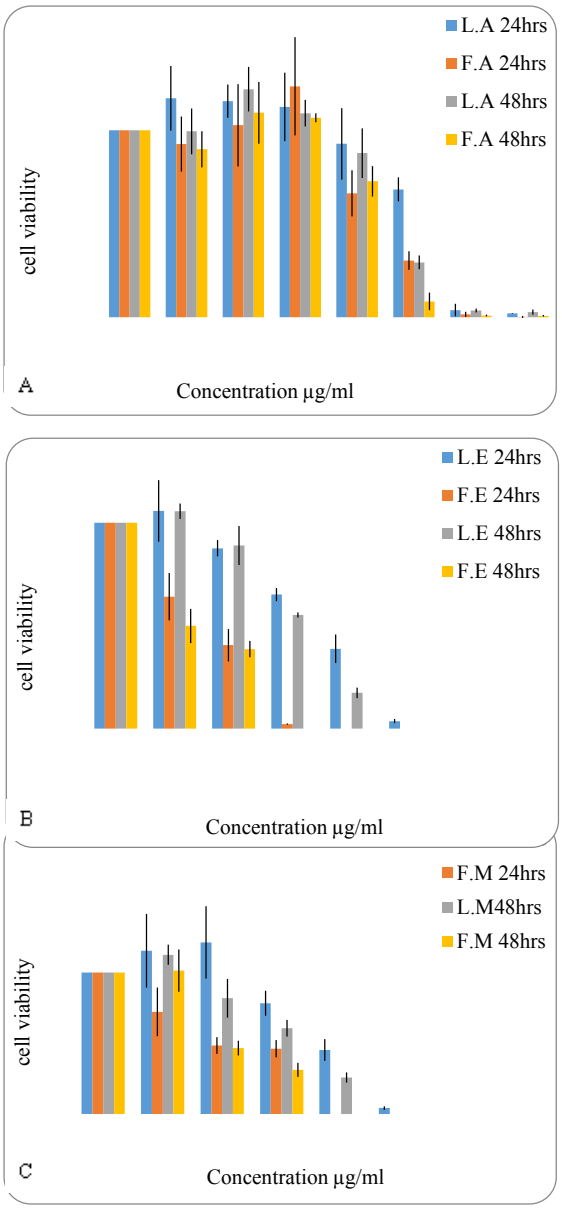

Figure 4. Comparative cytotoxic effects of the aqueous extract (A), ethanol extract (B) and methanol extract of *Achillea millefolium* leaves and flowers against MCF-7 cell line. L: leaf, F: flower, A: aqueous, M: methanol and E: ethanol.

Conflict of interest

The authors declared no conflict of interests.

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