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Original Article

Cytotoxic Effects of the Hydroalcoholic Extract of *Rorippa Nasturtium Aquaticum* on Hela Cell Line

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Regarding the high prevalence of cancer, research on medicinal plants have been increased due to their availability, cost, and the presence of anticancer compounds. *Rorippa nasturtium aquaticum*, also called watercress, is a plant from the Brassicaceae family which has large amounts of antioxidants. The present study aimed to examine the antioxidant properties and the phenolic, flavonoid, and anthocyanin contents of the watercress extract and its effect on the growth of cancerous Hela cells, and fibroblasts. Watercress extracts have been prepared through incubation and soxhlet methods in a hydroalcoholic solvent. The phenolic and flavonoid contents of the extract was determined through a spectrophotometric method. Anti-radical activity of the extract was examined by using the radical scavenging activity test of DPPH (2, 2-diphenyl-1 picrylhydrazil). The extract was applied on cancerous Hela cell line and fibroblasts at concentrations varying from 0.625 to 2 mg/ml and cell mortality rates were examined after 24, 48, and 72 h incubation using the MTT test. The extract obtained through the soxhlet method showed more antioxidant activity compared with the incubation method, and had more phenolic and flavonoid compounds. The survival rate of the cancerous Hela cells decreased with time and increasing concentrations of watercress extract. IC50 values after 24, 48 and 72 h were 373, 349, and 333 µg/ml, respectively. Based on the results of the study, the soxhlet method is better than the incubation method due to yielding more antioxidant and phenolic compounds. The hydroalcoholic extract of watercress can inhibit the growth of Hella cells and can be considered as an alternative for cervical cancer treatment in the future.

Keywords: Watercress, DPPH, cervical cancer, antioxidant activity, Hela cell line

Cervical cancer is the sixth most common type of cancer throughout the world, especially in developing countries, and the second most common type of cancer among women. Annually, around half a million women are diagnosed with invasive cervical cancer worldwide (1). Today, due to the high rate of cancer, using chemical drugs and radiation therapy is on the rise. The numerous side effects of these methods on the one hand, and their high costs on the other, have persuaded researchers to seek alternative methods to treat cancer. Recently, prevention and treatment of cancer using herbs has drawn the attention of many researchers (2). Use of herbal medicinal products in the treatment of disea-

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ses, especially cancer, is growing rapidly due to their availability, antioxidant content, and cost (3).

Watercress is a plant from the *Brassicaceae* family, which has been recommended throughout the history for the treatment and prevention of various diseases (4-5). Anticancer effects of the *Brassicaceae* and the active components in these plants have been widely examined both *in vitro* and *in vivo* and results of the researches suggest that the chemical agents extracted from the *Brassicaceae* have inhibitory effects on cancer at its initiation and progression phases, and the results of epidemiological studies and clinical experiments have confirmed these findings (6). Studies have shown that when the *Brassicaceae* plants are damaged, the glucosinolate released by the myrosinase enzyme is converted into isothioc-yanate, which has the ability to inhibit neoplastic effects in many cancerigenous factors. For this reason, the isothiocyanate present in *Brassicaceae* plants such as the watercress has been considered as an effective anticancer agent (7-8).

Due to the increasing prevalence of cervical cancer, the present study aimed to examine the antioxidant properties and the total content of phenolic, flavonoid, and anthocyanin compounds of the hydroalcoholic extract of watercress using two extraction methods of soxhlet and incubation, and studied its effect on growth inhibition of Hela cervical cell line and fibroblasts.

Materials and methods

Watercress extract preparation

Watercress plants were collected in Autumn 2015 from rice paddies of Babol-kenar area in Mazandaran province, Iran, and the plants were identified by herbology professors of Gilan University. The hydroalcoholic extract was extracted with two methods of soxhlet and incubation. In the soxhlet method, first the aerial part of the plant was powdered, and then 30 g of the powder was dissolved in 150 ml of 90% ethanol and 150 ml of distilled water. Then, extraction was performed using the soxhlet extraction device. In the incubation method, 10 g of the powder was dissolved in 50 ml of 90% ethanol and 50 ml of distilled water. The solution was, then, poured into a beaker and incubated by shaking for 48 h on 123 rpm. The extracts obtained from both methods were passed 3 times through normal filter papers in order to separate the solid particles. The hydroalcoholic extract obtained with soxhlet method was concentrated by evaporation after filtering. Then these extracts were stored at 4 °C.

Phenolic content evaluation

In order to measure the content of phenolic compounds of the extracts, the Folin-Ciocalteau method was used. (9) First, 250 µl of Folin-Ciocalteau and 1 ml of distilled water were added to 250 µl of each of the standards and extracts, and were mixed for 5 min. Then, 1 ml of sodium carbonate (35.7% Na2CO3) was added to the mixture. After an hour and 30 minutes, absorbance was measured at 760 nm. Distilled water was used in the blank solution and the standard curve was plotted using different concentrations (2.5, 1.125, 0.625, 0.312 mg/ml) of Gallic acid.

Flavonoid content evaluation

Flavonoids, the largest class of polyphenolic compounds, are good inhibitors of lipid oxidation. The color reagent in this method is aluminum chloride (10). To conduct this experiment, 250 µl of both hydroalcoholic watercress extracts, 750 µl of 90% ethanol, 50 µl of 10% aluminum chloride, 50 µl of 0.1% potassium acetate, and 1.4 ml of distilled water were mixed. After 30 min, absorbance was measured at 415 nm. The standard curve was plotted using different concentrations (1, 0.5, 0.125, 0.0625 mg/ml) of the quercetin solution.

Evaluation of DPPH free radical scavenging activity

In order to evaluate DPPH free radical scavenging, 8 mg of dried extracts obtained from the soxhlet and soaking methods were each brought to volume using 1000 ml of distilled water. Different concentrations (50, 100, 200, 400, and 800 mg/ml) of extracts were prepared and poured into a test tube

where 2 ml of DPPH solution was added. The solution was mixed and placed in darkness for 30 minutes, after which the absorption was measured at 517 nm frequency. The standard curve was plotted based on different concentrations (0.0312, 0.0625, 0.125, 0.25, 0.5, and 1 mg/ml) of quercetin and ascorbic acid solutions.

Cell culture

The Hela cell line was purchased from the cell bank of Pasteur Institute of Iran, and was placed in a culture medium containing RPMI 1640 supplemented with FBS 10% and penicillin/streptomycin 1% in an incubator under normal circumstances (37 °C temperature, containing 5% carbon dioxide, 95% moisture). Human dermal fibroblasts were enzymeaticcally extracted from foreskin of 1-1.5 month-old infants, and were placed in a culture medium containing RPMI 1640 supplemented with FBS 10% and penicillin/ streptomycin 1%, in a CO_2 incubator under normal circumstances. Cell passage was used to conduct the MTT test (11).

MTT test

The cytotoxic effect of the extract was determined using the MTT colorimetric method. In this method, the yellow MTT is transformed into the insoluble purple formazan compound by dehydrogenase enzymes found in mitochondria of active cells. The absorption of this compound is measurable at 570 nm after being dissolved (12).

10^4 cells from the Hela cell line and 8×10^3 human dermal fibroblast cells were seeded in each well of the 96-well plate (3 repeats for each concentration), and 24 h after cells adhesion to the bottom of the plate, 200 µl of different concentrations (0.0625, 0.125, 0.25, 0.5, 1, 2 mg/ml) of watercress hydroa-lcolic extract obtained through the soxhlet method was added to the wells in the culture medium. Then, the plates were placed in an incubator at 37 °C temperature, under 5% CO_2, 95% moisture. After 24, 48, and 72 h, the culture media containing the extracts were removed from the wells and 50 µl of 5 mg/ml MTT solution was added to each well. After 4 h, the MTT solution was removed and 200 µl of acidic isopropanol solution was added to each well in order to dissolve the formazan crystals and light absorption was measured at 570 nm by the ELISA reader (Ratio, China) device. Finally, using the following equation, cell survival rate was obtained for each concentration of the extract in comparison with the control group.

$$Viability\ \% = \frac{OD_{test} - OD_{blank}}{OD_{control}} \times 100$$

Where OD test is the light absorption of the test, OD blank is the light absorption of an empty well, OD control is the light absorption of a cell without the extract or with a concentration of 0 mg/ml. Viability % indicates the cell's viability rate in the test.

A concentration of the compound tested which decreases the viability rate to half is considered to be IC50 (the half maximal inhibitory concenteration).

Data analyzes

Statistical analyzes of the experimental results and the IC50 determination were performed using the SPSS software package. Analysis of variance (ANOVA) was performed to compare the results. The Tukey test was performed at the significance level of $P< 0.05$.

Results

Phenolic and flavonoid contents

The results show that phenolic and flavonoid contents of the hydroalcoholic extract of watercress are higher in the soxhlet extract. As shown in Table 1, the phenolic content in the hydroalcoholic extract of watercress obtained through the soxhlet method

Table 1. Phenolic and flavonoid contents in soxhlet and incubated extracts

Method	Incubation	Soxhlet
Phenolic content (mg/g)	0.96±16.80	0.61±23.53***
Flavonoid content (mg/g)	0.74±11.69	1.17±13.51

***: significance at P< 0.001

Table 2. Antioxidant activity of the soxhlet and incubated extracts of watercress

	Samples			Standards		
	Concentration mg/ml	Incubation	Soxhlet	Concentration mg/ml	Ascorbic acid	Quercetin
$IC_{50(\mu g.ml)}$	0.5	25.668	21.568	0.062	10.090	46.345
	1	37.789	26.916	0.125	62.714	55.814
	2	53.028	41.176	0.25	74.024	67.201
	4	78.074	50.802	0.5	77.718	80.213
	8	82.328	64.884	1	80.679	86.274
		105.20±2.28	108.68±5.41		7.07±0.33	5.22±0.63

is significantly higher than that of the extract obtained through the incubation method ($P< 0.001$). The flavonoid content in the soxhlet extract was also higher. However, the difference was not significant.

DPPH free radical scavenging activity

The results demonstrated a higher antioxidant activity rate of the extract obtained through the soxhlet method compared with the extract obtained through the incubation method. As shown in Table 2, the IC50 value in the hydroalcoholic extract of watercress was 105.20± 2.28 µg/ml for the soxhlet extract and 108.68± 5.41 µg/ml for the incubated extract. Statistical analysis show that the difference between the two values is significant ($P= 0.001$). Based on the results, the DPPH free radical scavenging activity of the soxhlet extract was higher than the incubated extract.

Cytotoxic effect of watercress extracts on cell viability

Cytotoxicity tests revealed a time- and concentration-dependent cell survival decrease of the cancerous Hela cells under the influence of the soxhlet hydroalcoholic extract of watercress. As shown in Figure 1, with the increase of the extract concentration, the survival rate of the cells decreased. Similarly, there was a decrease in dermal fibroblasts survival rate with increasing concentrations of the extract (Figure 2).

Anti-proliferative activities of the hydro-alcoholic extract of *Rorippa nasturtium aquaticum* were determined on both Hela cell line and fibroblasts after 24, 48 and 72 h. Dose and time-dependent effects of extract were observed on Hela cell line (Figure 3). There was a significant decrease

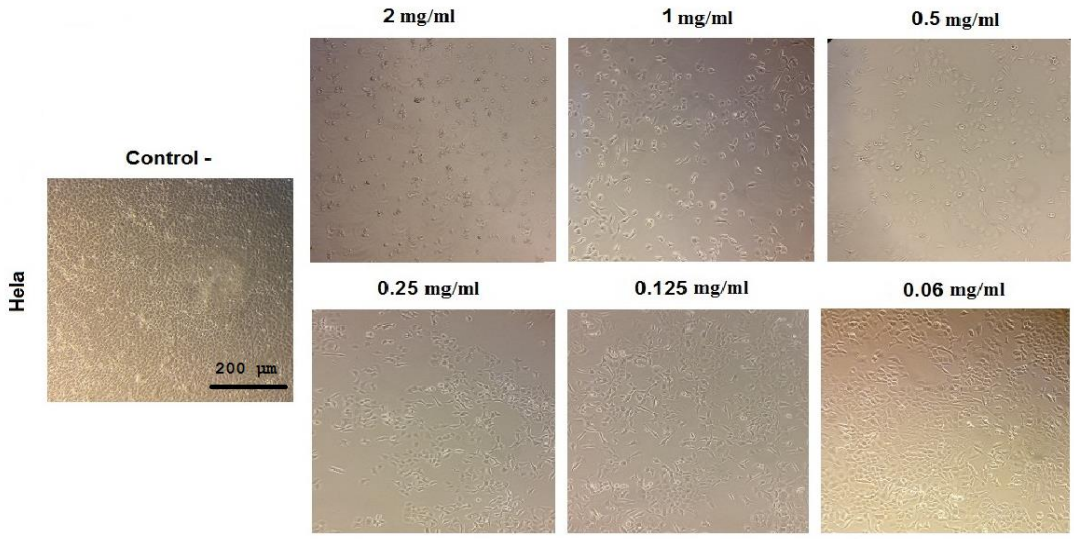

Figure 1. Survival of Hela cells under the watercress extract influence. Cells survival rate decreased gradually with extract concentration increase.

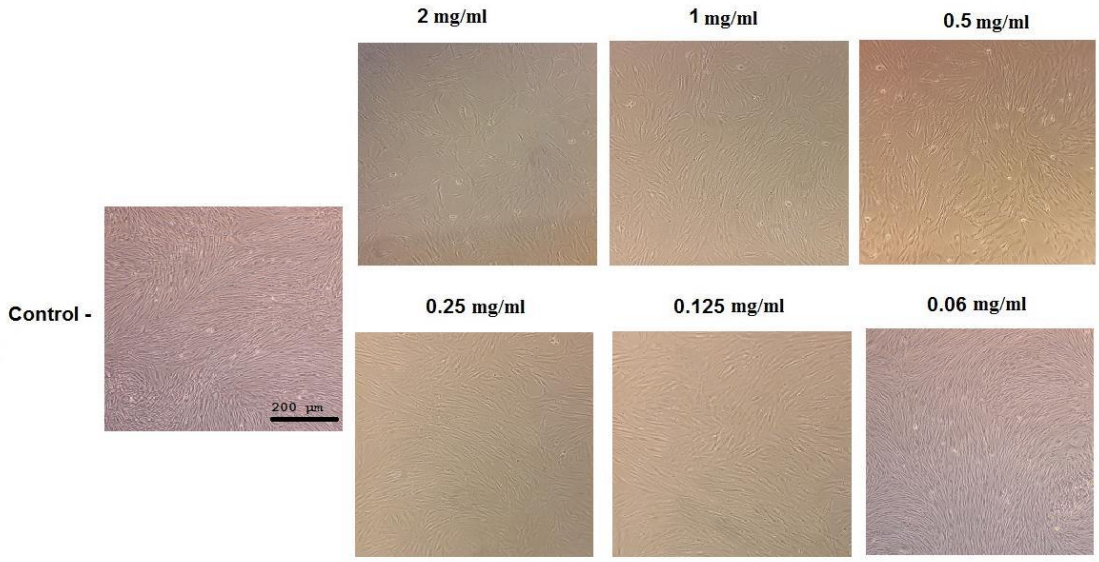

Figure 2. Survival of fibroblasts under the watercress extract influence. Cells survival rate decreased gradually with extract concentration increase.

in Hela cells viability between 62.5 µg/ml and 125 µg/ml ($P< 0.01$) and between 250 µg/ml and up to 2 mg/ml ($P< 0.001$). Moreover IC50 values after 24, 48 and 72 h were 373, 349, and 333 µg/ml, respectively. Similarly, fibroblasts showed significant cell viability decrease after 24, 48 and 72 h (Figure 4).

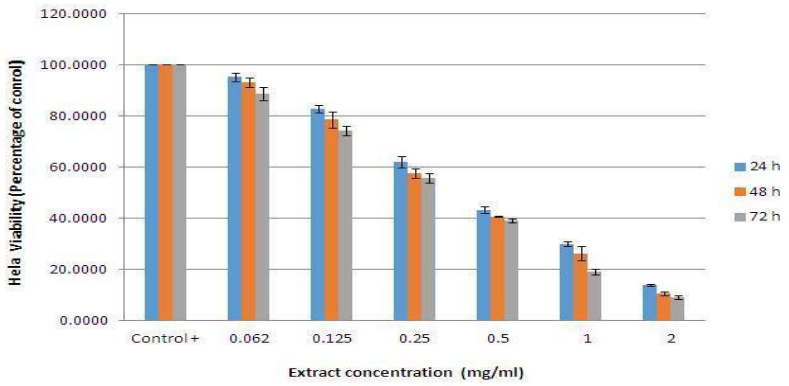

Figure 3. Hela cells survival rate according to concentrations and duration of extract application.

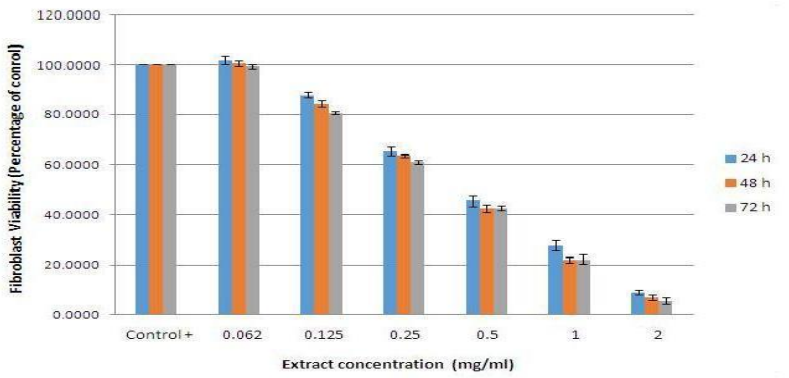

Figure 4. Fibroblasts survival rate according to concentrations and duration of extract application.

Discussion

Regarding adverse effects of conventional cervical cancer treatment methods on human body, such as blood cells decrease, hair loss, and menstrual and pregnancy disorders, the need for a low-risk, safe, and inexpensive alternative with the highest effectiveness and the lowest amount of side effects is strongly felt. Cisplatin is an of widely used drug in the treatment of this cancer which causes adverse side effects such as anemia, febrile neautropenia, gastrointestinal effects such as nausea and vomiting, infertility, and neurological effects (13). Given the high prevalence of cancer in the world and the increase in marriage and pregnancy age, using the above-mentioned method in the treatment of cervical cancer in pregnant women or those who had not yet become pregnant can have adverse effects on maternal and fetal health.

Watercress is a plant from the *Brassicaceae* family and substantial researches has been conducted on its therapeutic effects. For example, Hecht et al. showed that a compound named ethyl benzene cyanide iso-sulfate is found in the extract of watercress which can inhibit the enzymatic activity caused by carcinogens such as cigarette smoke in lung cancer and can contribute to the treatment of this cancer (14). It was shown that the extract of this plant contains materials such as beta-carotene, ascorbic acid, calcium, folic acid, iron, iodine, phosphorous, and amino acids such as arginine, glycine, lysine, and tryptophan (15). Researches conducted on rats demonstrated that the antioxidant property of the hydroalcoholic extract of watercress can reduce the infertility effect caused by the consumption of doxorubicin and improve ovulation in (16). Examining the genotoxic effect of watercress extract on peripheral blood lymphocytes showed that the extract has no cell toxicity and presents normal genotoxicity (17). The methanolic extract of watercress showed anticancer properties on T47D (breast cancer) and HT-29 (colon cancer) cell lines (18). Similarly, the hydroalcoholic extract of watercress caused a dose dependent survival decrease in the breast cancer cell line (MDA-MB-231) while no significant effect was observed on normal cell line (HF2FF) (19).

The present study investigated for the first time the effects of the hydroalcoholic extract of watercress on Hela cell line. Conducting DPPH, flavonoid, and Folin-Ciocalteu tests, large amounts of antioxidant, phenolic, and flavonoid compounds, which are important compounds in cancer treatment, were found in the extract. The results of the study showed that the hydroalcoholic extract of watercress obtained through the soxhlet method compared to the incubation method has more antioxidant, phenolic, and flavonoid compounds and is more suitable for future experiments.

The effectiveness of the hydroalcoholic extract of watercress at different doses and durations on the cervical cancer cell line (Hela) and dermal fibroblasts was evaluated, and a dose dependent decrease of the cell survival rate of the cancerous cell line was noticed.

Since watercress is readily and inexpensively available and contains large amounts of antioxidant, phenolic, and flavonoid compounds with anticancer properties and desirable effectiveness against cancerous cells, it can be considered as a legit alternative for cervical cancer treatment in the future.

Conflict of interest

The authors declared no conflict of interest.

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