



Comparison of Anticancer Effects of Hydroalcoholic Extracts of *Camellia sinensis* and *Lepidium sativum* L on HeLa Cell Line

Somayeh Jahani¹, Zahra Heidari^{1,2,*}, Mehdi Azami³ and Bita Moudi^{1,2}

¹Infectious Diseases and Tropical Medicine Research Center, Resistant Tuberculosis Institute, Zahedan University of Medical Sciences, Zahedan, Iran

²Department of Histology, School of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran

³Skin Diseases and Leishmaniasis Research Center, Isfahan University of Medical Sciences, Isfahan, Iran

*Corresponding author: Department of Histology, School of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran. Email: histology_iri@yahoo.com

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Abstract

Background: The antioxidative activity of green tea and garden cress extract is of interest in cancer.

Objectives: The current study aimed at evaluating the effect of hydroalcoholic extracts of *Lepidium sativum* (cress) and *Camellia sinensis* (green tea) on the culture medium of the HeLa cell line.

Methods: Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) was used to culture HeLa cells, which was exposed to the different concentrations of green tea and cress extracts at 24 hours and 48 hours. Cell viability and apoptotic cells were quantified by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT assay and propidium iodide, respectively.

Results: The highest percentage of growth inhibition (85%) was observed at 100 $\mu\text{g}/\text{mL}$ of the green tea extract after 48-hour treatment. The percentage of growth inhibition at 24 h after treatment was 83% for green tea ($P > 0.05$). The high growth inhibition percentage of HeLa cells at 100 $\mu\text{g}/\text{mL}$ of cress extract at 24 hours and 48 hours (49.8%) after treatment was 27.92% and 49.8%, respectively ($P > 0.05$). Additionally, the cell apoptosis assay indicated that green tea and cress extracts had toxic effects on the HeLa cells. This effect was highest at the concentration of 100 $\mu\text{g}/\text{mL}$ and more evident in green tea.

Conclusions: It can be concluded that green tea extract compared with cress had a more cytotoxic effect against cervical cancer.

Keywords: *Camellia sinensis*, *Lepidium sativum*, Uterine Cervical Neoplasm, HeLa Cell

1. Background

Herbal remedies are widely used in all areas of the world. Unlike the widespread use of these plants, there is little information about the safety and effectiveness of herbal remedies. Cancer is a serious disease that has a significant role in the mortality of humans. The death incidence rate from cancer was about 8.2 million people in 2012. Among the various types of cancer, more deaths arise from cancer of the stomach, lung, colon, liver, and breast. The rate of cancer-related deaths is increasing in the future and it is estimated that in the 2030s, approximately 12 million people worldwide lose their lives due to cancer (1-3). Almost 30% of cancer-related deaths could be inhibited and by controlling and harnessing carcinogens a lot of cancers can be prevented. Tobacco, poor diet, high body mass index, exposure to infectious agents, and ionizing radiation increase cancer incidence rates (1).

Cervical cancer is the fourth most common cancer in the world. It is created by increasing and irregular growth

of vaginal epithelial cells and continuous loss of cells is ongoing. The most well-known cause of this cancer is the human papillomavirus (HPV). In 2012, approximately 270 000 women worldwide died from cervical cancer and 85% of these deaths occurred in developing or underdeveloped countries (4). Surgery, chemotherapy, radiotherapy, and immunotherapy are the main methods of cervical cancer treatment. Most chemical treatments can cause various side effects, including decreased appetite, weight change, mouth or throat irritation, problems with teeth and gums, nausea and vomiting, depression, and fatigue. High incidence of cancer and ineffective chemical treatments show the need for access to new medicines and natural substances that have fewer side effects (2).

Studies have shown that plants, vegetables, and herbs can be effective as a resource for cancer prevention. Food also plays an important role in reducing the progression of cancer. The use of medicinal plants and traditional medicine has a long history in many countries, including Iran. Nowadays, using medicinal plants in the treatment of

various diseases is increasing due to better compatibility with human body physiology and having fewer side effects (5-11). Most plants and herbs contain substances to prevent cancer, which can exert their effects in different stages of the growth of cancer cells. The main purposes of cancer prevention by natural or chemical materials are slowing down or inhibiting the carcinogenic process. These approaches have been focused on the abnormal intracellular pathways that lead to abnormal cell functions (3).

Cell culture is one of the modern methods in study and research and is almost involved in all scientific fields. One of the main goals of cell culture is the study of the cells' growth, the need for food, and the inhibitors of the growth. Therefore, the need for cell culture outside the body is important and helps us to develop controlling methods for the cell cycle, cancer cell growth, and modulating gene expression (12).

Green tea is a popular drink in the world. It comes from the dried *Camellia sinensis* fresh leaves. It belongs to the Theaceae family. Green tea leaves are thermogenic, appetizer, digestive, carminative, and diuretic (13). Also, polyphenols of green tea have anticancer properties in humans. It has shown that green tea leaves are useful in the treatment of cancer of the duodenum, lung, liver, and breast (13). Moreover, it has been proposed that dietary agents, such as epigallocatechin gallate in green tea, can cause apoptosis and change the cell cycle process in malignant cells but not healthy cells (14, 15). Green tea contains important and active catechins, which have been identified as active components responsible for the antioxidant property (16). Epicatechins can remove free radicals and act as electron donors inside the cells. The antioxidative and anti-peroxidative effects of epicatechins are induced through enzymatic and non-enzymatic mechanisms, which have protective effects on the cells (17). Free radicals can induce the oxidation of nucleic acids and lipid peroxidation and destroy the biomembranes, which lead to changes in cell function and cancer incidence. Catechins are protective compounds against diseases by contributing along with antioxidant vitamin E and enzyme-like superoxide dismutase, catalase to the total antioxidant defense system (18).

Cress or *Lepidium sativum* L (*L. sativum*) is a plant from the Brassicaceae family, which has antioxidant ingredients with protective effects against cancers. Active ingredients of cress can control the apoptosis process in malignant cells, eliminate diseased cells, and maintain the survival of healthy cells (19, 20). As an important feature of this plant, it has phytochemicals such as phenolic compounds, terpenoids, alkaloids, and organosulfur compounds, which can treat and/or prevent cancer (21). Besides, phytosterols in cress can induce protection against cancer via antioxi-

dant and anti-inflammatory activities (22).

2. Objectives

The main goal of the current study was to examine the effect of *Camellia sinensis* and *L. sativum* as powerful antioxidants against cancer cells and comparing their effects. The present study investigated the anticancer effects of green tea and cress on the HeLa cell line and determined that which of them were more effective in the prevention of growth of the HeLa cell line.

3. Methods

3.1. Preparation of Extracts

The leaves of the desired plants were purchased from the Institute of Medicinal Plants of Karaj and were air dried and grounded to a fine powder. The maceration technique was used to preserve the original content of the plant. Firstly, 50 g of dried powder was prepared from each plant, put inside the bags, and transported to 2 separate Erlenmeyer flasks. To each flask, approximately 1500 mL of a solution containing water/Ethanol 96% with a concentration of 1:1 was added. The flasks were packed with aluminum foil and put in the shaker with 90 rpm for 48 hours. The solutions were, then, separated by filter paper. Finally, the solvent was removed from the extracts, using a rotary apparatus. The extracts were kept in the refrigerator in sterile containers until the experiment.

3.2. Cell Lines and Culture Medium

HeLa cells were purchased from Iran University of Medical Sciences (IUMS) and cultivated in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Cells were maintained in the following conditions: the temperature was 37°C with 5% CO₂ and airflow was 95% with 100% relative humidity. The passage was done weekly by 2 changing the culture medium every week (23).

3.3. Treatment of Cell Line

Initially, monolayer cells were changed to a suspension medium containing single cells using trypsin-Ethylenediaminetetraacetic acid (EDTA). Cell viability was measured by a hemocytometer and a solution consisting of 1×10^5 cells/mL produced in the 5% FBS. Cells were seeded in 96-well plates. In each well, 100 μ L of suspension containing 10 000 cells was placed. After 24 hours and the cells' attachment to the plates, methanolic extracts of *Camellia sinensis* or *Lepidium sativum* were added to the plates with serial concentrations (1, 10, 50, 100 μ g/mL). Plates were incubated for 48 hours. Cell-free media were used as controls and each test was repeated 3 times for each concentration (23).

3.4. Cell Viability Assay

MTT assay protocol was used to determine cell viability (23). Initially, a suspension containing 7×10^3 cells/well were seeded into the flat-bottom 96-well culture plates. After 24 hours, different concentrations (1, 5, 10, 50, 100 $\mu\text{g}/\text{mL}$) of extracts were added. Then, the medium was removed and MTT solution containing 5 mg/mL in PBS was added. After 4 hours, formazan dissolved in 100 μL of dimethyl sulfoxide (DMSO). An enzyme-bound immunosorbent analysis (ELISA) reader was used to measure the absorption at 570 nm; 620 nm was referred to as a reference. All tests were repeated 3 times. Finally, the percentage of cellular inhibition was calculated by the following formula (24):

$$\text{Cell inhibition (\%)} = 100 - \frac{\text{Absorption (Sample)}}{\text{Absorption (Control)}} \times 100$$

3.5. Cell Apoptosis Assay

The apoptosis process was evaluated by the Annexin V–FITC/PI staining kit (Miltenyi). Cells were treated in 24-well plates for 24 hours and 48 hours at 1×10^5 cells/mL cell density. The cells were, then, transferred to polypropylene tubes and centrifuged ($500 \times g$, 5 min). The precipitate was washed with binding buffer and centrifuged ($300 \times g$, 10 min). The washing step was repeated. The cell suspension was prepared in binding buffer and, then, the staining process was performed, using Annexin V-FITC (1 μL , 25 $\mu\text{g}/\text{mL}$) and propidium iodide (PI) (5 μL ; 250 $\mu\text{g}/\text{mL}$). Finally, the samples were evaluated as soon as possible by flow cytometry.

3.6. Statistical Analysis

Statistical calculations were performed by SPSS (Version 16). In the current study, all tests were repeated 3 times and the results were reported as the mean of these repetitions \pm standard deviation (SD). Data were analyzed, using ANOVA and t test. $P < 0.05$ was considered statistically significant. Non-linear regression and graph pad prism software was used to show cell inhibition and half-maximal inhibitory concentration (IC_{50}).

4. Results

4.1. In Vitro Cytotoxic Activity

Table 1 shows the effects of different concentrations of extracts on growth inhibition of HeLa cells.

Figure 1 shows the cytotoxic curves from the MTT assay, depicting the growth inhibition of HeLa cells grown for 48 hours in the presence of increasing the concentrations of extracts.

The hydroalcoholic extract of green tea exhibited a significant cytotoxic effect against HeLa cells in the first and second days, IC_{50} ; 81 $\mu\text{g}/\text{mL}$ and 43 $\mu\text{g}/\text{mL}$, respectively. The hydroalcoholic extract of cress did not show active cytotoxic activity against the HeLa cell lines on the first day ($\text{IC}_{50} > 100 \mu\text{g}/\text{mL}$) and revealed a mild cytotoxic effect screened against HeLa cells on the second day ($\text{IC}_{50} > 100 \mu\text{g}/\text{mL}$) (Table 1 and Figure 1).

As shown in Table 1, the growth inhibition percentage of cells treated with green tea extract was increased with increasing concentrations of green tea extract. The highest percentage of growth inhibition (85%) was observed at a concentration of 100 $\mu\text{g}/\text{mL}$ at 48 hours after treatment with the green tea extract. Statistically, the percentage of growth inhibition at 24 hours after treatment (83%) with green tea was not significantly different ($P > 0.05$). The results showed that green tea extract increased the growth inhibition percentage of cells in a dose-dependent manner (Table 1).

As shown in Table 1, the growth inhibition percentage of cells treated with cress extract was increased with incubation time and increasing concentrations of cress extract. The highest growth inhibition percentage of the cell (49.8%) was observed at a concentration of 100 $\mu\text{g}/\text{mL}$ at 48 hours after the treatment with the cress extract; the percentage of growth inhibition at 24 hours after the treatment (27.92%) with cress extract was significantly different ($P > 0.05$). The results showed that cress extract increased the growth inhibition percentage of cells in a time- and dose-dependent manner (Table 1).

The results of t test indicate a statistically significant difference between the total results of cell growth inhibition of various concentrations of the extracts of green tea and cress against HeLa cell lines ($P < 0.05$).

4.2. Microscopic Characterization of Nuclear Morphology Upon Plant Extracts Treatment

Significant morphological changes and cell membrane damage occurred during 48 hours incubation with 100 $\mu\text{g}/\text{mL}$ of both extracts on the HeLa cells. The control cell has normal morphology. The extracts cause the lost normal shape and show a shrunken cytoplasm and condensed chromatin. These changes have been shown in Figure 2 for green tea.

4.3. Analysis of Apoptotic Cells as a Measure of Membrane Changes by AnnexinV-FITC

After treatment with the increasing concentrations of the *C. sinensis* and *L. sativum* extracts for 48 hours, AnnexinV-FITC and PI were used for staining the cells. As shown in Figure 3A related to the Fluorescence Associated

Table 1. In Vitro Cytotoxicity of *C. sinensis* and *L. sativum* on HeLa Cell Lines ^a

Plant Extract Concentration ($\mu\text{g/mL}$)	Mean of Inhibition Percentage		IC ₅₀ ($\mu\text{g/mL}$)		Mean of the Total Inhibition Percentage
	1st Day	2nd Day	1st Day	2nd Day	
<i>Camellia sinensis</i>			81	43	
1	19.68 \pm 2.39	20.1 \pm 1.85			19.89
10	21 \pm 2.64	24 \pm 2			22.5
50	25.38 \pm 1.90	55.58 \pm 3.66			40.48
100	82.52 \pm 3.36	85.13 \pm 2.68			83.82
<i>Lepidium sativum</i>			-	100	
1	2.3 \pm 1.1	8.3 \pm 1.5			5.3
10	5.6 \pm 1.3	19.7 \pm 2.7			12.65
50	14 \pm 1.52	42.7 \pm 2.5			28.35
100	27.92 \pm 1.8	49.8 \pm 4.03			38.86

Abbreviation: IC₅₀: half maximal inhibitory concentration.

^aThe results are shown by the mean of 3 independent repetitions of the tests \pm standard deviation.

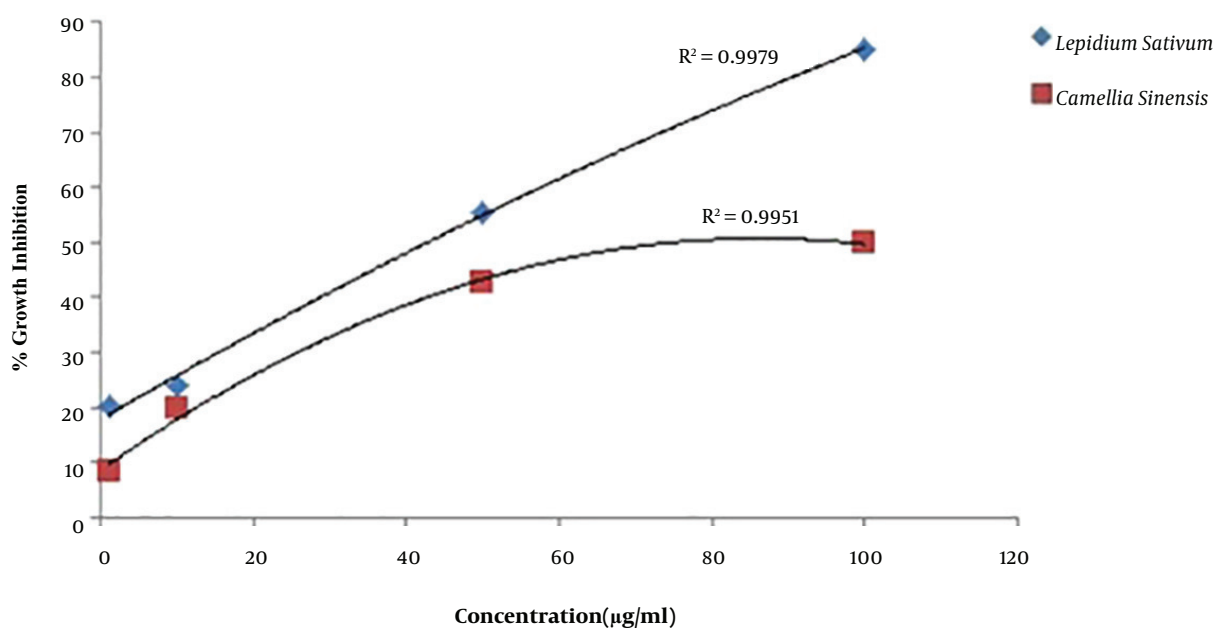


Figure 1. Representative graph of HeLa cells growth inhibition after 48 hours of cell growth in the presence of increasing concentrations of investigated extracts.

Cell Sorter Scan (FACSscan), control untreated cells were viable with no apoptosis. Applying different concentrations of these two extracts resulted in 4 states in the cells: Q1) PI⁺ related to the dead cell, Q2) AnnexinV-FITC⁺/PI⁺ related to the late apoptosis or necrosis in cells, Q3) AnnexinV-FITC⁻/PI⁻ related to the normal cells, Q4) AnnexinV-FITC⁺/PI⁻ related to the early apoptosis in cells. The percentage apoptosis of 11%, 22.43, 37.7%, and 46.9% at a concentration of 1, 10, 50, and 100 $\mu\text{g/mL}$ of *L. sativum* extract was observed in

HeLa cell (Figure 3B to Figure 3E). As demonstrated in Figure 3, it was clear that the sensitivity to *C. Sinensis* in HeLa cells was significantly more than *L. sativum* in these cells.

It is interesting to note the green tea with concentration (10 $\mu\text{g/mL}$), as shown in Figure 3G, that the most prominent cellular state was early apoptotic phenotype (56.4%) and late apoptosis/necrotic mode (5.5%). With increasing concentration and in a dose-dependent manner (20.1% and 65.9% for 50 and 100 $\mu\text{g/mL}$, respectively), most

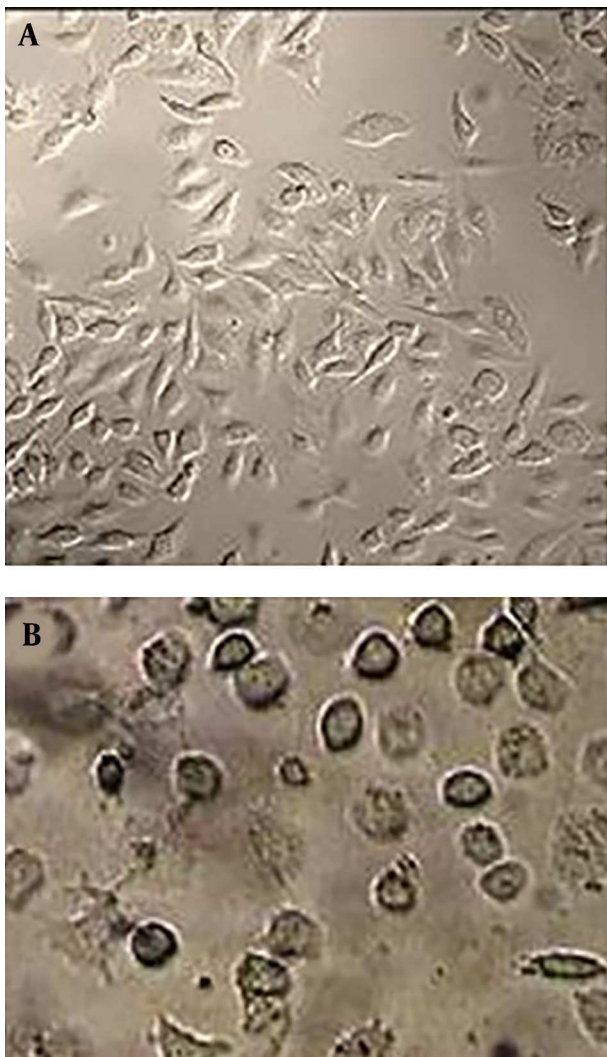


Figure 2. Light microscopy of HeLa cells cultured with or without the 100 $\mu\text{g}/\text{mL}$ of extract: (A) control; (B) *Camellia sinensis* 48 hours after the addition of drug (Magnification 40X).

cells appeared in quadrant 2, which indicates a late apoptotic or necrotic population (Figures 3H and Figures 3D).

5. Discussion

Many herbs and spices have pharmacological and biochemical properties, including antioxidant and anti-inflammatory effects and it seems that the plants are involved in anti-mutagenic and anti-cell malignancy activities. Given that cancer is closely associated with inflammation and oxidative stress, the compounds with antioxidant and anti-inflammatory properties can be an anti-cell malignancy. Nowadays, it is easy to study the behavior and

performance of cancer cells, using cell culture in simplified and controlled environments. In this method, we can organize the conditions of the experiment, which leads to the discovery of anticancer drugs (25).

Due to its antioxidant polyphenols, green tea has attracted the attention of scientists. Antioxidant effects can protect the cells against cancer; therefore, the evaluation of green tea extract on cell lines can be useful in cancer treatment (26).

This investigation is the first study that compares the effects of *Lepidium Sativum* L and *Camellia sinensis* extracts on the cervical cancer cell line. In the current study, we have analyzed cytotoxic and anti-proliferative effects of hydroalcoholic extracts of *Lepidium sativum* L and *Camellia sinensis* on the HeLa cell line. We have found a dose- and time-dependent cytotoxic effect for green tea against the HeLa cell line. The HeLa cell line was more sensitive to the green tea extract than cress extract. The cytotoxic effect of the cress on the cancer cell line was milder.

In this study, the effect of different concentrations of extracts was evaluated on the HeLa cell line, using MTT assay and PI staining. The results showed that the IC_{50} of both extracts on the HeLa cell line was 100 mg/mL, which significantly reduced cell proliferation.

Also, in a study conducted by Spavieri et al. (27), the IC_{50} of green tea extract was 100 mg/mL. Therefore, the hydroalcoholic extract of green tea leaves reduces the growth of the HeLa cell line in a time- and dose-dependent manner. It can be revealed that green tea extract compared with cress had a more protective effect against cervical cancer. Also, green tea has potential anti-proliferative power against cervical cancer.

The leaves of *Camellia sinensis* also have antibacterial activity and are used to treat asthma, angina pectoris, peripheral vascular disease, and coronary artery disease. Besides, *Camellia sinensis* has become interested in the production of organic food and the prevention of bacterial infections. In this regard, Moghbel et al. revealed that green tea extract was useful against halitosis infection induced by bacteria (28).

Moreover, Roomi et al. (29) found that green tea extract could inhibit matrix metalloproteinases (MMP-2 and MMP-9) expression in the HeLa cell line in a dose-dependent manner, which led to the inhibition of matrix invasion. The improvement of the strength of the connective tissue can control the matrix invasion, which involves the encapsulation of the tumor. Green tea probably enhanced the stability of the connective tissue via the synthesis of collagen fibrils. In other words, they suggested that green tea extract has a potential effect on the treatment of cervical cancer by inhibiting cancer development and spread. According to the results of this study, it can be concluded that

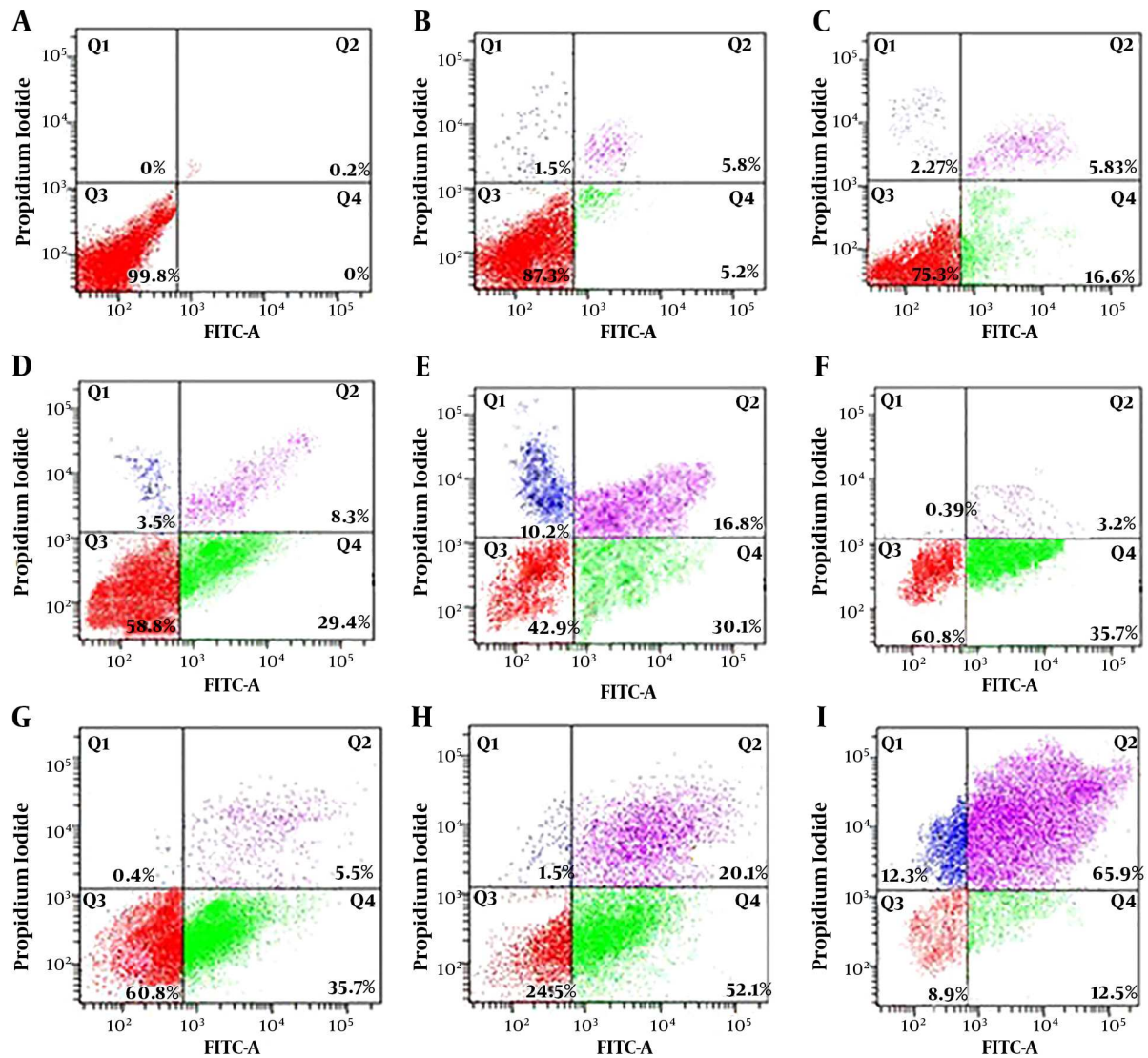


Figure 3. Induction of apoptosis by *C. sinensis* and *L. sativum* extracts and confirmation by AnnexinV-FITC/PI dual staining. HeLa cells were treated with 1 μg/mL (B), 10 μg/mL (C), 50 μg/mL (D), and 100 μg/mL (E) of *L. sativum* extract for 48 hours. HeLa cells were treated with 1 μg/mL (F), 10 μg/mL (G), 50 μg/mL (H), and 100 μg/mL (I) of *C. sinensis* extract for 48 hours. A represents experiment controls of untreated cells alone. Normal healthy, early apoptotic, late apoptotic, and dead/necrotic cell populations are shown as a percentage of total cells in the quadrants Q3, Q4, Q2, and Q1, respectively.

the hydroalcoholic extract of green tea can effectively prevent cervical cancer.

In this study, *Lepidium sativum* was also investigated in vitro to assess its cytotoxic effect against cervical cancer. Considering the results, it can be understood that the hydroalcoholic extract of cress has a cytotoxic effect on the HeLa cell line. Kutacek et al. (30) reported a high rate of cytotoxicity in a low concentration of cress extract in combination with antioxidant compounds such as vitamins and glucosinolates. In other words, phytochemi-

cals in cress can act as antioxidants and induce cytotoxic effects on cancer cell lines. In this regard, the current findings demonstrated the occurrence of apoptosis and necrosis in the HeLa cell line following the addition of the cress extract. Maximum cytotoxic effects were induced when cells were treated with 100 μg/mL of cress extract. At this concentration, the majority of cells died, and severe morphological changes were observed in them after 48 hours of exposure to the cress extract. This is in contrast to the 100 μg/mL concentrations of green tea, where maximum

cytotoxic effects were observed after 24-hour and also 48-hour, which were not significantly different. It means that the observed inhibition by green tea was more effective than cress, the green tea and cress reduced cell viability by approximately 85%, 49.8%, respectively. Our results confirmed that green tea possessed more potent antiproliferative properties against the HeLa cell line compare to the cress. This study indicates that green tea is a more effective therapeutic anti-carcinogenic agent against human cervical cancer.

5.1. Conclusions

The findings of the present study revealed that green tea has better anti-proliferative properties than cress. Moreover, to support the hypothesis that hydroalcoholic extracts of *Lepidium sativum* L and *Camellia sinensis* might have a preventive role in cancer, some animal models and randomized controlled trials are needed. An important goal of studies can be an investigation of the synergistic anticancer effect of green tea, cress, and vitamin E on several human cancer cell lines, which probably may lead to the production of a plant extract jelly that can be useful for different medical purposes. As another suggestion for future studies, the research can be continued by using nanotechnology methods.

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Footnotes

Authors' Contribution: ZH and MA were responsible for the experimental design of the study; MA and SJ were responsible for the execution techniques and examination. SJ and BM were responsible for the statistical analysis and drafting of the manuscript. All authors reviewed and contributed to the writing of this manuscript and its final approval.

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Ethical Approval: Ethical approval was obtained from the Ethics Committee of Zahedan University of Medical Sciences (IR.ZAUMS.REC.1394.451).

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