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Evaluation and Comparison of Antimicrobial and Anticancer Effects of Aqueous and Ethanolic Extracts of Viola Odorata

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Introduction: According to studies conducted on Viola odorata, many medical properties, including antimicrobial, anti-cancer, antioxidant, anti-inflammatory, etc. have been mentioned. Recent results have shown the strong cytotoxic effects of Viola odorata cycloidal compounds on different cancerous cell lines and bacteria. In addition, pathogen resistance has necessitated the study of new antimicrobial compounds. This study attempted to evaluate the antimicrobial and anticancer effects of aqueous and alcoholic extracts of Viola odorata.

Methods: Aqueous and alcoholic extracts of Viola were prepared using maceration method. After culturing Gram-positive Staphylococcus aureus and Gram-negative Escherichia coli, the minimum lethal concentration was measured by minimum bactericidal concentration (MBC) method, and the minimum inhibitory concentration of different treatments was measured by minimum inhibitory concentration (MIC) method. To evaluate the cytotoxicity, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed on human foreskin fibroblasts (HFF) and A549 cells. The obtained data were statistically analyzed, and the results were considered significant with a $P<0.05$.

Results: The results showed antimicrobial effects of Viola extract against tested bacteria, MIC 25mg/ml and MBC 50mg/ml in Escherichia coli, and MIC 50mg/ml and MBC 100mg/ml in Staphylococcus aureus were obtained. MTT test results showed that the cytotoxicity of ethanolic extract in both cell lines was higher than the aqueous extract. Moreover, the concentration of ethanolic extracts at 1000 and 1500mg/ml in A549 and HFF cell lines reduced the viability to 50%.

Conclusions: Aqueous and ethanolic extracts of Viola have an inhibitory effect on Gram-negative and positive bacteria and cancer cell proliferation. The lower side effects of Viola aqueous extract (VOA)/Viola ethanolic extract (VOE) on normal cells (HFF) have indicated that it can be considered as candidate for further studies in the field of new drug production.

INTRODUCTION

Viola odorata are very important in traditional medicine, and all parts of Viola odorata, including leaves, roots, flowers and seeds, have medicinal uses. Anti-inflammatory, analgesic, anti-rheumatic, anti-tumor, laxative and diuretic traits are among the properties of Viola [1, 2]. In traditional medicine, Viola odorata is used to treat and relieve ailments such as colds, pneumonia, flu and other hot flashes such as fever, sore throat, pleurisy, pneumonia, colds and lung infections. The medicinal properties of this plant are related to its secondary compounds and active ingredients, including flavonoids, glycosides, peptides, alkaloids, steroids, saponins and its tannins [3, 4]. Viola cyclotides (check the spelling of this word) play a vital role in defending against pathogens. They have also exhibited antimicrobial, anticancer and insecticidal properties in other research work. Staphylococcus aureus is one of the most important species of Staphylococcus medically [5]. This Gram-positive bacterium has been reported as a pathogenic bacterium in most studies because it causes a wide range of infections from simple skin infections such as pimples, boils to more threatening infections such as pneumonia, meningitis, osteomyelitis, endocarditis, etc. [6]. Since this bacterium has a flexible genome and its pathogenic and drug-resistant strains are expanding, we have seen an increase in studies on this bacterium in recent years [7]. Escherichia coli Gram-negative bacteria are also considered as pathogenic Gram-negative strains. Although this bacterium is part of the normal microbial flora of healthy people, it is also an opportunistic pathogen. Having resistance to common antibiotics, it is considered as a cause of urinary and gastrointestinal infections, neonatal meningitis, and diarrhea. In addition to studying the antimicrobial effects of Viola odorata, other therapeutic aspects of Viola odorata have also received much attention. Its analgesic and sedative properties along with anti-tumor effects have led to the use of this plant in the prevention and treatment of cancer, especially gastrointestinal cancers and metastases after tumor surgery [8].

This study intended to investigate the lethal and inhibitory effects of Viola aqueous extract (VOA) and Viola alcoholic extracts (VOE) on Gram-positive Staphylococcus aureus and Gram-negative Escherichia coli. Anti-cancer properties of the extracts were also evaluated in lung cancer cells (A549) and skin fibroblasts (HFF). It was expected that the difference between the compounds in VOA and VOE would lead to the difference in the antitumor and antibacterial effects.

METHODS

Preparation of Viola Extract

In the present experimental study, Viola flower (Viola odorata) was prepared from Kimia Medicinal Plants Preparation Center (Tehran Province). Extraction of aerial parts of the plant was performed by maceration method (soaking in solvent at room temperature). For this purpose, 100 grams of Viola odorata were weighed and extracted separately in two solvents, water and ethanol. Extraction was performed in a period of 5 days with a solvent to tissue ratio of 3:1. The obtained extract was then separated by filtration method. The clear liquid was decanted off, and the sediment was re-filtered through a high-purity filter paper, and then dried at 45°C. The extracts were stored in sterile plates in a freezer (-20°C) for various stages of the experiment.

Cultivation and Preparation of Bacterial Samples

Bacterial strains of Escherichia coli PTCC1399 and Staphylococcus aureus PTCC1112 were prepared from the Bank of Microorganisms of the Industrial Research Organization of Iran. In all assays, specific antibiotic standards were used to ensure the uniformity of the conditions of all tests. The specific standard antibiotic, streptomycin, was used for the Gram-negative sample (Escherichia coli), and vancomycin for the Gram-positive sample (Staphylococcus). Before to use, all strains were stored at -70°C in medium containing (v/v) 11% dimethyl sulfoxide. Before the experiments, bacterial strains were grown on Mueller-Hinton agar plates (Merck) at 37°C. A single colony of bacterial samples was cultured linearly in N-agar plates (NB 8g+agar 16g) and incubated at 37°C for 24 hours.

Determination of Minimum Inhibitory Concentration (MIC) Inhibitory Concentration

The microdilution method recommended by the

National Committee for Clinical Laboratory Standards (NCCLS, 1997) was used to determine the minimum bacterial growth inhibitory concentration (MIC). For this purpose, 96-well microplates were used. For MIC testing, 200µl of each row was poured into the first well and 100µl of Mueller Hinton Broth (Merck, Germany) was poured into the other wells. Afterwards, the desired concentration of the sample was prepared in the first wells of each row, and 100µl was transferred from the first well to the second well. Then, in the same way, a 1:2 dilution was created from the extract to the last well, and 10µl of inoculum fluid prepared from the studied bacteria was added to all wells. Finally, the microplates were incubated at 37°C for 24 h. To determine the minimum inhibitory concentration, the concentration of the first well in which turbidity was not observed was used as the MIC number. To remove ethanol, due to the initial dissolution of the samples in ethanol (10%), the plates incubated for 24 hours at 37°C.

Determination of Lethal Concentration

To determine the minimum bactericidal concentration (MBC), dilution of the MIC representative and at least two of the most concentrated dilutions of the test product were counted to identify the appropriate colony-forming unit (CFU). MBC is the lowest concentration indicating a predetermined decrease (such as 99.9%) in CFU/ml compared to dilution MIC. Therefore, to perform the test, 96 houses were planted with swaps from plate wells. The plates were incubated at 37°C for 18 hours. All experiments were performed in three replications.

Cell Culture and Cytotoxicity Study

A549 and HFF cells purchased from the National Center for Genetic Resources of Iran were grown in cell culture flasks in completely sterile conditions in low glucose-dulbecco's modified eagle medium (L-DMEM) medium containing 10% fetal bovine serum (FBS) (Gibco, Brazil) at 37°C and 5% (v/v) CO2. After the cells reached at least 70%, they were separated by trypsin (Gibco) and transferred to 96 cells after centrifugation and counting. Different concentrations of the extract were added to the wells and kept in an incubator for 48 hours. After 48 hours of cell treatment with the extract, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the toxicity and determine the percentage of viable cells. For this purpose, to prepare MTT solution at a concentration of 5mg/ml, which is commonly used in MTT assay, 50mg of MTT powder (SIGMA, USA) was dissolved in 10ml of phosphate buffer and then sterilized by 0.22µm filter. 200µl of MTT solution was added to plate wells and incubated for 3 hours. After removal of the supernatant, 100µl of dimethyl sulfoxide (DMSO) was added. When the crystals were dissolved by pipetting, they were incubated for 20 minutes. The amount of light absorption was measured at 570nm with a spectrophotometer and cell viability percentage was calculated according to the formula.

Measurement of Antioxidant Activity by 2,2-Diphenyl-1-Picrylhydrazyl Assay

In this method, which is based on trapping 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals using antioxidant agents and reducing light absorption at 520nm, the antioxidant activity of plant samples was measured. In the current study, the antioxidant activity of Viola odorata aqueous and ethanolic extracts was measured at 520nm using a spectrophotometer.

RESULTS

Antibacterial Effect of VOA and VOE

When Viola (VOA and VOE) extracts were prepared, the antibacterial effect was determined by MIC and MBC methods. The antibacterial effect of different concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.562 and 0.781mg/ml) was measured in 96-well plates. Wells containing bacteria and medium were considered as a positive control for bacterial growth and wells without bacteria but containing other compounds used as controls for sterile test conditions. The results of the inhibitory effect of Viola odorata extract with spectrophotometer at 630nm showed a favorable decreasing trend with increasing the concentration of the extract. In the case of Staphylococcus bacteria, a concentration of 25mg/ml was obtained as an inhibitory concentration of MIC of Viola extract. In Escherichia coli bacteria, a concentration of 50µg of the extract showed inhibitory properties. For

S.aureus MBC 50µg and for E.Coli MBC100µg were obtained. Similar results were obtained for both aqueous and ethanolic extracts (Table 1).

Table 1: Results of Lethal and Inhibitory Concentrations of Aqueous and Ethanolic Extracts of Violet on Escherichia Coli and Staphylococcus.

	Violet Aqueous Extract	Violet Ethanolic Extract
Staphylococcus Aureus	MIC 25µg	MIC 25µg
	MBC 50µg	MBC 50µg
Escherichia Coli	MIC 50µg	MIC 50µg
	MBC 100µg	MBC 100µg

The Effect of Aqueous and Ethanolic Extracts of Viola Odorata on Cell Proliferation

We performed the MTT assay with different concentration of Viola extracts to assess whether VOA and VOE act on the invitro viability of A549 and HFF cells. As shown in Figure 1, treatment with 125µg/ml to 250µg/ml of VOE did not affect the cell viability after 24 hours. While concentration of 500, 1000 and 1500µg/ml of VOE significantly decreased the cell viability in A549 cell line.

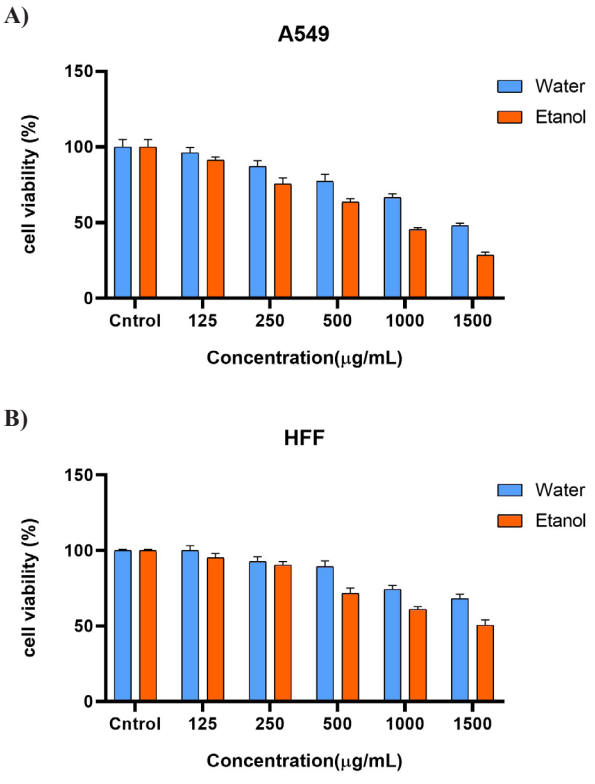

Figure 1: Effect of Different Doses of Aqueous and Ethanolic Extracts of Viola Odorata on A549 and HFF Cell Proliferation

According to the results, the A549 cell is highly sensitive to ethanolic extract of Viola. In HFF cell, as normal cell, treatment with 125, 250 and 500µg/ml of VOA did not affect the cell viability after 24 hours. While concentration of 1000 and 1500µg/ml of VOE decreased the cell viability in HFF cell line. The survival rate of cells decreased with increasing dose and the lethal effect of ethanolic extract (VOE) was higher in both cell lines than aqueous extract (VOA). In A549 cell line, the lethal effect of the extract was higher than HFF cell line.

Antioxidant Effect of Viola Extract

The results of the evaluation of the free radical restraining power of aqueous and alcoholic extracts of Viola odorata are shown in Figure 2. According to the results, the IC50 value is 3.847 for aqueous extract and 6.758 for ethanolic extract.

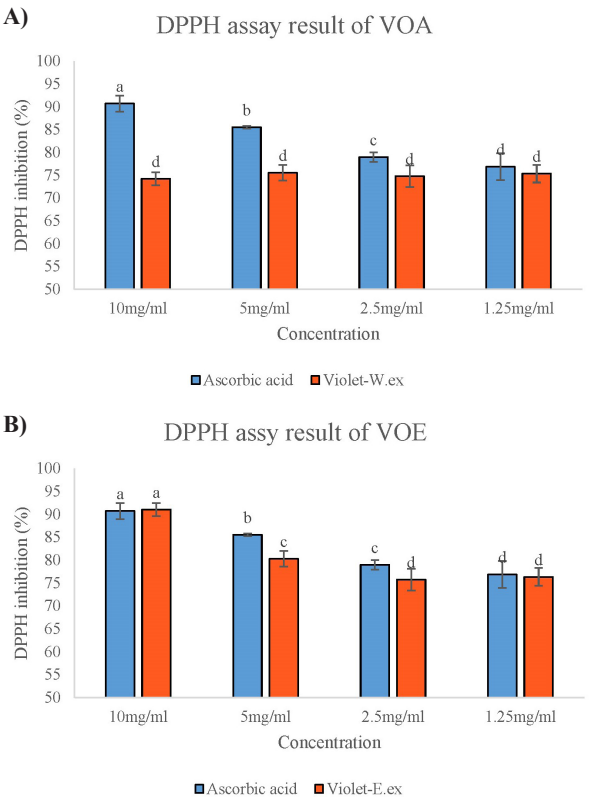

Figure 2: 2,2-Diphenyl-1-Picrylhydrazyl Test Results of Aqueous and Ethanolic Extracts of Viola Odorata in Comparison With Salicylic Acid

DISCUSSION

Today, medicinal plants are valuable resources for the production of new drugs in the treatment of various diseases, including cancer. Many studies in in vivo and in vitro conditions have shown the antitumor effects of various medicinal plants.

Helps to repair deoxyribonucleic acid (DNA), increase immunity, and increase the activity of antioxidant enzymes are among the effects of these compounds [9]. Due to the fact that there is still no complete cure for most of diseases such as cancer, it is essential to identify effective medical compounds. Examination of MTT assay showed that Viola odorata extracts can significantly reduce the proliferation of A549 cancer cells. In fact, the survival rate of A549 cells decreases with increasing the concentration of the extract. While the inhibition of proliferation of Viola extracts in HFF cells, which was used as control, was significantly lower than A549 cancer cells. Other studies have shown the strong inhibitory effect of extracts of various Viola odorata species, including the inhibitory effect of Viola odorata on MDA-MB-468 cell line, the inhibitory effect of Viola tricolor on cervical cancer cells [10]. The differences observed in the results of the inhibitory effect of cell proliferation of aqueous and ethanolic extracts in this study could be related to the solubility and extraction of compounds by alcohol and water. Marinal and Vigestella showed that ethanolic extracts contained more phenolic compounds than aqueous extracts [11]. Therefore, it can be said that Viola odorata compounds that have less solubility in water (such as flavonoids and cycloids) are able to inhibit the growth of lung cancer cells and because these compounds are more soluble in alcoholic media, Viola odorata alcoholic extracts showed more anti-proliferative effects than aqueous extracts.

Since one of the problems of human societies is the genesis of antibiotic-resistant infections, today we are witnessing a growing number of laboratory studies in the design and manufacture of new antibiotics. Plant compounds were considered in this field along with chemicals. Many studies have investigated the effect of plant compounds on microbial agents, including the inhibitory effect of Zataria multiflora [12], the inhibitory effect of Hypericin [13], Rosemary [14], Nanocurcumin and Turmeric [15, 16]. Regarding the antimicrobial effect of different species of Viola odorata plant, we can mention the study of the antibacterial effect of aqueous extract by the method of growth inhibition zone on Escherichia coli, Staphylococcus aureus and Bacillus subtilis by Ashfaque Khan [17]. The results of the study carried out by Bahram Bibak et al., on the antibacterial effects of Viola odorata aqueous and ethanolic extracts on chronic sinusitis-causing bacteria using well and disc immobilization method showed the inhibitory effect of Viola odorata aqueous and ethanolic extracts on Staphylococcus epidermis and Staphylococcus aureus bacteria, while no effect was observed on Enterobacter bacteria [18]. Studies have been done in the other species of Viloa such as Hybanthusenneasperus [19] and Paypayrola grandiflora [20]. Also, the purification of cycloids, and its antimicrobial effects on the Gram-positive bacterium Staphylococcus have also been performed [21]. Like previous studies, the results obtained in this study have shown the antibacterial effects of Viola odorata. Although the difference in the type of extract and bacteria has caused a difference in the level of antibacterial properties of this plant, it can be concluded that according to the data of previous studies and the present study, as well as the recommendations of traditional medicine, this plant has the ability to inhibit bacterial infections.

The difference in inhibitory effect on Escherichia coli and Staphylococcus aureus studied in this research could be due to differences in Gram-positive and Gram-negative cell wall structure as well as differences in resistance mechanisms in these two bacteria. Despite having secondary compounds such as flavonoids, glycosides, peptides, alkaloids, steroids, saponins and tannins and cycloids, etc., and the results obtained in this study it can be claimed that this plant has high antioxidant potential and with this antioxidant property can play an important role in antimicrobial and antitumor activities. In the case of Viola odorata extract, it is important to note that it had almost no adverse effects on normal human cells and only inhibitory effects were seen on cancer and bacterial cells. Finally, due to the low production costs and side effects of plant compounds, it is suggested that more attention be paid to the Viola odorata plant in the design and manufacture of new drugs in further studies.

Aqueous and ethanolic extracts of Viola have an inhibitory effect on Gram-negative and positive bacteria. Antibacterial results of aqueous and alcoholic extracts of Viola were similar, while cytotoxicity results in ethanolic extracts of Viola were more lethal than Aqueous extract. VOE and VOA have more inhibitory effect on the proliferation of lung cancer cell (A549 cell line) than normal

cell (HFF cell), so due to the lower side effects of Viola, this plant can be used in further studies for the synthesis of drugs with less side effects.

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CONFLICT OF INTEREST

The authors have no conflicts of interest.

ETHICS APPROVAL

The project was found to be in accordance with the ethical principles and the national norms and standards for conducting medical research in Iran.

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