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# Evaluation of Antibacterial Properties and Cytotoxicity of Ethanolic Extract of *Alhagi Maurorum*

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## ABSTRACT

**Background and Objective:** Cervical cancer is the fourth most common cancer in the world. The most important issue in cancer treatment is the destruction of cancer cells in the presence of normal cells. For this reason, it is necessary to use natural resources such as plants to treat cancer. The aim of this study is to evaluate the antibacterial effects of the ethanolic extract of *Alhagi maurorum* on *Staphylococcus aureus* and *Pseudomonas aeruginosa* and cytotoxicity on HeLa cervical cancer cell line.

**Methods:** In this experimental study, first the ethanolic extract of *Alhagi maurorum* was prepared, and then the two standard strains of *Staphylococcus aureus* (ATCC: 25923) and *Pseudomonas aeruginosa* (ATCC: 27853) were lyophilized by culturing in nutrient medium. In order to confirm the standard strains, biochemical tests were performed. Microdilution method was used to determine the minimum inhibitory concentration and after obtaining the minimum inhibitory concentration, the minimum bactericidal concentration was evaluated. Furthermore, the effects of the cytotoxicity of the extract at concentrations of 0.1, 10, 50, 100, 500 and 1000 µg/ml was evaluated on the HeLa cell line in a period of 48 hours using the MTT method and comparing its toxicity with the cisplatin group (positive control group).

**Findings:** Ethanol extract of *Alhagi maurorum* at a concentration of 50 µg/ml reduced the growth of cancer cells, and in the statistical comparison, 50, 500 and 1000 µg concentrations revealed significant differences ($p<0.05$). According to minimum inhibitory concentration results, the minimum growth inhibitory concentration of the extract on the standard strain of *Staphylococcus aureus* and *Pseudomonas aeruginosa* was reported to be 4000 and 16000 µg/ml, respectively, and according to minimum bactericidal concentration results, the minimum bactericidal concentration of the extract was found 4 times the minimum inhibitory concentration (16000 µg/ml) in *Staphylococcus aureus* (ATCC: 25923), but it was not lethal in *Pseudomonas aeruginosa* (ATCC: 27853).

**Conclusion:** The results of the study showed that the ethanolic extract of *Alhagi maurorum* affected HeLa cells through antioxidant activity and inhibited their growth, and according to minimum inhibitory concentration and minimum bactericidal concentration results, it was also shown that the most inhibitory effect was on the standard strain of Staphylococcus aureus while it showed no effects on the strain of *Pseudomonas aeruginosa*.

**Keywords:** *Alhagi Maurorum Extract, Cell Viability, Staphylococcus Aureus, Pseudomonas Aeruginosa.*

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## Introduction

Cervical cancer is the fourth most common cancer in the world (1). Due to the increasing prevalence of cancer, multiple treatments have been considered. The initial response to chemotherapy and surgery is generally positive, but patients commonly complain of tumor recurrence (2). The most important issue in cancer treatment is the destruction of cancer cells in the presence of normal cells. For this reason, it is necessary to use natural resources such as plants for cancer treatment (3). It is also very important to eliminate bacterial infections, especially those caused by *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which cause a wide range of infections in hospital and non-hospital environments and show resistance to antibiotics with a variety of innate, adaptive and acquired resistance strategies. Some strains of these bacteria cause biofilm infection (4). Irregular, inappropriate and irrational use of antibiotics has led to the emergence of resistance of microorganisms to antimicrobial compounds. The creation of resistance in microorganisms in causing acute infections has caused the use of plant metabolites to benefit from their antimicrobial and antioxidant effects (5).

According to the results of Moore's research, HeLa cancer in the world is caused by the increasing and irregular growth of cervical epithelial cells and the continuous shedding of cells, and the most important factor is the human papilloma virus. HeLa cancer cells are used for research studies in the field of cancer, whose advantages include the ability to multiply indefinitely and tolerate long passages (6).

*Alhagi maurorum* is widely used for several medicinal purposes. *Alhagi maurorum* is a perennial plant of the Fabaceae family and Fabales order which grows abundantly in Saudi Arabia, the Middle East, and other regions of the world, and has common names such as Alhagi maurorum, Persian mannaplant, or Caspian manna (7). This plant and its flower are used in traditional medicine as a laxative, diaphoretic, stimulant, diuretic and expectorant drug and its extracts are used as medicine to treat warts and migraines. Its leaf extract is used as an oil to treat rheumatism, and its root extract is used to remove kidney stones by relaxing the ureter (8). This plant is rich in flavonoids, phenolic compounds, glycosides, anthraquinones, saponins, steroids, tannins and alkaloids. These are compounds that inhibit lipid peroxidation. Flavonoids are secondary metabolites that have antioxidant, anti-allergic, anti-inflammatory, antibacterial, antifungal and antiviral roles (9). This plant has peripheral and central analgesic activity with a dose of 400 mg/kg, which acts as an antioxidant (10). It has been reported that due to the presence of these complex compounds, the total extract of *Alhagi maurorum* has anti-cancer, anti-bacterial, anti-inflammatory, anti-allergy, anti-viral and anti-clotting properties. Various flavonoids of this plant cause anti-cancer effects and plant tannins have antibacterial and antiviral effects (11). In previous studies, they found that *Alhagi maurorum* extract is an effective medicine for liver and urinary tract diseases (12).

According to the above explanation, this study was conducted in order to evaluate the antibacterial and cytotoxicity properties of the ethanolic extract of *Alhagi maurorum* on HeLa cell line and *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacteria.

## Methods

This experimental study was carried out after being approved by the Sana University with the code 1712618 in order to evaluate the antibacterial properties and cytotoxicity of the ethanolic extract of *Alhagi maurorum* on the HeLa cell line, the standard strain of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. In this study, two standard bacterial strains were used, including the standard gram-positive strain of *Staphylococcus aureus* ATCC: 25923 and the standard gram-negative strain *Pseudomonas aeruginosa* ATCC 27853, both prepared from Pasteur Institute Microbial cell bank as well as a cervical cancer cell line

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(HeLa) also prepared from Pasteur Institute Microbial cell bank. HeLa cells (CCL-2TM) are the first immortalized human cells grown in culture. This cell was isolated from cervical cancer in a 31-year-old patient in 1951. This study was conducted based on 8 groups:

**The first negative control group:** cell suspension

**The second positive control group:** cell suspension + cisplatin 3 μM (cisplatin 50 mg/50 ml VIAL, Sobhan Oncology Pharmaceuticals)

**The third to eighth treatment groups:** cell suspension + 0.1, 10, 50, 100, 500 and 1000 μg/ml concentrations of the ethanolic extract.

In all groups, the experiments were performed in triplicate and the duration of the study was 48 hours.

**Preparation of the sample of *Alhagi maurorum* and condensation of the extract:** *Alhagi maurorum* plant was purchased from the market, and after drying, it was ground into powder as much as possible. After preparing the aerial parts of the plant and separating its impurities, 800 grams of the plant was crushed by a grinder and mixed with 96% ethanol at a ratio of 30 to 70 and then placed on a shaker for 72 hours and every 24 hours, the extract was separated, the resulting extract was filtered through filter paper and a funnel. Then, the extract was distilled in a vacuum distillation apparatus at 60°C and 70% rotation until the remaining volume reached a quarter of the original volume. In this case, the extract tank was separated from the apparatus and the remaining extract was poured into a Petri dish and dried in an oven at a temperature of 50°C. The concentrated extract was transferred to a freeze dryer to dry and turn into powder (13-15).

**Maintenance and culture of HeLa cell line:** In this experimental study, HeLa cancer cell line was used, which was obtained from the cell bank of Pasteur Institute of Iran. Then, the cell line was kept in DMEM culture medium containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin antibiotics in an incubator at 37°C with sufficient humidity and 5% carbon dioxide, and when its growth reached 70%, the cells were separated from the bottom of the flask by trypsin and centrifuged at 1500 rpm for 5 minutes, and the resulting cell sediment was prepared in the form of a suspension and to determine the percentage of cell viability, trypan blue dye and hemocytometer were used by light microscope (16, 17).

**MTT Test (Microculture Tetrazolium Test):** Evaluation of the cytotoxic effects of the ethanolic extract of *Alhagi maurorum* on the target cell line was performed by formazan dye based on the reduction of yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) in comparison with the control group. The MTT test is based on the mitochondrial enzyme succinate dehydrogenase (SDH) to convert the water-soluble yellow salt of MTT into purple formazan crystals insoluble in water, which is measured by the spectrophotometric method by adding DMSO as a detergent solvent (18, 19)

**MTT cytotoxicity assay methodology:** After incubation of cervical cancer cells (HeLa) with concentrations (0.1, 10, 50, 100, 500 and 1000 μg/ml) of the ethanolic extract of *Alhagi maurorum* in a period of 48 hours, MTT was used for assessment. For this purpose, some MTT powder was diluted with PBS solution and then sterilized with a filter, 50 microliters of it was added to the plates and placed in a CO2 incubator. After 4 hours, the plates were removed from the incubator, the medium containing MTT was removed, and 200 microliters of DMSO was added to each well. After about 10 minutes, it was read by ELISA Reader at a wavelength of 570 nm and the OD (Optical Density) was obtained (18, 20, 21).

**Properties and reduction of bacteria:** Standard strains of *Staphylococcus aureus* (ATCC: 25923) and *Pseudomonas aeruginosa* (ATCC:27853) were prepared from Bahar Afshan Company in lyophilized form. In order to reduce the bacteria, the bacterial suspension was inoculated into BHI (Brain Heart Infusion) broth culture medium and incubated for 18-24 hours at 37°C. Then, one loop of *Staphylococcus aureus* (ATCC: 25923) was cultured in blood agar medium and one loop of *Pseudomonas aeruginosa* (ATCC: 27853) was cultured in MacConkey agar (MAC) in four zones. Then, it was incubated for 18-24 hours at 37°C.

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**Diagnostic tests:** to confirm bacteria, gram staining and differential tests of catalase, coagulase and mannitol salt agar for *Staphylococcus aureus* (ATCC: 25923) and biochemical tests of catalase, oxidase, Simon citrate, urea test and TSI (Triple Sugar Iron), MRVP (Methyl Red-Vagoes Proskauer), SIM (Sulfide, Indole, Motility) were used.

**Minimum inhibitory concentration (MIC) of bacteria:** To determine the minimum inhibitory concentration of the ethanolic extract of *Alhagi maurorum*, first, 100 µl of Muller Hinton Broth medium was added to the wells of the 96-well microplate. Then, successive diluted concentrations of the ethanolic extract of chert were added to the Muller-Hinton broth medium in the form of serial dilutions. Then $1.5\times10^5$ cfu/ml of isolates *Pseudomonas aeruginosa* and *Staphylococcus aureus* (ATCC: 25923) was inoculated separately in the medium and after 18-24 hours of incubation at 37 degrees Celsius, the bacterial growth in the medium was evaluated and recorded. The lowest concentration at which no growth is observed is considered as MIC. It should be noted that concentrations of 1000, 2000, 4000, 8000 and 16000 micrograms/ml of the ethanolic extract of *Alhagi maurorum* were used to perform the MIC method. The MIC of vancomycin antibiotic was determined at concentrations of 1, 2, 4, 8, 16, 32, 64, 125, 250, 500 µg/ml as a control for the desired bacterial strains (22).

**Minimum bactericidal concentration (MBC):** After obtaining the minimum inhibitory concentration of the extract of *Alhagi maurorum* on the strains, to determine the minimum bactericidal concentration of the ethanolic extract of *Alhagi maurorum*, the concentrations of MIC, 1.2 MIC, 1.4 MIC, 2 MIC, and 4 MIC on Mueller Hinton Agar medium were cultured by swap in sterile conditions and the plates were placed in an incubator at 37°C for 18-24 hours. The lack of growth of bacteria indicates the minimum bactericidal concentration (22).

Prism 8 was used for statistical analysis, and one-way analysis of variance was used for statistical testing, and Tukey post hoc test was used for comparison within groups, and $p<0.05$ was considered significant.

## Results

### The results of the MTT test:

**The effect of the cytotoxicity of the ethanolic extract of *Alhagi maurorum* on the cervical cancer cell line (HeLa):** HeLa cells, when exposed to cisplatin at IC50 concentration, reduced the growth of cells by 50%. In addition, the exposure of cells to the ethanolic extract of *Alhagi maurorum* in different concentrations has caused a decrease in the growth of cervical cells in a dose-dependent manner; the growth of the cells has reached from 96% in the lowest concentration to 23% in the highest concentration of the extract. In a statistical comparison with the positive control group, it was found that the concentrations of 0.1 and 10 µg/ml ($p<0.001$), the concentration of 50 µg/ml ($p<0.05$) and the concentrations of 500 and 1000 µg/ml ($p<0.01$) showed a significant difference (Table 1).

**Table 1. Mean MTT test results of different treatment groups in HeLa cell line**

| Groups | HeLa Mean±SD |
|---|---|
| Control | 100.6±0.14 |
| Cisplatin | 48.60±3.91 |
| Alhagi 0.1 | 96.40±2.07 |
| Alhagi 10 | 87.00±1.87 |
| Alhagi 50 | 63.40±2.48 |
| Alhagi 100 | 46.40±2.05 |
| Alhagi 500 | 39.00±3.67 |
| Alhagi 1000 | 23.40±2.40 |

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### MIC and MBC results of ethanol extract:

**Diagnostic test:** In *Staphylococcus aureus* (ATCC: 25923), catalase, coagulase positive and growth in mannitol salt agar were observed. In *Pseudomonas aeruginosa*, catalase and oxidase, citrate and methyl red tests were positive, indole and VP were negative, and ALK/ALK and H2S were negative in TSI medium.

**The minimum inhibitory concentration of bacteria (MIC):** the minimum growth inhibitory concentration (MIC) of *Alhagi maurorum* in the standard strain of *Staphylococcus aureus* (ATCC: 25923) and *Pseudomonas aeruginosa* was 4000 and 16000 µg/ml, respectively, and the minimum growth inhibitory concentration (MIC) of the antibiotic vancomycin in standard strain of *Staphylococcus aureus* (ATCC: 25923) and *Pseudomonas aeruginosa* (ATCC: 27853) was 2 and 250 µg/ml, respectively.

**Minimum bactericidal concentration (MBC):** The minimum bactericidal concentration (MBC) of *Alhagi maurorum* was not able to kill bacteria in the standard strain of *Staphylococcus aureus* (ATCC: 25923) at 4 MIC (16000 µg/ml) and in *Pseudomonas aeruginosa*.

The minimum bactericidal concentration (MBC) of vancomycin antibiotic for *Pseudomonas aeruginosa* strain (ATCC: 27853) did not have the ability to kill bacteria, but in the standard strain of *Staphylococcus aureus* (ATCC: 25923) at concentrations 2 and 4 MIC (4 and 8 µg/ml), it reduced the number of bacterial colonies.

## Discussion

The results of the study showed that the chemical compounds present in the ethanolic extract of *Alhagi maurorum* have antibacterial and antioxidant properties due to the presence of phenol and flavonoids. According to the studies conducted in this research, in terms of cytotoxicity (MTT) on the HeLa strain, the highest effect in the high dose of the extract (500 and 1000 µg/ml) led to the reduction of cancer cells, and in terms of antibacterial properties in our study, the highest effectiveness of the extract was reported against *Staphylococcus aureus* (ATCC: 25923) at a concentration of 4 times MIC (16000 µg/ml).

Cervical cancer is the fourth most common cancer in the world (23). On the other hand, cancer cell lines have defeated treatment due to drug resistance to chemotherapy drugs, such as cisplatin, doxorubicin, and melphalan. Thus, cancer research has focused on natural compounds such as plant extracts to develop effective and selective cytotoxic agents for the treatment of many cancers, including cervical cancer (24) and the results of the present study showed that the ethanolic extract of *Alhagi maurorum* inhibited the growth of cervical cancer cells. In general, the findings of the study showed that the combination of *Alhagi maurorum* and docetaxel has synergistic antitumor effects on 4T1 cells, which may be a valuable choice for complementary and alternative treatments, and further results revealed that *Alhagi maurorum* alone also had an acceptable antitumor effect (25).

As a very valuable plant, the extract of *Alhagi maurorum* has numerous secondary metabolites and flavonoids and has shown many medicinal properties. Over the past years, several experimental studies have shown that diethyl ether and petroleum ether extracts have IC50 values of 114.7 µg/ml and 105.7 µg/ml against human breast cancer cell line MCF7. IC50s > 100 µg/ml of extract against A549, MCF7, HepG2, HT-29 and MDBK cell lines have been shown in studies (26, 27). The results of cytotoxicity test (MTT) show that the ethanolic extract of *Alhagi maurorum* in high doses led to a decrease in the number of cancer cells.

The results obtained from a research show that the extract of some plants has reduced the growth of gram-positive and gram-negative bacteria (17). The results of this research are in line with the present research. Furthermore, in terms of investigating the antibacterial property of the ethanolic extract, the results of the minimum inhibitory concentration and the minimum bactericidal concentration showed that the strain

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of *Pseudomonas aeruginosa* (ATCC: 27853) is resistant to the ethanolic extract of *Alhagi maurorum*, while the strain of Staphylococcus aureus (ATCC: 25923) has shown sensitivity to the extract. According to the obtained MIC, the minimum inhibitory concentration of *Staphylococcus aureus* (ATCC: 25923) and *Pseudomonas aeruginosa* (ATCC: 27853) is reported as 4000 and 16000 µg/ml, respectively. According to the MBC results, the extract of *Alhagi maurorum* in the standard strain of *Staphylococcus aureus* (ATCC: 25923) compared to 4 MIC (16000 µg/ml) positive control group showed lethality, but the extract did not have the ability to kill the bacteria in *Pseudomonas aeruginosa* (ATCC: 27853). In this study, vancomycin antibiotic was used as a positive control in comparison with the properties of the ethanolic extract, where the strain of *Pseudomonas aeruginosa* (ATCC: 27853) showed its resistance, and this antibiotic did not have the ability to kill this strain. The standard strain of *Staphylococcus aureus* (ATCC: 25923) at 2- and 4-times MIC (4 and 8 µg/ml) decreased the number of bacterial colonies.

Considering the significant cytotoxic and antibacterial effect of the ethanolic extract of *Alhagi maurorum* on the cervical cancer cell line and the strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacteria that play a role in causing various harmful and hospital infections, this extract can be used as a natural herbal therapeutic product and be considered as a complementary option.

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