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Anti-bacterial Property and Cytotoxicity of *Allium sativum* and *Myrtus communis* Extracts Against Nosocomial Bacterial Infections

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ABSTRACT

Background: Microbial infections and the resistance to antibacterial drugs are on the rise, and scientists are in search of the safest and most effective approach to overcome them. Medicinal plants are potentially effective against many microorganisms. Therefore, this study was planned to examine the antibacterial properties and cytotoxicity of the extracts of *Allium sativum* and *Myrtus communis* against bacteria that cause nosocomial infections.

Methods: *A. sativum* and *M. communis* were collected from the northern regions of Iran during the spring. After preparing the hydroalcoholic extracts of *A. sativum* and *M. communis*, the minimum inhibitory and bactericidal concentrations (MIC & MBC) were determined. The cytotoxicity of the extracts was asseyed in normal cells and Hu02 fibroblast cell line.

Results: The MIC and MBC of *A. sativum* (62.5 mg/mL) against *Salmonella enterica* were similar. Also, the MIC of *A. sativum* and *M. communis* against *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were similar (62.5 & 15.6 mg/mL), respectively. The viability percentage of skin fibroblasts after treatment for 48 hours with the extract of *A. sativum* was significantly higher than that of *M. communis*.

Conclusion: Both extracts from *A. sativum* and *M. communis* demonstrated good antimicrobial properties. Based on the results, the safe antibacterial potential of the extracts may be used as alternative agents to fight against nosocomial bacterial infections.

Keywords: *Allium sativum*, Toxicity, Nosocomial infections

Introduction

The acronym ESKAPE represent a group of life-threatening pathogens responsible for nosocomial infections, including *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Enterobacter* spp [1].

In recent years, the growing outbreak of resistance against conventional antibiotics, especially ESKAPE has caused much concern among the medical community in the treatment of infectious diseases [1, 2]. According to the list of global priorities for antibiotic-resistant pathogens issued by the World Health Organization (WHO), the antibiotic resistance against the *Enterobacteriaceae* family is a top concern. Thus, *Enterobacteriaceae* was added to the list of ESKAPE bacteria [3, 4]. Through horizontal gene transfer, ESKAPE pathogens

are able to escape the biocidal effects of antimicrobial agents, hence their strong resistance to antibiotic treatments. The resistance is caused by the exchange of genetic materials of antibiotic resistance genes from one bacterium to another [5, 6].

Due to the global emergence of multi-drug resistance (MDR) among Gram-negative and Gram-positive bacteria and difficulties with treating them, the resistance control in bacteria that are resistant to drugsis one of the main approaches to prevent infections [7, 8]. There are numerous problems with the application of conventional antibiotics, such as antimicrobial resistance, environmental problems, carcinogenicity, side effects, and the high costs to patients. Thus, using natural medicinal agents have been considered to replace the synthetic and conventional antibiotics particularly against nosocomial infections [9]. A major group of these alternative agents are plant-based products. They have been used against resistant bacteria for a long time and have been known as antimicrobials against numerous microorganisms [5, 10]. In this context, we need to find anti-bacterial agents that do not cause resistance to antibiotics [11].

Garlic with the scientific name being *Allium sativum* is a member of the *Alliaceae* family. Garlic which has been known for its potent antibacterial properties, can be an appropriate alternative to synthetic drugs. In this context, the inhibitory effect of garlic on Gram-negative and Gram-positive bacteria, such as *Staphylococcus aureus* and *Escherichia coli* has already been established [9, 10]. The inhibitory effects of garlic, i.e. its antimicrobial, antioxidant, and an enhancer of immune system are due to its active contents of sulfur (allicin) and polyphenol compounds. The antibacterial mechanisms of efficacy of garlic are believed to be associated with degradation and induction of oxidative stress on microorganisms [11, 12].

In addition, *Myrtus communis* is a member of *Myrtaceae* family which grows widely in Iran and numerous tropical nations [13, 14]. Various parts of this plant have medicinal and anti-inflammatory properties, and are used in the treatment of certain diseases globally [15]. According to research findings on parts of this plant, especially its leaves and limbs, it contains specific chemical compounds. For instance, the leaves contain essential oils, phenolic acids, flavonoids, and tannins [16] while the dried leaves contain different compounds [17]. The plant's secondary metabolites play an important role in its survival and defense system. The main secondary metabolites of this plant are essential oils and polyphenols. The greatest antimicrobial and antifungal effects are related to the essential oil in different parts of the plant, especially in the leaves [18]. The *M. communis* leaves have antimicrobial activity against some bacteria, especially *Salmonella enterica*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Candida albicans* [13]. We did not find any published articles comparing the antibacterial toxicity of the extracts from *A. sativum* and *M. communis*. Thus we believe that conducting the current study is justified.

Aim of the study: The main objective of this study was to look for natural agents with antibacterial properties without toxicity to human cells. Thus, we evaluated the plant's extracts for its antibacterial properties against major bacteria responsible for nosocomial infections (ESKAPE). Also, we selected *S. enteric*a from the *Enterobacteriaceae* family due to its significant resistance to physical and chemical agents, and the difficulty we face for its destruction [19]. This study determined the antibacterial properties and cytotoxicity of the hydroalcoholic extracts of *A. sativum* and *M. communis* against bacteria that cause nosocomial infections.

Materials and Methods

Bacteria samples: Strains of *S. enterica* (ATCC 6962), *E. faecalis* (ATCC 29212), *S. aureus* (ATCC 25922), *A. baumannii* (ATCC 19606), and *P. aeruginosa* (ATCC 27853) were purchased from the Iranian Biological Resource Center (Tehran, Iran).

Extraction materials: The 95% ethanol was obtained from Merck (Darmstadt, Germany). The hydroalcoholic extracts of *A. sativum* and *M. communis* were prepared each at a concentration of 100 mg/mL.

Cell line for MTT assay: Hu02 fibroblast cell line (from healthy skins) was obtained from the Iranian Biological Resource Center (Tehran, Iran). High glucose cell culture medium (DMEM), trypsin, fetal bovine serum (FBS) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

Preparation of garlic hydroalcoholic extracts: The garlic species of *A. sativum* and *M. communis* were collected from the northern regions of Iran in the spring. They were washed and dried under appropriate heating conditions without direct sunlight. The decoction was performed through a percolation method and the dry garlic samples were crushed to powder, using an electric shredder. Next, a sample of the garlic powder from eithr *A. sativum* or *M. communis* origin was suspended in a

hydroalcoholic solution (1:4 v/v) to prepare the initial extracts. The extract solution was placed in a water bath at 45-50°C. The extract solution was dehydrated, using an evaporator, filtered, and stored at 4°C [20]. The final garlic extract solution was prepared at a concentration of 100 mg/mL and stored in a dark glass bottle.

Minimum inhibitory concentration: The minimum inhibitory concentration (MIC) of the garlic extracts was determined by the micro-dilution method, based on the clinical and laboratory standard guidelines. We used 96-well microplates to detect the lowest concentration of the extracts at 100 mg/mL that inhibited the visible growth of a typical microorganism [12, 19]. For each series of the tests, a 100 µL aliquot of Mueller-Hinton (MHB) medium was added to 10 wells. Then, a 100 µL aliquot of the extract was added to the first well, and this process continued by a decreasing dilution up to the tenth well.

Finally, a 100 µL aliquot of the microbial suspension (1.5×10^8) was added to each well. The bacterial batches that were used in these tests were *E. faecalis*, *S. aureus*, *A. baumannii*, *P. aeruginosa*, and *S. enterica*. The eleventh well was considered as a positive control that contained the media only, and the twelfth well was designated as a negative control that contained the bacterial suspension only. After an overnight incubation of the microplate at 37°C, the turbidity of the solution in each well was checked. A well without showing any turbidity would be considered as the MIC for the bacterial growth [21, 22].

Minimum bactericidal concentration (MBC): After selecting the lowest dilution of the antimicrobial agent as MIC, the first clear well was considered as the MBC for the given bateria. But for better certainty, a 100 µL aliquot of all of the clear wells were transferred to Mueller-Hinton agar medium and cultured. After overnight incubation at 37°C, the last dilution in which no sign of bacterial growth was observed on the agar surface was designated as the MBC [23].

Cytotoxicity assessment by MTT assay: The MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) assay is a colorimetric method based on the reduction and breaking of yellow tetrazolium salt crystals by the enzyme succinate dehydrogenase. The final products are dehydrogenase and the formation of insoluble purple crystals. As part of the MTT assay and to examine the cytotoxicity of the extracts, MIC assays were performed in 96-well microplates, using an Hu02 fibroblast cell line. Initially, cell suspensions in 10% FBS, consisting of 5×10^3 cells in 180 µL high glucose DMEM were seeded per well on 96-well plates.

After incubation overnight at 37°C and 5% CO_2 in an incubator, the cells were treated with the extracts of *A. sativum* or *M. communis* at the highest effective dose, based on the data from theMIC assays (62.5 mg/mL) for 48 or 72 hours. Each test was performed in triplicate. A 20 µL aliquot (5 mg/mL) of the MTT solution was added to each well and the incubation continued for another four hours. During the incubation, MTT was regenerated by succinate dehydrogenase, one of the enzymes in the respiratory cycle of living cell mitochondria. As a result, the breaking of the tetrazolium ring by the action of living cells leads to the formation of an insoluble formazan. The amount of formazan dye produced is directly proportional to the number of cells that are metabolically alive. Aiming to colorize the formazan crystals, 150 µL of DMSO was dissolved in the solution per well. Finally, the absorbance of the solution was read at 570 nm, using an ELISA reader [2, 24].

Results

This study evaluated the antibacterial and toxicity properties of the extracts from *A. sativum* and *M. communis* against five different bacterial strains. The cytotoxicity of the plant extracts with normal cells was also evaluated, using a Hu02 fibroblast cell line, the results of which are presented below.

Assessment of MIC and MBC: The MIC and MBC values of the extracts from *A. sativum* and *M. communis* were evaluated in mg/mL against ESKAPE pathogens, such as *S. enterica*, *E. faecalis*, *S. aureus*, *A. baumannii*, and *P. aeruginosa*. The results are presented in Table 1.

Cell cytotoxicity based on MTT assay: We investigated the cytotoxicity of *A. sativum* and *M. communis* extracts at the highest effective dose based on the MIC assay (62.5 mg/mL) against five bacterial strains, using a Hu02 fibroblast cell line (Table 2). The mean viability percentage of the cell line was measured separately with MTT assay after 48 or 72 hours following treatment with each extract. The values are reported as the Means±SD, and the differences among the means were considered statistically significant at $P<0.05$ based on student t-test. The results indicated that the viability percentage of the fibroblast cell line was significantly higher for the *A. sativum* extact than for *M. communis* ($P=0.00$). However, the percent viability of the cells was almost identical for both extracts after the 72-hour treatment ($P=0.91$).

Table 1. MIC and MBC in extracts of *A. sativum* and *M. communis* against ESKAPE pathogens

Species	*A. sativum* (mg/mL)		*M. communis* (mg/mL)	
	MIC	MBC	MIC	MBC
S.enterica	62.5	62.5	7.8	15.6
S. aureus	7.8	15.6	15.6	15.6
P. aeruginosa	15.6	31.2	15.6	31.2
E. faecalis	15.6	15.6	15.6	31.2
A. baumannii	62.5	125	62.5	125

Table 2. Comparison of mean viability percentage of skin normal fibroblast cells (Hu02) after 48 h and 72 h treatment measured by MTT assay (based on statistical method t-test)

A. sativum (62.5 mg/mL)		*M. communis* (62.5 mg/mL)	
48 h	72 h	48 h	72 h
58.33±1.52	85±7	47±1	86±14

A. sativum with *M. communis* (48 h); P=0.00

A. sativum with *M. communis* (72 h); P=0.91

Discussion

This study examined the antibacterial properties and cytotoxic effects of the extracts from *A. sativum* and *M. communis* against pathogens such as *S. enterica*, *E. faecalis*, *S. aureus*, *A. baumannii*, and *P. aeruginosa* (ESKAPE).

Daka and Awole evaluated the antibacterial activity of *A. sativum* extract against *Enterobacteriaceae* group, including *E. coli*, *Shigella* and *Salmonella* strains, and found that the MIC values for the tested organisms was above 30 mg/mL [25]. In the current study, the MIC and MBC of *A. sativum*'s extract against *S. enterica* were also above 30 mg/mL. The antimicrobial activity of the garlic extracts against *E. coli*, *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *Shigella sonei*, and *Salmonella typhi* has been investigated by Shobana et al. They demonstrated that both Gram-positive and Gram-negative bacteria were susceptible to the extracts [26]. Also, numerous studies have shown that the susceptibility of Gram-positive bacteria versus the extracts was higher than that of Gram-negative bacteria. The proposed reasons for the susceptibility are: a) the presence of flavonoids and volatile oils [27], and b) the easy penetration of the extract into Gram-positive bacteria due to the lack of hydrophilic lipopolysaccharides (LPS) in their plasma membrane.

Gram-positive bacteria have a cell wall, consisting of thick peptidoglycan and teichoic acid, while Gram-negative bacteria have a periplasmic space containing thin peptidoglycan and proteins, making it difficult for the essential oil to pass through [28]. Conversely, one study by Azzouny et al. has shown that the aqueous garlic extracts have a greater antibacterial effect on Gram-negative than on Gram-positive bacteria, because of their outer membrane [29, 30]. The different conclusion by the above studies might be due to the effects of varying methods of preparing antimicrobial extracts. In the current study, we obtained almost similar results. On the other hand, in this study, the antimicrobial activities of *A. sativum* extract against some Gram-positive and Gram-negative bacteria were similar. For instance, the effects of *A. sativum* extract against *E. faecalis* and *P. aeruginosa* were similar at 15.6 mg/mL. Also, the effects on *S. enterica* and *A. baumannii* were identical at 62.5 mg/mL.

It appears that the differences in the MIC values of the plant extracts may arise from differences in the extraction methods. The antibacterial range of plant extracts is also likely to be associated with the culture environment and the climatic conditions. Evidently, the chemical compounds present in the plant extracts have an inhibitory effect to bacteria. Also, garlic extracts are rich in polyphenols and allicin, which exert their inhibitory effect on the pathogens, depending on the concentra-

tion used [31]. The inhibitory mechanism of allicin is believed to be related to the delay or inhibition of the synthesis of DNA, RNA, proteins, and lipids [32]. Also, high concentrations of monoterpene hydrocarbons, such as 1,8-cineole, α-pinene, and linalool are known to exist in *M. communis* oil, acting as strong antimicrobial substances [13].

In a study conducted by Teimoory et al., the strongest inhibitory effect of *M. communis* extract was demonstrated against *S. aureus* [33]. The findings in the current study also showed the greatest inhibitory effect by the extracts of *M. communis* and *A. sativum* was against *S. aureus*. Also, the inhibitory effect of Myrtaceae extract against *S. enterica* (MIC: 7.8 mg/mL) was more than that of *A. sativum* (MIC: 62.5 mg/mL). The Myrtaceae extract contains 2.3% polyphenol [33], which may justify its stronger efficacy compared to that of garlic extract against *S. enterica*. Polyphenolic compounds, such as phenolic acids, tannins, and flavonoids, contribute to the antibacterial activity of *M. communis* extract [34]. For instance, tannins, which are water-soluble phenolic polymers, readily inhibit free radicals [35]. Also, oxidized phenolic compounds inhibit enzymes by reacting to their sulfhydryl group or interact with proteins through non-specific pathways [36].

Studies have suggested that the antibacterial activity of *M. communis* is linked to the compounds contained in the extract such as 1,1,8a-trimethyloctahydro-2,6-naphthalenedione (27.6%) and pyrogallol (9.1%) [28, 37]. Of note, pyrogallols are also found in polyphenols. These compounds have been shown to possess strong antibacterial effects against various species [38]. In the current study, the viability of the skin fibroblasts was significantly higher after being treated for 48 hours with *A. sativum* extract than with that of *M. communis*. Also, the results of the treatment with either extract for 72 hours were almost identical (85% vs 86%) as determined by our cytotoxicity assays.

The cytotoxic effects of plant extracts normally operate by stopping the cell cycle and causing apoptosis. These mechanisms lead to cell death through the activation of caspases and disruption of mitochondrial membrane potential (MMP) [39]. Experiments have shown that raw plant extracts may be toxic and exert mild toxicity against cells, reducing their viability [40].

The data provided by a former study [34] have demonstrated that garlic extracts at low doses has no toxic effect on normal human cells. However, at higher concentrations than required for IC_{50}, the extract inhibits cell growth in fibroblasts or even induces cell necrosis. Other morphological alterations in cells include spindle-shaped cells convert to round cells, development of vesicles inside the affected cells, dissociations in the intercellular connections, and creating dark-colored nuclei [31, 41]. Also, in the experiments performed by Hassanzadeh, et al. [41], significant lethal effects were not induced in fibroblasts after a 24-hour treatment.

However, lethal effects emerged at a concentration of 12 mg/mL of the extract after 72 hours of exposure. Also, the survival rate of the cells was about 81% [41], which was consistent with the cell viability documented in the current study. Thus, garlic extract has shown very little lethal effects on normal human cells. Conversely, the sulfur compounds, such as allicin, diallyl sulfides, diallyl disulfides, diallyl trisulfides, flavonoids, and saponins in garlic extract can cause cytotoxicity and cell apoptosis. Available data from research indicate that diallyl trisulfides binds to reactive oxygen species, causing cytotoxicity and lead to apoptosis via caspase activation pathway [42, 43].

According to reports, certain doses of garlic extract do not have significant toxic effects on normal cells. Interestingly, the extract at low doses is believed to stimulate fibroblasts proliferation [44]. Conversely, the results from a previous study have shown that the percent- age of survival and proliferation of gingival fibroblasts increase at any concentrations of garlic extract, when treated for 24, 48, or 72 hours. Variations in cell survival may be associated with the effect of garlic's active compounds, such as flavonoids, and allicin [45]. Previous research has suggested that Allicin heals human skin wounds by inhibiting inflammatory cells, and increases the fibroblasts density and collagen synthesis [46, 47]. The presence of flavonoids leads to increased proliferation of fibroblasts in the wound by activating various growth factors [48]. Therefore, it may be noted that *A. sativum* extract has differing functions depending on the cell types and the dosage used [31, 41].

Based on earlier research, the essential oil in the *M. communis* extract has a mild antioxidant effect but a significant cytotoxic property [49]. In addition, it has been found that the viability percentage (47%) of *M. communis* extract after a 48 hr treatment was low compared to that of *A. sativum* extract. This effect might be associated with the polyphenol (2.3%) in Myrtaceae extract that may be toxic to some cells [33].

Ample studies have not been attempted to fully elucidate the toxicity of *M. communis* extract on human fibroblasts. In a previous study [50], it has been shown that *M. communis* extract does not adversely affect fibroblasts [50]. However, other studies [31, 39] have demonstrated that *M. communis* extract has active compounds, such as polyphenols, myrtucommulone, semi-myrtucommulone, 1,8-cineole, a-pinene, myrtenyl acetate, limonene, linalool which may induce cell death.

Conclusions

Both extracts from *A. sativum* and *M. communis* demonstrated good antimicrobial properties.

Overall, using plant-based products, i.e. pharmaceutical plants as antimicrobial agents against numerous microorganisms could be one of the main ways to prevent infections and treat pathogens strongly resistant to antibiotics. Based on the results of this study, the safe antibacterial potential of *A.sativum* and *M. communis* may be used as potential agents to fight against nosocomial infections.

Ethical Considerations

Compliance with ethical guidelines

The protocol of this study was reviewed and approved by the Ethics Committee, Iran University of Medical Sciences (Code: IR.IUMS.REC1394.26604).

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Authors' contributions

Writing of the original draft and laboratory activities: Somayeh Soleymanzadeh Moghadam and Samaneh Mazar Atabaki; Methodology: Pedram Ebrahimnejad and Zahra Mohammadi; Project Administration: Maliheh Nobakht; Supervision: Maliheh Nobakht and Pedram Ebrahimnejad; Review and final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

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