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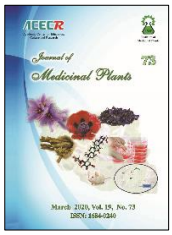

Research Article

The effect of subinhibitory concentrations of *satureja* spp. essential oils on the biofilm formation and urease activity of *Klebsiella pneumoniae*

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ABSTRACT

Background: *Klebsiella pneumoniae* is one of the most important bacteria that cause nosocomial infections. This opportunistic pathogen has a high potential for antibiotic resistance and can generate a thick layer of biofilm. Nowadays, antibiotic resistant strains are emerging and widely spreading worldwide. Thus, it is necessary to combat drug-resistant strains through the use of novel drugs (such as medicinal plants). **Objective:** This study aimed to evaluate the in vitro antibacterial activity of *Satureja rechingeri* Jamzad, *S. khuzestanica* Jamzad, *S. bachtiarica* Bunge, and *S. mutica* Fisch. & C.A.Mey. essential oils against *K. pneumonia* ATCC 700603. **Methods:** For evaluation of the minimal inhibitory concentration (MIC) of essential oils, broth microdilution method was used. The microtiter plate assay method was used for the assessment of anti-biofilm activities of sub-MIC value of essential oils. Colorimetric and Iodometric assays were used for determination of urease and beta-lactamase activity. **Results:** According to data collected, the MIC value of essential oils was 4096 µg/mL. sub-MIC value of essential oils inhibited biofilm formation and urease activity of *K. pneumoniae*. However, *S. khuzestanica* had more activity. None of the essential oils caused a significant decrease in beta-lactamase activity of *K. pneumoniae*. **Conclusion:** Based on our analysis *S. khuzestanica* had a good antibacterial, anti-biofilm activities and urease inhibitory effects against *K. pneumoniae*, but additional studies are required to investigate the exact mechanisms of the antibacterial action and functions of this phytocompound.

1. Introduction

Klebsiella pneumoniae is one of the most important nosocomial pathogens, causing a variety of infections such as urinary tract infections, hospital-acquired pneumonia (HAP), ventilator-associated pneumonia (VAP), surgical-wound infection, bacteremia, and septicemia [1]. It has been reported that, among

Abbreviations: BHI, Brain heart infusion; CLSI, Clinical and Laboratory Standards Institute; ESBLs, extended-spectrum beta-lactamases; MIC, Minimum inhibitory concentration; NB, nutrient broth; PBS, phosphate-buffered saline.

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immunocompromised patients or persons with certain debilitating medical conditions such as diabetes mellitus or bladder neuropathy, *K. pneumoniae* was recovering with high frequency and caused severe opportunistic infections in such cases [2]. *K. pneumoniae* has important virulence factors which contribute to its pathogenesis such as capsular polysaccharides, type 1, and type 3 pili and urease. Moreover, it has a high capacity of biofilm formation among *K. pneumoniae* isolates [1]. Therefore, it can produce a thick layer of extracellular biofilm, which is considered as a virulence factor. Biofilm formation can help the bacteria to attach to living or abiotic surfaces and protect the bacteria from the immune system and also effects on antimicrobial agents [3]. This capacity of *K. pneumoniae* leads to treatment failure in some cases. Moreover, almost all clinical strains of *K. pneumoniae* have different types of beta-lactamase enzymes such as extended-spectrum beta-lactamases (ESBLs), AmpC beta-lactamases and carbapenemases, which are contributing to emergence and distribution of drug resistance strains worldwide [2, 4].

There is an increased interest in utilizing natural products for medicinal purposes. Many studies were conducted on medical herbs and this field is expected to expand in the future [5]. Among the studies performed on medical herbs, Lamiaceae (Labiatae) family are of pharmaceutical interest because of their potential antimicrobial properties. This family is distributed throughout the world and is consisted of more than 7000 species [6]. The geographical analysis showed that *Satureja* species (members of the Lamiaceae family) are found in mountainous areas in Iran, mainly in Western and Northern parts of the country [6]. These species were formerly used as a traditional medicine to treat various diseases such as cramps, muscle pains, nausea, indigestion, diarrhea, and infectious diseases. It has been reported that these species have many effects, such as antispasmodic, antidiarrheal, antioxidant, sedative, and antimicrobial activities [7].

Therefore, this study was initiated to evaluate the *in vitro* antibacterial and anti-biofilm activities and also inhibitory effects of four Satureja spp. essential oils on urease and beta-lactamase enzymes of *K. pneumoniae*.

2. Materials and Methods

2.1. Essential oils and Bacterial strain

Satureja rechingeri Jamzad, *S. khuzestanica* Jamzad, *S. bachtiarica* Bunge, and *S. mutica* Fisch. & C.A.Mey essential oils were donated by Research Institute of Forests and Rangelands, Agricultural Research Education and Extension Organization (AREEO), Tehran, Iran. *K. pneumoniae* ATCC 700603 standard strain was donated by Pasteur Institute of Iran. This bacterium was kept at -70°C in nutrient broth containing 15% glycerol.

2.2. Determination of Minimum inhibitory concentration (MIC)

For determination of MIC values of herb's essential oils, broth microdilution method was used according to the Clinical and Laboratory Standards Institute (CLSI) guideline with a few modifications [8]. Serial dilutions of the essential oils were prepared in Mueller-Hinton Broth (Himedia, India), from 16 to 8192 µg/mL concentrations. The bacterial suspension was prepared in Mueller-Hinton Broth, and the turbidity was adjusted to 0.5 McFarland. Then, the suspension diluted to achieve approximately, 5×10^5 CFU/mL in wells. After 24hrs incubation at 37°C, the MIC was defined as the lowest concentration of essential oil that completely inhibits the growth of the organism.

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2.3. Determination of the anti-biofilm activity of essential oils

To evaluate the anti-biofilm activities of the essential oils, 96-well flat-bottomed sterile polystyrene microplates were used. First, the bacterium was cultured in Brain heart infusion broth (BHI broth) for 24 hours. This culture was diluted 1:100 with BHI broth medium containing the sub-MIC values of essential oils and the number of cells in suspension was adjusted to 10^8 CFU/ml. Then, 200 µL of this suspension was transferred into wells of microplate and incubated at 37 °C for 24 hours. Following incubation, the microplates were gently washed using 200 µL phosphate-buffered saline (PBS, pH: 7.4). After the washing, the microplates were stained with 0.1% (w/v) crystal violet for 20 min at room temperature, and then washed twice with distilled water. The biofilm was solubilized using 200 µL of 33% Acetic acid for 20 min. Finally, the optical density of each well was quantified at 570 nm using a microplate reader. BHI broth with and without bacterial suspension was incorporated as a positive and negative control, respectively [9].

2.4. Determination of the inhibitory effect of essential oils on urease activity

Inhibitory effect of essential oils on urease activity of tested strain was determined by modified procedure of the colorimetric assay described by Hamilton-Miller and Gargan [10]. Accordingly, bacteria were grown overnight on urea broth (0.1 g/L yeast extract, 9.1 g/L KH_2PO_4, 9.5 g/L Na_2HPO_4, 20 g/L urea, phenol red 0.01 g/L; Merck, Germany) containing sub-MICs of essential oils (1/2× to 1/8×MIC). Urea broth with and without essential oils was used as a positive and negative control, respectively. All tubes were incubated for 24 hours at 37 °C. Following incubation, the suspensions were centrifuged for 3 min at 5000×g, and the supernatant was used for determination of urease activity. Color intensity was measured using a spectrophotometer at 560 nm.

2.5. Determination of the inhibitory effect of essential oils on beta-lactamase activity

To determine the inhibitory effect of essential oils on beta-lactamase activity, Micro-Iodometric Assay was used [11]. The beta-lactamase producing *K. pneumoniae* ATCC 700603 was grown overnight at 37°C in nutrient broth (NB) supplemented with amoxicillin and sub-MIC concentration of four essential oils separately. Bacterial suspension supplemented with amoxicillin but without essential oils and NB supplemented with amoxicillin but without bacteria were used as the positive and negative controls respectively. Following incubation, bacterial cells were harvested and re-suspended in phosphate buffer (pH 7) and lysed by ultrasonicator. Afterwards, cell lysates, which contained cell enzymes were centrifuged and used for further assays. Cefetoxime and penicillin were used as substrate. According to the protocol, substrate and lysate were mixed in starch-iodine solution; finally, the optical densities of starch-iodine solutions were measured at 630 nm using a microplate reader.

2.6. Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) test with the SPSS software version 22.0. All experiments were performed in triplicate. The results are reported as mean ± standard deviation (SD). pvalues less than 0.05 were considered statistically significant.

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3. Results

The MIC value of four essential oils against *K. pneumoniae* ATCC 700603 were found 4096 µg/mL. In this regards, 2048 µg/mL of each essential oil was considered as ½ MIC.

The biofilm formation of *K. pneumoniae* ATCC 700603 was significantly inhibited by ½ and ¼ MIC concentrations of all four essential oils compared to the control (P<0.05) (table 1 and Fig. 1). *S. khuzestanica* had more activity than others because, at 1/8 MIC concentration, only this essential oil caused a significant reduction in biofilm formation.

The urease activity of *K. pneumoniae* ATCC 700603 was inhibited by ½ to $^{1}/_{8}$ MIC concentration of all tested oils, except for *S. mutica* whereby its activity was inhibited by ½ and ¼ MIC concentrations (P < 0.05). However, the most inhibition effect was for *S. khuzestanica* essential oil (P < 0.05) (Table and Fig. 2).

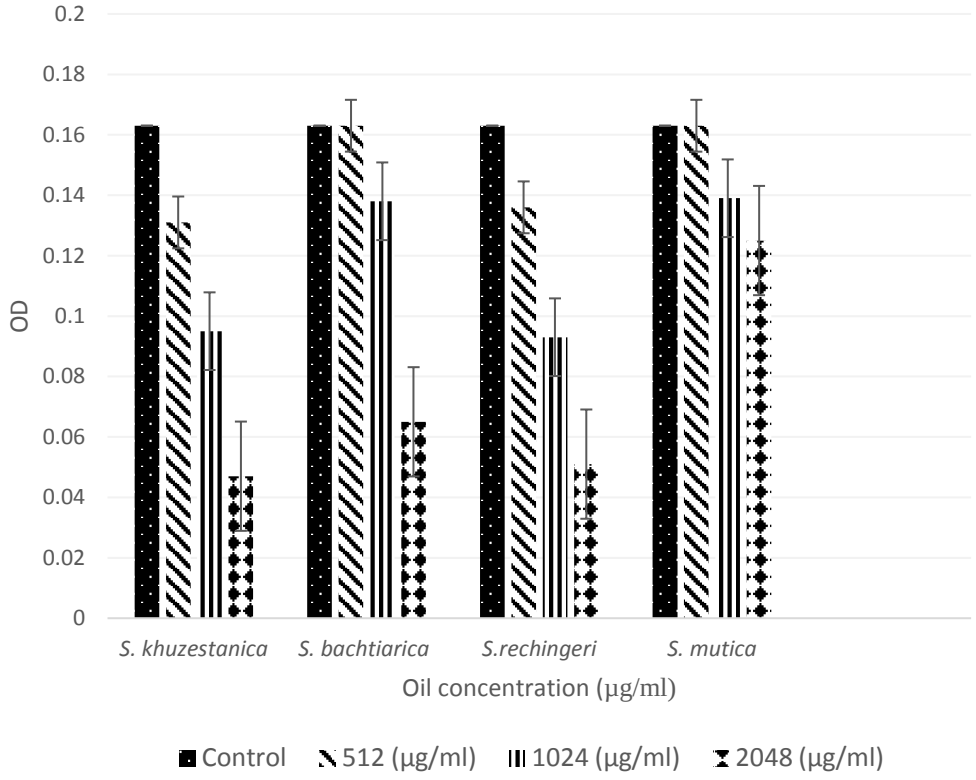

Fig. 1. The anti-biofilm activity of different *Satureja* spp. essential oils against *K. pneumoniae* in 1/2 to 1/8 MIC concentration

Table 1. The anti-biofilm activates of four *Satureja* spp. essential oils against *K. pneumoniae*

Sample	Positive Control	*S. bachtiarica*			*S. khuzestanica*			*S. rechingeri*			*S. mutica*		
		1/2 MIC	1/4 MIC	1/8 MIC	1/2 MIC	1/4 MIC	1/8 MIC	1/2 MIC	1/4 MIC	1/8 MIC	1/2 MIC	1/4 MIC	1/8 MIC
Mean OD	0.163	0.065*	0.138*	0.164	0.047*	0.95*	0.131*	0.051*	0.093*	0.136	0.125*	0.139*	0.164

*P<0.05

Table 2. Inhibition of *K. pneumoniae*'s urease activity by sub-MIC concentrations of four *Satureja* spp. essential oils

Sample	Positive Control	*S. bachtiarica*			*S. khuzestanica*			*S. rechingeri*			*S. mutica*		
		1/2 MIC	1/4 MIC	1/8 MIC	1/2 MIC	1/4 MIC	1/8 MIC	1/2 MIC	1/4 MIC	1/8 MIC	1/2 MIC	1/4 MIC	1/8 MIC
Mean OD	0.433	0.046*	0.093*	0.18*	0.036*	0.087*	0.16*	0.04*	0.085*	0.21*	0.21*	0.3*	0.43

*P<0.05

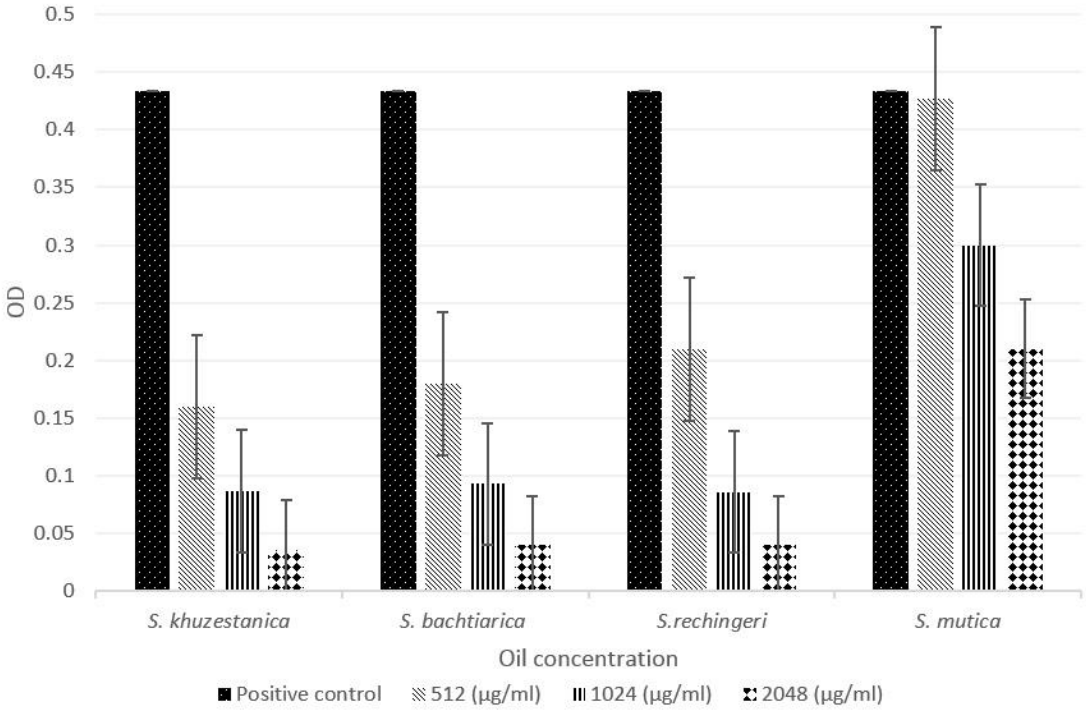

Fig. 2. Inhibition of *K. pneumoniae*'s urease activity by sub-MIC concentrations of four *Satureja* spp. essential oils

4. Discussion

In the present study, the antibacterial and anti-biofilm formation activities and also inhibitory effects of essential oils of four Satureja species on urease and beta-lactamase enzymes of *K. pneumoniae* were analyzed. According to our results, the MIC value of essential oils was 4096 µg/ml. Sub-MIC value of essential oils inhibited biofilm formation of *K. pneumoniae*. However, *S. khuzestanica* had more activity, and *S. mutica* had less effect than others.

Gulluce *et al*. conducted a study on antibacterial and antifungal properties of *S. hortensis*' essential oil against 23 and 15 pathogenic bacteria and fungi isolates, respectively. They reported that the essential oil has a great antimicrobial effect against all tested organisms [7]. The maximum MIC value of this essential oil ranged from 31.25 to 500 µg/mL, which is comparable to our finding but it had more effect than *S. khuzestanica*.

Taebi *et al*. in 2017 evaluated the antibacterial activity and biofilm inhibition of *S. khuzestanica* against *Streptococcus mutans* [12]. According to their result, the extract of this herb had significantly antibacterial activity against *S. mutans*. The MIC value of *S. khuzestanica* against *S. mutans* was 3000 µg/mL which is similar to our results but the main difference is related to different bacterial species and there used extract. Also, they reported that this herb could inhibit biofilm formation of *S. mutans* in >1500 µg/mL of concentration. According to our analysis, the biofilm formation of *K. pneumoniae* was inhibited by *S. khuzestanica* in 512 µg/mL. Therefore, *S. khuzestanica* oil showed more anti-biofilm formation activity against *K. pneumoniae* than *S. mutans* which is also reported by Taebi *et al*. [12].

Recently, the anti-biofilm activity of *S. khuzestanica* is confirmed by bioinformatics tools. It is reported that phytochemical compounds, especially gamma-terpinene, carvacrol and beta-bisabolene can interfere and also inhibit glucansucrase enzyme. The glucansucrase enzyme has a crucial role in biofilm formation of *S.mutans* which can be targeted by *S. khuzestanica* oil and extract very well [12].

In this regards, some studies reported the antibacterial and anti-biofilm activities of different essential oils [5, 13]. Amaral et al. (2015) evaluated the effects of carvacrol and thymol on *Salmonella* spp. biofilm [13]. Carvacrol is a major component of the essential oils of *Satureja* species [7]. According to these studies, the greatest reduction in carvacrol-treated biofilms was observed in 156 and 312 µg/mL. In another study, effect of *Satureja* essential oils on biofilm formation of *Staphylococcus aureus* were evaluated by Taghian et al [14]. According to their report, sub-MIC value of *S. khuzestanica* and *S. rechingeri* were 128 µg/mL, while *S. bachtiarica* and *S. mutica* were 256 and 512 µg/mL, respectively. Biofilm formation was inhibited by sub-MIC value of all tested essential oiles, but *S. mutica* was reported as a greatre effect than others. It is not sourprisingly because the sub-MIC value of *S. mutica* was more than another oils. In comparing to their results, *S. mutica* oils had more effects on Gram positive bacteria, but still *S. khuzestanica* and *S. bachtiarica* are have the most antibacterial effects on both Gram positive and negative bacteria.

It should be noted that different results and reported are related to the nature of herbal species. The chemical compounds of essential oils depend on climatic, seasonal, and geographic conditions, harvest period, and distillation technique. In addition, their antibacterial properties of herbs depending on the type, effective compounds and its concentration in the essential oils, and is even influenced by the processing and the storage conditions [15].

According to a research report, each antimicrobial agent which considered as a disinfectant must have ability to reduce 3 logs of the microbial population attached to a surface [16]. Interestingly in the present study, *S. khuzestanica* showed more than 90% activity against biofilm formation of *K. pneumoniae*.

In *K. pneumoniae* 13 genes have been identified that encode factors required for in vivo colonization of the gastrointestinal tract using signature-tagged mutagenesis. Among these genes, nitrogen metabolism genes, specifically urea metabolism gene is crucial. Therefore, urease targeting as an antibacterial strategy is responsible for impairing the colonization capacity of *K. pneumoniae* [17]. In this case,

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recently, urease inhibitors or blocker have designed and developed as potential new antibacterial drugs [18]. Drakhshande *et al*. (2008) reported that essential oil alcoholic extract of *Cuminum cyminum* inhibits urease activity of *K. pneumoniae* [19]. According to our analysis, all of the tested oils in sub-MIC concentrations could inhibit urease activity compared to the control.

5. Conclusion

As a conclusion, according to the results, it can be said that all *Satureja* species essential oils evaluated in this study had antimicrobial activity and were able to decrease the production of *K. pneumonia* virulence factors. Among the essential oils tested, *S. khuzestanica* had the most inhibitory effect on reduction of *K. pneumonia* virulence factor production. Therefore, further studies on the antimicrobial effects of the compounds of these plants are recommended.

Author contributions

Fateme Yazdani participated in set up and perform tests. Iraj Rasooli participated in consulting and design. Fatemeh Sefidkon participated in preparation of essential oils. Navid Saidi participated in set up and perform tests. Parviz Owlia participated in providing and executor.

Conflict of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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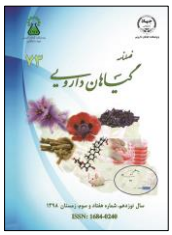

مقاله تحقیقاتی

اثرات مهاری گونه‌های مرزه علیه تشکیل بیوفیلم و فعالیت اوره‌آزی *کلبسیلا پنمونیه* در غلظت‌های کمتر از غلظت بازداری

فاطمه یزدانی[۱]، ایرج رسولی[۱]، فاطمه سفیدکن[۲]، نوید سعیدی[۱]، پرویز اولیاء[۱،*]

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اطلاعات مقاله

گل‌واژگان:
کلبسیلا پنومونیه
مرزه
بتالاکتاماز
بیوفیلم
اوره آز

چکیده

مقدمه: کلبسیلا پنومونیه یکی از مهم‌ترین باکتری‌هایی است که موجب عفونت‌های بیمارستانی می‌شود. این پاتوژن فرصت طلب، دارای پتانسیل بالایی در ایجاد مقاومت آنتی‌بیوتیکی بوده و می‌تواند تشکیل لایه ضخیمی از بیوفیلم بدهد. امروزه سویه‌های مقاوم به آنتی‌بیوتیک در حال ظهور و گسترش در دنیا می‌باشند. بنابراین مقابله با سویه‌های مقاوم به دارو به وسیله داروهای نوین (مانند گیاهان دارویی) ضروری است. **هدف:** این مطالعه با هدف بررسی اثر ضد باکتریایی اسانس‌های *Satureja rechingeri* Jamzad ،*S. khuzestanica* Jamzad ،*S. bachtiarica* Bunge و *S. mutica* Fisch. & C.A.Mey. در شرایط آزمایشگاهی علیه کلبسیلا پنومونیه ATCC 700603 انجام شد. **روش بررسی:** برای بررسی حداقل غلظت بازدارندگی (MIC) اسانس‌ها، روش براث میکرودایلوشن استفاده شد. روش میکروتیتر پلیت برای ارزیابی اثرات ضد بیوفیلمی مقادیر sub-MIC اسانس‌ها استفاده شد. از روش رنگ‌سنجی و یدومتریک برای تعیین فعالیت‌های اوره‌آزی و بتالاکتامازی استفاده شد. **نتایج:** بر اساس نتایج جمع‌آوری شده، مقدار MIC اسانس‌ها ۴۰۹۶ µg/ml بود. مقادیر sub-MIC اسانس‌ها تشکیل بیوفیلم و فعالیت اوره‌آزی کلبسیلا پنومونیه را مهار نمود، اسانس *S. khuzestanica* بیشترین تاثیر را از خود نشان داد. هیچ‌کدام از اسانس‌ها نتوانستند به صورت معناداری فعالیت بتالاکتامازی کلبسیلا پنومونیه را کاهش دهند. **نتیجه‌گیری:** بر اساس بررسی انجام شده *S. khuzestanica* دارای اثر ضد باکتریایی، ضد بیوفیلمی و مهارکنندگی اوره‌آز خوبی بر علیه کلبسیلا پنومونیه بود، ولی مطالعات بیشتری برای شناخت عملکرد دقیق و مکانیسم ضد باکتریایی این ترکیب گیاهی مورد نیاز است.

مخففها: (BHI) Brain heart infusion; (CLSI) Clinical and Laboratory Standards Institute; (ESBLs) extended-spectrum beta-lactamases; (MIC) Minimum inhibitory concentration; (NB) nutrient broth, (PBS) phosphate-buffered saline

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