Naturally Derived Efflux Pump Inhibitor among Tetracycline-Resistant *Acinetobacter baumannii* Isolates

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Abstract

Background: The antimicrobial resistance of *Acinetobacter baumannii* is of major concern. It is one of the commonly distributed nosocomial pathogens, and its strains are frequently reported to demonstrate resistance to the most routinely prescribed antibiotics in varying degrees. **Objective:** This study aimed to detect the inhibitory effect of chamomile extract against the efflux pump of *A. baumannii.* **Materials and Methods:** Clinical samples (125) were collected from different sites (urine, sputum, wound, and burn swabs), followed by *A. baumannii* isolation and identification, and detection of tetracycline susceptibility test by disk-diffusion. Later on bacterial efflux pump activity was detected by ethidium bromide cartwheel assay and water extract of chamomile was used as an inhibitor of efflux pump *A. baumannii*. **Results:** The percentage of *A. baumannii* isolation 23/125 (18.4%) that distributed as 25% isolates were obtained from sputum, 17.9% isolates from burns, whereas 16% and 12.5% isolates from urine and wounds respectively. It showed various resistances against different members of the three generations of tetracyclines, with the highest resistance against minocycline 47.8% followed by resistance to tetracycline and doxycycline as 43.5% and 39%, respectively. Efflux pump was detected in all cases, water extract of chamomile showed a significant inhibitory effect especially at 80 mg/mL concentration. **Conclusion:** Efflux pump is an important mechanism for tetracycline resistance among *A. baumannii* isolates, and the watery extract of chamomile can be considered a prompt inhibitor for this pump.

Keywords: *Acinetobacter baumannii*, chamomile, efflux pump, tetracycline resistance

Introduction

Acinetobacter species are found in abundance in the environment, as *A. baumannii* is responsible for approximately 90% of all *Acinetobacter spp*. clinical infections in humans. Its ability to withstand harsh conditions, such as desiccation and disinfection, contributes to its longevity and spread in hospital settings. *Acinetobacter spp*. have developed a variety of resistance strategies. *A. baumannii* is resistant to dehydration, ultraviolet (UV) radiation in addition to common chemical sanitizers and detergents, making it extremely difficult to eradicate the increasing resistance of *A. baumannii* to antimicrobial drugs, with persistence in hospital environments and propensity to cause outbreaks, as it can persist for a prolonged period in harsh environments (walls, surfaces, and medical devices) in the hospital settings.[1] The nosocomial infections that are mostly caused by *A. baumannii* isolates include ventilator-associated pneumonia, endocarditis, secondary meningitis, urinary tract infection (UTI), and bloodstream infections. Also, it can cause septicemia, pneumonia, burns, wounds, and soft-tissue infections especially in the intensive care unit (ICU) and burn unit. *A. baumannii* may colonize the skin, oropharynx, and gastrointestinal tract without causing infection. However, among immunocompromised hosts, particularly patients in ICU settings, *A. baumanniii* may cause serious infections including bacteremia and wound infections.[2]

A. baumannii resists antibiotic action through various mechanisms, including target alteration, drug inactivation,

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decreased permeability, and increased efflux. *A. baumannii* is known to have intrinsic resistance to several classes of antibiotics, including penicillins, first-generation cephalosporins, aminoglycosides, and fluoroquinolones. This resistance is primarily due to the presence of efflux pumps, which actively pump out antibiotics from the bacterial cell before they can exert their antimicrobial effect. In addition, *A. baumanniii* has a low permeability outer membrane, which restricts the entry of hydrophilic antibiotics, such as beta-lactams, and contributes to their reduced susceptibility.[3]

Tetracyclines are a class of antibiotics that have been used to treat *A. baumanniii* infections, but their efficacy has been limited by the emergence of resistant strains, and the most important mechanisms of resistance to tetracyclines in *A. baumannii* isolates are efflux pumps followed by ribosomal protections and enzymatic inactivation.[4]

Resistance to the first and second generations of tetracyclines in *A. baumannii* isolates mainly resulted from the acquired major facilitator superfamily efflux pumps, including *tetA*, *tetB*, *tetG*, *tetH*, *tetL*, and *tet39,* resistance–nodulation–division (RND) family efflux pumps nominated as *adeABC*, *adeIJK*, *adeFGH*, *adeM*, and *adeDE*, and, finally, ribosomal protection *tetM* and enzymatic inactivation. *tetA* is responsible for the resistance to tetracycline and doxycycline, whereas *tetB* has been found in the isolates that were also resistant to minocycline. It has also been found that Tet(A) pump acts synergistically with the RND superfamily of efflux pumps, such as *adeABC* and *adeIJK*, serving as an important resistance mechanism of resistance to tigecycline in *A. baumannii*.[5]

This continuous emergence of antibiotic-resistant bacteria urges the search for alternative therapeutic options. A combination therapy of efflux pump inhibitors (EPIs) and antibiotics can be a promising option for the efflux-mediated bacterial resistance. For any compound to be used as an EPI, it must be selective, not target any eukaryotic efflux pumps, and have ideal pharmacological properties, such as nontoxicity and a high therapeutic effect. Many EPIs have been discovered, but none of them was clinically approved because of their low potency, narrow activity spectrum, inappropriate pharmacokinetics, or high toxicity.[6]

Matricaria chamomilla also called *Matricaria recutita* is from the *Asteraceae* family. In various cultures, the so-called chamomile is one of the prominent plants in traditional medicine as it is rich in active ingredients; it was reported to treat colic and diarrhea as well as mouth, throat, and ear infections and infections in general. It, similar to other medicinal herbs, is a rich source of various biologically active compounds that are produced as secondary metabolites.[7]

Chamomile has exhibited bactericidal activity, with 120 chemical constituents having been identified as secondary metabolites, including 28 terpenoids, 36 flavonoids, and 52 additional compounds with potential pharmacological activity and anti-inflammatory activities that have been attributed to some of chamomile's active ingredients, especially the flavonoids (anthemidin, apigenin, and luteolin, among others), bitter glycosides, and coumarins (herniarin and umbelliferone) and its volatile oils (containing alpha bisabolol and matricine, among others).[8]

Thus, according to the problem of widespread of *A. baumannii* infections and its tetracycline resistance due to efflux pump, this study aimed to attain natural alternatives for antibiotics.

Materials and Methods

Sample collections

One hundred twenty-five (125) clinical specimens were collected during the present study period extended from the beginning of November 2022 to the end of January 2023, from patients who were attended to hospital; it was collected from different clinical sites suggested to have infection such as urinary and respiratory infections, and burn and wound infections. Mid-stream urine from patients suffering from symptoms of UTIs, swabs from burns and wounds, and sputum were collected from patients before they take any antibiotics or cleaning. Each sample was cultured on different media including MacConkey and nutrient agar then incubated aerobically at 37°C for 24 h, followed by Gram staining and biochemical tests for identification of *A. baumannii*.

Antibiotic susceptibility test

The Kirby-Bauer method was used to perform the antibiotic susceptibility test for four tetracyclines' members; where the bacterial suspension was prepared, and turbidity was adjusted to obtain approximately 1.5×10^8 colony forming unit/mL (MacFarland 0.5 tube). By a sterile cotton swab, a portion of bacterial suspension was transferred and evenly spread on Mueller-Hinton agar medium. Then, after the antimicrobial disks were placed on the agar surface, it was incubated at 37°C for 24 h. Inhibition zones that developed around the disks were measured by millimeter unit using a metric ruler. The isolate was interpreted as susceptible, intermediate, or resistant to a particular antibiotic according to clinical and laboratory standard institute.[9]

Detection of efflux pump activity

Efflux pump activity was evaluated using the ethidium bromide-agar Cartwheel method as prescribed by Martins et al.[10] Plates of Trypticase Soy Agar (TSA) were prepared, and before it was solidified, Ethidium Bromide (EtBr) was added in different concentrations

ranging from 0.0 to 2.5 mg/L. The TSA plates should be prepared freshly on the same day of the experiment and kept protected from light.

Overnight cultures of the bacterial isolates were prepared in liquid media, and on the following day, their concentration was adjusted to McFarland 0.5. The TSA plates are then divided into as many as eight sectors by radial lines, forming a cartwheel pattern. The adjusted bacterial cultures are then swabbed on the EtBr-TSA plates starting from the center of the plate to the margin. The TSA plates were then incubated at 37°C overnight. After this period, the TSA plates will be examined using a UV transilluminator for fluorescence, and the TSA plates were photographed. Isolates without fluorescence indicated active efflux pump activity, whereas those that fluoresced lacked efflux pump activity. Also, the minimum concentration of EtBr that produces fluorescence to be recorded.[11]

Preparation of aqueous extract of *Matricaria chamomilla*

The dried flowers of chamomile were bought from the market and primed for the preparation of its aqueous extract, and the stock solution was prepared as w/v suspension in a flask by adding hot boiled water. The flask was then placed on a shaker (200 rpm) for 4 h, the temperature was maintained at 37°C, and containers were covered to keep all active elements. Later on, the solution was filtered using filter paper, sterilized using Millipore filter (0.45), and kept at 4°C until the time for further applications, where multiple dilutions were prepared as 5, 10, 20, 40, and 80 mg/mL to be used as antimicrobial drugs and antiefflux pumps.[12]

Efflux pump–inhibitory effect of Matricaria *chamomilla*

Ethidium bromide-agar Cartwheel method used to detect efflux pump activity was repeated with the application of different concentrations of aqueous Chamomile extract to detect if there is an efflux pump–inhibitory effect of the Chamomile extract.

Ethidium bromide efficiently effluxes out and only accumulates in cells in the presence of an EPI and emits strong fluorescence. Six isolates positive for efflux pump were applied in this part of the study, with the application of only two concentrations of the extract (40 and 80 mg/mL).

Ethical approval

The necessary ethical approval was taken from the ethical committee of the College of Medicine/University of Babylon and Al-Hilla General Teaching Hospital according to the document number 310 (on October 26, 2022), Moreover, agreement from the family and patients for sampling and carrying out this work was obtained.

Results

The present study showed that A. baumannii isolation rate was 23/125 (18.4%), that distributed as 10/40 (25%) isolates were obtained from sputum, 5/28 (17.9%) isolates from burns, whereas 4/25 (16%) and 4/32 (12.5%) isolates were obtained from urine and wounds respectively, as shown in Figure 1.

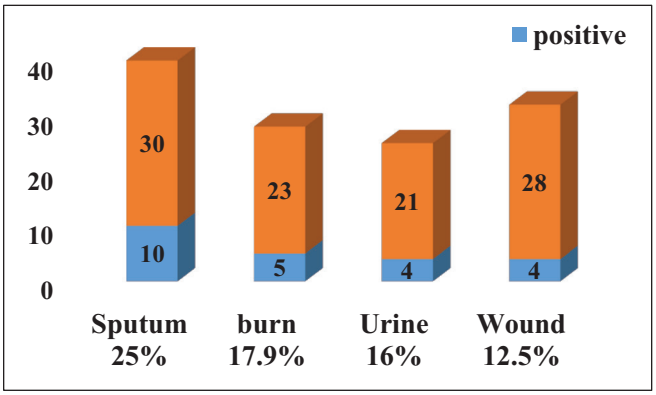

Figure 1: Distribution of *A. baumanii* according to clinical specimen

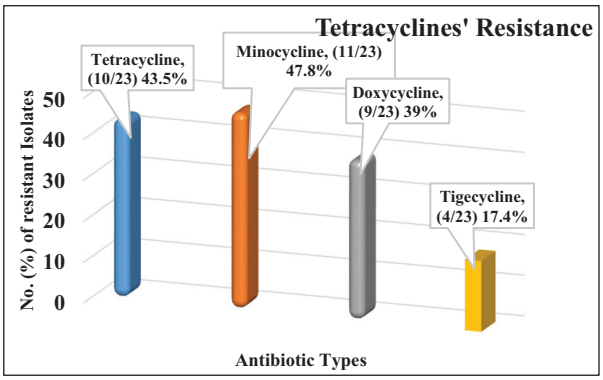

Figure 2: Frequency distribution of tetracyclines resistance among *A. baumannii* clinical isolates

Isolates of *A. baumannii* grow well on blood agar and MacConkey agar. On blood agar, it forms colorless, nonhemolytic, shiny mucoid colonies, smooth in contexture with a diameter of 1–2 mm after 18–24 h of incubation at 37°C. It produces colorless colonies on MacConkey agar, which are shiny mucoid colonies, indicating its nonlactose fermenting ability.

These results showed various distribution patterns of resistance against different members of the three generations of tetracyclines, with the highest resistance against minocycline 11/23 (47.8%) followed by resistance to tetracycline and doxycycline as 10/23 (43.5%) and 9/23 (39%), respectively. Moreover, resistance to tigecycline about 4/23 (17.4%), as in Figure 2.

Results in Figures 3 and 4A and B showed that 2/10 (20%) isolates exhibited fluorescence at all plates with different concentration gradients of EtBr in comparison with controls, which mean that these two isolates had no phenotype of efflux pump, whereas the other 8/10 (80%) isolates showed no fluorescence at lower concentration gradients and started to fluorescence 5/8 at 2 mg/L

and more, but the remaining 3/8 did not fluorescence even at 2.5 mg/L of EtBr similar to the positive control *A. baumannii* American type culture collection, which mean that these eight isolates expressing an active efflux pump that has the ability to expel its substrate (EtBr) outside the bacterial cell and, thus, did not fluorescence on exposure to UV light.

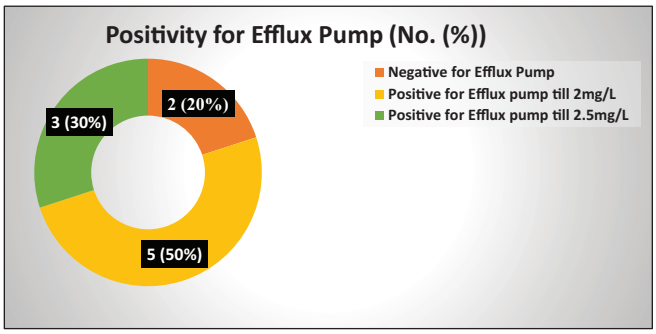

Figure 3: Positivity for efflux pump (No. [%]) phenotype production by *A. baumannii* clinical isolates by the application of Cartwheel assay

Application of aqueous extract of *M. chamomilla* in different concentrations (5, 10, 20, 40, and 80 mg/mL) for each one of the *A. baumannii* isolates and their effect was expressed in percentage value and compared by Chi-square as *P* value, as revealed in Figure 5, and there were statically significant differences ($P = 0.032$) between the inhibitory effects of different concentrations. The first two lower concentrations showed a little or no effect in comparison with other higher concentrations and with the effect of antibiotics. These results showed that higher antimicrobial inhibition effect of Chamomile at (80 mg/mL) concentration; as the inhibition for *A. baumannii* growth was in a percentage (78.3%) and (69.6%) for (80 and 40 mg/mL), respectively; while (20 mg/mL) concentration made (47.8%) inhibition effect of *A. baumannii* growth.

Thus, these results showed that the inhibitory activity of the aqueous extract of *M. chamomilla* is proportional

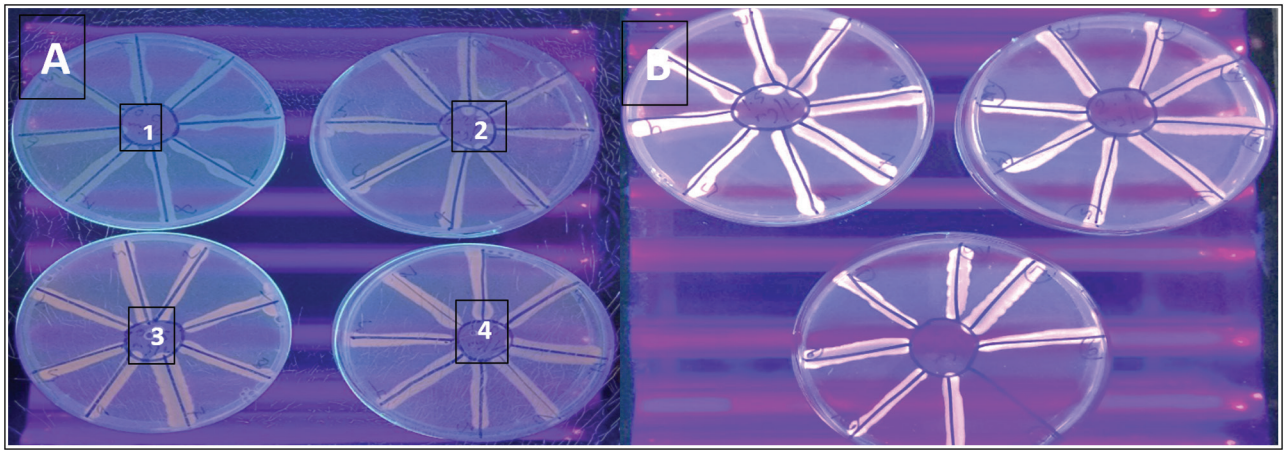

Figure 4: (A and B) The Ethidium Bromide-agar Cartwheel method for detection of efflux pump phenotype production applied to different *A. baumannii* clinical isolates. As A1 is free of Ethidium Bromide, A2, 3, and 4 refer to positive efflux pump with no fluorescence. (B) Negative for efflux pump with fluorescence on UV light visualization. (A) 1 = 0.0 mg/L, 2 = 0.25 mg/L, 3 = 0.5 mg/L, 4 = 1 mg/L. (B) 1.5, 2, 2.5 mg/L

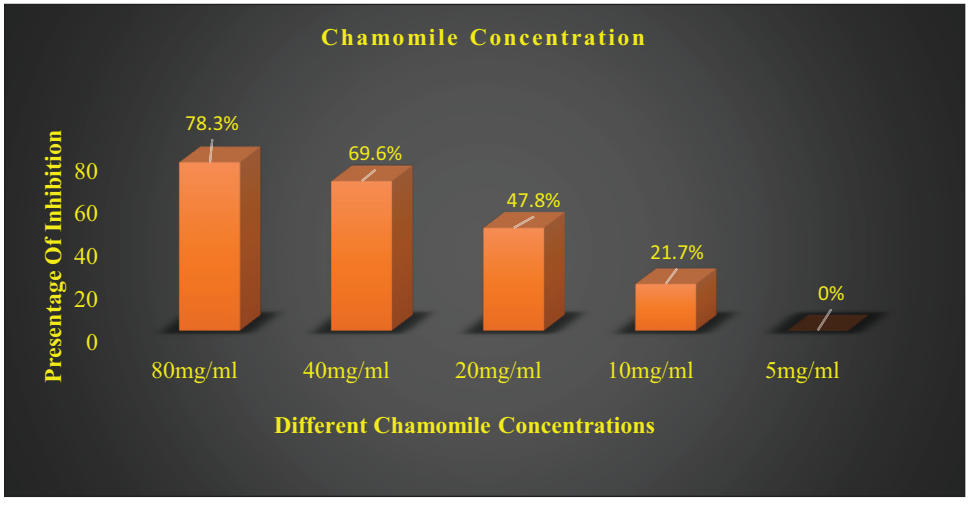

Figure 5: Inhibitory effect of aqueous extract of *Matricaria chamomilla* against *A. baumannii*

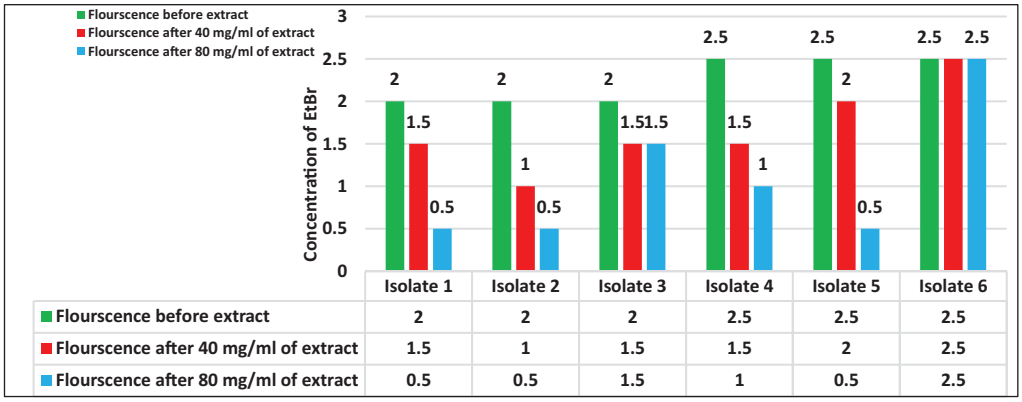

Figure 6: Efflux pump-inhibitory effect of aqueous extract of *Matricaria chamomilla* against *A. baumannii*

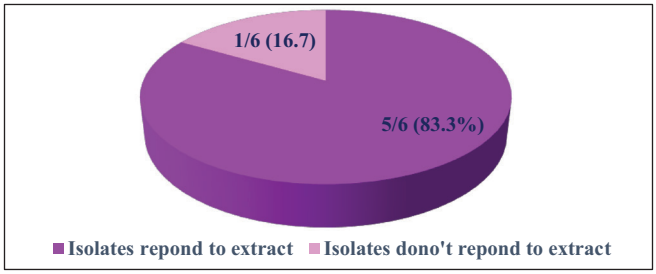

Figure 7: Number and percentage of *A.* baumannii isolates responding to efflux pump-inhibitory effect of aqueous extract of *Matricaria chamomilla*

linearly with its concentration, as it is increase when the concentration increase.

Results of Figures 6 and 7 showed that the fluorescence against UV light appeared more frequently among isolates even with the lower concentrations of EtBr, which is an indicator of loss of efflux pump function, as there was a decreased outflow of ethidium bromide from the bacteria, which increased fluorescence compared with the control. Where 80 mg/mL concentration of the extract inhibited efflux pump of 5/6 (83.3%) isolates, as the inhibition started to some extent at 40 mg/mL and appeared more frankly at the higher concentration. Only one of six (16.7%) isolates does not show any inhibition at both concentrations indicating pronounced efflux activity.

Discussion

The dissemination of *A. baumannii* in clinical samples could be due to its ability to cause different nosocomial infections and resistance to a wide range of antibiotics where most cases in this study were collected from the ICU as their patients are usually on ventilators or other invasive supportive devices as the hospital environment itself is an increasingly important reservoir of *A. baumannii*

In addition, these percentages and variations could be attributed to the collection methods, differences in sample type and size, and antibiotic usages with the hygienic and protective hospital measures to prevent dissemination of infections whether applied efficiently.

A. baumannii are widely distributed in different environments, especially in hospital environment, they reside on medical devices and equipment with their capacity to survive for prolonged periods on inanimate objects. The factors that are responsible for their persistence in a hospital environment are resistance to key antimicrobial drugs and disinfectants and their ability to survive in desiccants.[13]

Different works concerning the study of *A. baumannii* from various clinical sites revealed varying frequencies, where the study by Bayram and Al-Shukri[14] at Babylon City, Iraq, showed that *A. baumannii* was mostly isolated from respiratory tract infection with 27% followed by 14% and 10% from burns and urine, respectively, whereas the lowest from wound was 8%. Also, another study was carried out in Babylon, Iraq, in the same year resulting in 20/100 (20%) overall isolation divided into 7/20 (35%) from burn swabs, 8/20 (40%) from wound swabs, whereas 5/20 (25%) from urine.[15] In comparison with these two studies, a certain study identified only 20 isolates out of 600 specimens (3.33%),[16] Mirazae *et al*.[17] got 9.51%, and Ribeiro *et al*.[18] showed 55.6% of isolation frequency.

A. baumannii has become a common cause of infections associated with high mortality and morbidity and is typically more prevalent in those that are immunocompromised, in ICU and from war wounds. Furthermore, it has become a multidrug-resistant microorganism worldwide leading to a great concern in the medical community. The high resistance rates are likely to be associated with a wide range of empirical and therapeutic use of antibiotics at hospitals. The employed selective pressure by multidrug resistant (MDR) strains is emerging, which, in turn, may have led to the gene encoding resistance mechanisms.[19,20]

Infections by antibiotic-resistant bacteria, especially multiresistant bacteria, are challenging to treat, resulting in serious health issues and even death due to extended hospital stays and unsuccessful treatment attempts. Thus,

in spite of the importance of tetracyclines as a part of *A. baumannii* treatment lines, results showed resistance to all members with varying degrees, even the member of the third generation. Regarding the first and second generations, doxycycline appeared as the effective one (susceptibility rate 61%) compared with both tetracycline and minocycline, whereas tigecycline was the most effective (susceptibility rate 82.6%) in comparison with other tetracyclines' members.[21]

A study by Bayram and Al-Shukri,[14] in Hilla City, showed a resistance rate to tetracycline of 50%, which is close to the present study result, also it is nearly identical to the results of Mahich *et al*.[22] and Sehree *et al*.[23] that were 50%–60% and 31.7%, respectively.

Al-Tamimi *et al*.[24] suggested that clinical *A. baumannii* isolates showed a low resistance rate of 7.2% for tigecycline and 23.8% for minocycline but higher for tetracycline at 65.4%. Also, Sepehr *et al*.[25] showed high resistance to tetracycline and tigecycline.

Efflux pump is involved in resistance to tetracyclines, tigecycline, and many other different classes of antibiotics, and unfortunately, the nosocomial infections caused by bacteria harboring these pumps and resistances have increased over the past decade, especially in patients admitted to ICUs, burns, and surgery wards.[26] Cartwheel-Ethidium bromide assay was used by many researchers for the detection of efflux pump, where Sepehr *et al*.[25] showed the nine tested isolates with tetracyclines' resistance resulting in the increased outflow of ethidium bromide from the bacteria, which decreased fluorescence compared with the control; also, it was beneficial to detect the effect of EPIs.

The treatment of bacterial infections is currently a great challenge for the medicine. Antibiotics that have been used for decades are not as effective due to multidrug resistance, and new ones rarely appear. Therefore, research focuses on adjuvant substances that can support the activity of antibiotics without the risk of developing secondary resistance. These adjuvants can directly inhibit the resistance mechanism in nontoxic concentrations or modulate virulence factors and, thus, reduce the danger of bacterial strains. Plant extracts are a promising source, because they contain a wide range of substances. There are many biological activities studied on *M. chamomilla*. Among these studies, antimicrobial, antioxidant, and anti-inflammatory activity studies are the most common, where many antimicrobial activity studies on *M. chamomilla* are mainly performed with its extracts that are obtained using various solvents and extraction techniques or essential oil of the plant. *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Bacillus cereus*, and *Salmonella typhi* bacterial strains, and *Candida albicans, Aspergillus flavus*, and *Aspergillus niger* fungal strains were the most common strains in these studies.[27-29]

Several studies had studied the antimicrobial activity of Chamomile and many other plants' extracts and gave various minimal inhibitory concentration values that are mainly due to different extraction methods, types of extract, and concentrations series that are applied. Also, the presence of active compounds in the extract is also influenced by the conditions under which the plant grows (soil conditions, amount of rain, and sunshine) and in which generation period the plant is at the time of collection (germination and full bloom). However, all extracts tend to be more active against Gram-positive than Gram-negative bacteria at lower concentrations, which may be caused by poor permeation of active substances through the complicated membrane of Gram-negative bacteria.[30] Also, the use of different bacterial strains can also lead to different minimum inhibitory concentration values.

The sensitivity of bacteria to antibiotics is weakened not only by the expression of resistance genes but also by the formation of biofilms. The bacteria in biofilms can be up to 100 times resistant to antibiotics,[31] and thus, extracts that have the ability to destroy and overcome biofilm formation can also potentiate and prevent bacterial growth and inhibit antimicrobial resistance.

Ali *et al*.[32] revealed that watery extract of Chamomile has an inhibitory effect on the microorganisms under investigation. Extract of chamomile was detected to be contain azulene and apigenin compounds, after detection and quantitation using high performance liquid chromatography techniques; as the extract was used against both Gram positive and negative isolates, extract showed no inhibition in the control treatments, with slight inhibition at 10 and 20 mg/mL while caused an inhibition zone with 15.2 mm at the concentration 40 mg/mL with more inhibition with concentrations increment.

Ismail *et al*.[33] showed that the water extract of chamomile is more effective than the ethanolic one against different types of bacterial isolates, where higher concentrations gave better effects than the lower ones. *M. chamomilla* contains many active compounds and is attributed to antimicrobial activity as terpene compounds, azulen and abisabolo. The dried flowers of chamomile contain many terpenoids and flavonoids contributing to its medicinal properties.

Jafarzadeh *et al*.[34] explained that the analysis of the chemical ingredients of *M. chamomilla* showed the presence of flavonoids, phenylpropanoids, polyyne compounds, coumarins, polysaccharides, and essential oils. *M. chamomilla* shows a wide range of biological activities, which are most often due to its phenolic compounds. It has been shown that the spasmolytic and

anti-inflammatory activities of *M. chamomilla* are mainly related to its flavonoids.

A combination therapy of EPIs and antibiotics could be a favorable for the efflux pump- mediated bacterial resistance; as EPI must be selective, not target any eukaryotic efflux pumps, and has ideal pharmacological properties, such as the nontoxicity and high therapeutic effect. Inhibition of these systems can cause an increase in the concentration of antibiotics within the bacterial cell. Furthermore, it can complicate intercellular communication and, thus, reduce the production of virulence factors. Křížkovská *et al.*[35] suggested that *M. chamomilla* at nontoxic concentrations can inhibit MDR-Gram-negative efflux systems, and it is able to reduce the virulence of both Gram-positive and Gram-negative bacteria by inhibiting efflux pumps.

Even though drug efflux pumps play a critical role in the emergence of tetracycline resistance and even MDR bacterial strains, there is no clinically approved EPI with which to inhibit these efflux pumps. Efforts are being made to develop a more effective EPI with fewer adverse effects and increased efficacy. Thus, suppression of efflux pumps is a promising strategy for overcoming this problem.

Conclusion

Tetracycline-resistant *A. baumannii* is considered a healthy and community hazard, efflux pump has a great role in this problem, and chamomile extract could be an efficient EPI.

Conflict of interest

There is no conflict of interest.

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