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ACTIVITY OF THYME OIL (*OLEUM THYMI*) AGAINST MULTIDRUG-RESISTANT *ACINETOBACTER BAUMANNII* AND *PSEUDOMONAS AERUGINOSA*

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Abstract: Almost as soon as antibiotics were introduced to treat infectious diseases, it could be observed that bacteria were able to develop resistance against them. Currently, multidrug-resistant strains are being isolated mainly in the hospital environment. These are primarily non-fermenting Gram-negative bacilli, which exhibit both natural and acquired resistance to multiple antibiotics and disinfectants rendering them difficult to eradicate. The development of new, effective and safe substances that prevent troublesome infections is greatly needed to provide alternative therapeutic options for patients. There is increasing interest in drugs of natural origin, including essential oils. It is of particular interest that, although active against many bacterial strains, they do not contribute to antibacterial resistance against their components. The aim of our study was to evaluate the *in vitro* antibacterial activity of thyme oil against multidrug-resistant strains of *A. baumannii* and *P. aeruginosa* using the disc diffusion and macrodilution methods. The strains were isolated from patients hospitalized in the years 2013-2014. The *in vitro* antibacterial activity of thyme oil was assessed by the disc diffusion method and the inhibition zones for the oil at different concentrations, produced against *A. baumannii*, ranged from 7 to 44 mm. Low level of activity of thyme oil was observed against *P. aeruginosa* strains. The results of serial dilution tests confirmed the high activity of thyme oil against *A. baumannii* isolates, expressed as MIC values ranging from 0.25 to 2 µL/mL. These results suggest the need for further studies of antibacterial activity of essential oils, especially against multidrug-resistant bacterial isolates.

Keywords: thyme oil, activity, non-fermenting bacilli, multidrug-resistant strains

The healing powers of essential oils, the secondary metabolism products of oil plants, have been known for centuries. Despite the long history of their use, chemical composition of essential oils was not analyzed until the nineteenth century, when the development of chromatographic techniques allowed researchers to study the plant products in far greater detail, but only chromatography combined with mass spectrometry could be used in more advanced analysis of the compounds (1). In chemical terms, oils are mixtures of carbohydrates, alcohols, aldehydes, ketones as well as esters and ether derivatives of both terpenes (mainly mono- and sesquiterpenes) and propylbenzene. The specific chemical composition of an essential oil depends on the plant species but the relative percentages of the different components will vary according to the climate, growing conditions, stage of vegetation as well as the part of the plant used (roots, rhizomes, herbs, leaves, flowers, fruits, seeds or bark). The rich composition of essential oils determines their extremely wide range of biological activity, including activity against bacteria, viruses and fungi which depends mainly on the nature of the leading chemical component. Essential oils containing phenols (thymol and carvacrol), eugenol and cinnamaldehyde exhibit the strongest antibacterial activity. Oils have lipophilic properties, which allow them to rapidly penetrate the cell wall and cytoplasmic membrane causing damage to the cell. Essential oils interfere with the transport proteins, which leads to leakage of bacterial cell components, inhibits the hydrogen pump and ATP formation, and affects the cytoplasmic enzymes, which ultimately causes cell lysis (2-4). The above-mentioned activity of essential oils and their major constituents shows significant therapeutic potential in the treatment of infections. Antioxidant, immunostimulatory and anti-inflammatory properties of the oils are of equal importance (5).

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An outstanding achievement of the 20th century was the discovery of antibiotics which revolutionized the treatment of infectious diseases but unfortunately, shortly after their introduction to clinical practice, the emergence of bacterial resistance to antimicrobial agents began. Recently, a progressive increase in drug resistance among pathogenic microorganisms has been confirmed, which adversely affects the outcomes of infected patients.

Among the bacterial strains, the following groups of multidrug-resistant microorganisms can be currently distinguished: MDR (multidrug-resistant), XDR (extensively drug-resistant) and PDR (pandrug-resistant), referred to as alert pathogens. Multidrug-resistant microorganisms are most commonly found in the hospital environment, where they can cause serious nosocomial infections, occurring particularly in the following hospital units: intensive care, burn therapy, surgical, hematological and oncological (6). These are primarily non-fermenting Gram-negative bacilli belonging to the species *Acinetobacter* (*A.*) *baumannii* or *Pseudomonas* (*P.*) *aeruginosa*. Microorganisms, which can survive in nutrient-poor conditions, colonize the hospital environment including sanitation facilities, ventilation and the medical equipment, mainly respirators, inhalators and venous or urinary catheters (7, 8). They are responsible for surgical site infections and infections of burns in patients hospitalized in surgical wards and burn units, whereas in the intensive care units (ICU) they are the most important species that cause infections of the respiratory system and urinary tract. Their ability to form biofilm contributes to their survival and colonization of respirators and urinary catheters (9, 10). Resistance to many antibiotics and disinfectants, primarily acquired but also natural, exhibited by many harmful pathogens makes them difficult to eradicate, therefore the search for new, effective and safe substances, that would prevent troublesome infections, is becoming increasingly more urgent. It should be noted that recently drugs of natural origin, including essential oils, have been the subject of growing interest. Essential oils, although active against many bacterial strains, do not contribute to antibacterial resistance against their components. It cannot be ruled out that using essential oils in the treatment of infections caused by multidrug-resistant microorganisms could be a powerful alternative to synthetic antibiotics whose effectiveness has declined over the last decade. In the search for compounds or substances that prevent infections or the spread of infectious agents in hospital environment, we studied the effect of thyme oil on some selected multidrug-resistant clinical strains of non-fermenting bacilli.

The aim of the study was to evaluate the *in vitro* antibacterial activity of thyme oil (*Oleum Thymi*) against the selected multidrug-resistant strains of *A. baumannii* and *P. aeruginosa*, using the disc diffusion and macrodilution methods.

EXPERIMENTAL

Material

We studied the natural thyme oil (Bakalland, Warszawa, Poland) certified according to the National Institute of Hygiene standards (PZH certificate No. HŻ/15994/96).

The original, 100% oil was serially diluted in dimethyl sulfoxide (DMSO; Merck, Warszawa, Poland) to achieve the final concentrations of 50, 25, 12.5, 6.25 and 3.125%. We examined the following strains of *A. baumannii*: 59 clinical isolates and the reference strain ATCC 19606 and *P. aeruginosa* strains: 29 clinical isolates and the reference strain ATCC 27853. The strains were collected from subjects hospitalized between 2013 and 2014 at the L. Rydygier Memorial Specialized Hospital in Kraków, in the following wards: anesthesia and intensive care, burn therapy, multiple trauma care, orthopedics and neuro-orthopedics, neurology and brain injuries, plastic surgery, nephrology, cardiology and internal medicine as well as toxicology.

Acinetobacter baumannii isolates were cultured from endotracheal aspirates (22 strains), blood samples (17), wound swabs (12), urine (4), and catheters (4), whereas *P. aeruginosa* isolates were cultured from the wound swabs (11), endotracheal aspirates (9), urine (4), swabs of ear (4) or conjunctival sac (1).

All the examined *A. baumannii* and *P. aeruginosa* strains were multidrug-resistant. Susceptibility testing of *A. baumannii* isolates (59) was performed against imipenem (IPM), meropenem (MEM), amikacin (AMK), gentamicin (GEN), tobramycin (TOB), ciprofloxacin (CIP), colistin (CST), and trimethoprim/sulfamethoxazole (SXT). The following antibiotics were used to determine the susceptibility of *P. aeruginosa* strains (29): piperacillin (PIP), piperacillin/tazobactam (TZP), ceftazidime (CAZ), cefepime (FEP), imipenem, meropenem, aztreonam (ATM), amikacin, gentamicin, tobramycin, ciprofloxacin, and colistin.

Methods

The antimicrobial susceptibility of *A. baumannii* and *P. aeruginosa* strains was evaluated by disc

diffusion technique (Oxoid Ltd., Hampshire, United Kingdom) and, to selected antimicrobials, with the use of the automated system Vitek 2 Compact (bioMérieux, Marcy l'Étoile, France) and the minimum inhibitory concentrations (MICs) were determined. The obtained results suggest that these isolates should be classified as multidrug-resistant (Figs. 1 and 2).

Antibacterial activity of thyme oil was determined by disc diffusion and tube macrodilution methods in order to find the minimum inhibitory concentration capable of inhibiting the growth of bacteria.

The isolates were inoculated onto solid medium, Trypticasein Soy Agar (bioMérieux, Marcy l'Étoile, France) and incubated at 35°C for 20 h. Next, the colonies were suspended in 0.85% saline solution in order to obtain an equivalent of 0.5 McFarland units.

The disc diffusion tests were performed on Mueller-Hinton agar (bioMérieux, Marcy l'Étoile, France) in 90 mm-diameter Petri dishes. Bacterial suspensions were inoculated onto the culture plates and then 6 mm sterile filter paper discs, containing decreasing concentrations of thyme oil (15 µL), were placed on the surface of the plates. After 20 h of incubation at 35°C, the diameters of the growth inhibition zones were measured in mm.

Minimum inhibitory concentrations were determined by a broth macrodilution method in Trypticasein Soy Broth (BioCorp, Warszawa, Poland). The original thyme oil was diluted in

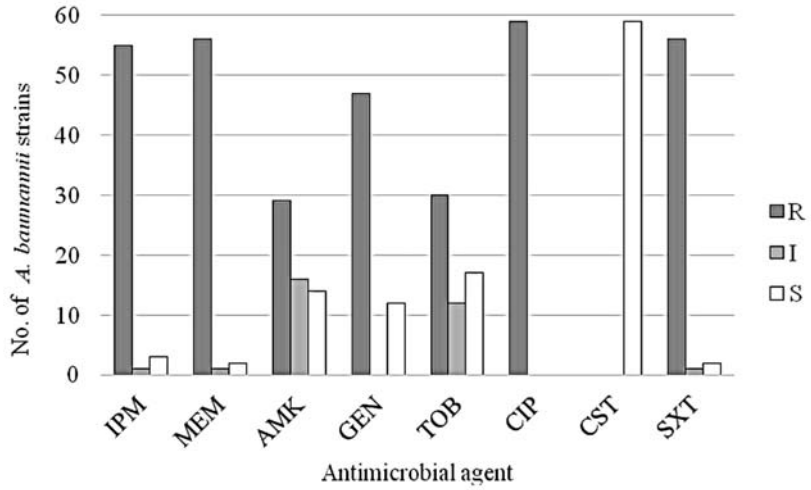

Figure 1. Susceptibility of *Acinetobacter baumannii* strains to selected antimicrobial agents

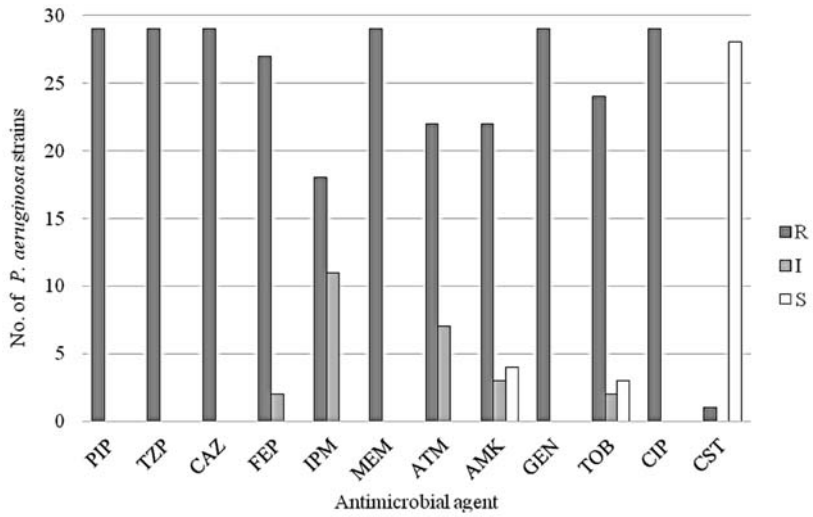

Figure 2. Susceptibility of *Pseudomonas aeruginosa* clinical strains to selected antimicrobial agents.

dimethyl sulfoxide (DMSO). The volumes were matched in a way that the solvent volume was the smallest possible. First, a stock solution was prepared by dissolving the original thyme oil in DMSO to the concentration of 16 µL/mL, and then serial dilutions ranging from 8 µL/mL do 0.125 µL/mL were made in test-tubes containing 1 mL of broth. Next, 100 µL of bacterial suspension, equivalent to 0.5 McFarland units, was added to each tube and incubated at 35°C for 20 h. After this time, the minimum inhibitory concentration (MIC µL/mL) was determined as the lowest thyme oil concentration at which no bacterial growth could be observed. Controls of the broth, thyme oil and bacterial growth were run in parallel with all tests.

Table 1. Range of zone diameters of inhibition of *Acinetobacter baumannii* (clinical and reference strains) growth in relation to the tested thyme oil concentrations determined using the disc diffusion technique.

Tiyme oil concentration [%]	Range of inhibition zones [mm]
100	26 – 44
50	19 – 30
25	11 – 22
12.5	8 – 17
6.25	7 – 11

RESULTS

In the present study we investigated *in vitro* the antimicrobial activity of thyme oil against bacterial reference strains. Disc diffusion tests were performed and the diameters of zones of inhibition

Table 2. Range of zone diameters of inhibition of clinical *Pseudomonas aeruginosa* strains growth in relation to the tested thyme oil concentrations determined using the disc diffusion technique.

Tiyme oil concentration [%]	Range of inhibition zones [mm]
100	0 – 12
50	0 – 7
25	0

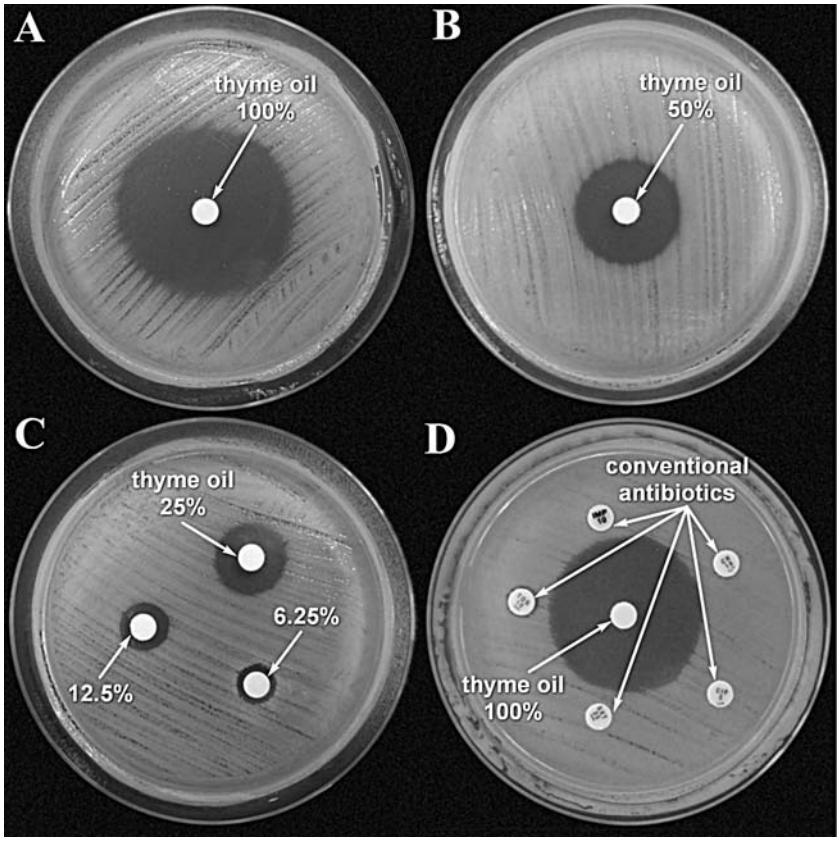

Figure 3. Growth inhibition zones against an *Acinetobacter baumannii* clinical strain, thyme oil at the concentration of: A. 100%; B. 50%; C. (25%, 12.5%, 6.25%). Comparison of the susceptibility to thyme oil with the resistance to the selected antibacterial agents: IPM, GEN, TOB, CIP, SXT (D).

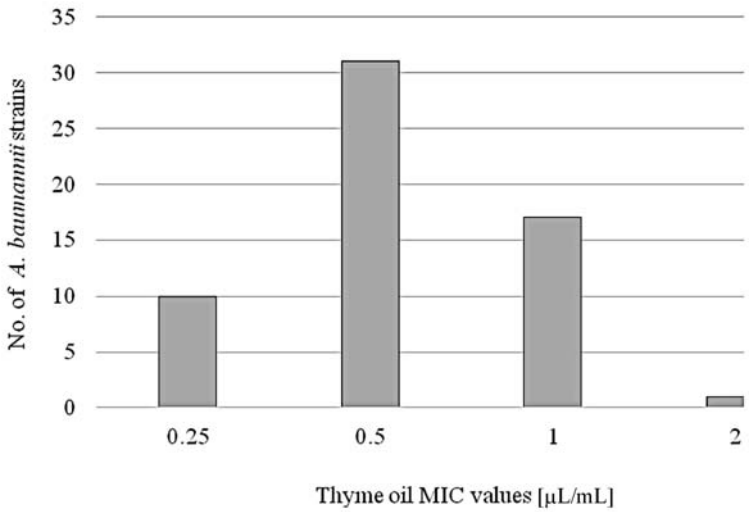

Figure 4. Thyme oil MIC values (µL/mL) determined against clinical strains of *Acinetobacter baumannii*

around the discs impregnated with thyme oil were measured. The zone diameters for the reference strain ATCC 19606 of *A. baumannii* ranged from 9 to 38 mm at the oil concentrations of 100, 50, 25, 12.5 and 6.25%, whereas at the oil concentrations of 100, 50 and 25%, the reference strain ATCC 27853 of *P. aeruginosa* produced inhibition zones ranging from 7 to 18 mm. The same concentrations were used to evaluate the antibacterial activity of thyme oil against pathogens isolated from inpatients. Our results confirmed that the antibacterial activity of thyme oil against the studied strains of *A. baumannii* was high. The size of the inhibition zones ranged from 26 to 44 mm for thyme oil at the concentration of 100%, from 19 to 30 mm for 50%, from 11 to 22 mm for 25% and from 8 to 17 mm for 12.5%. The smallest inhibition zones, ranging from 7 to 11 mm, were produced at the oil concentration of 6.25% (Table 1). The results of bacterial susceptibility testing of thyme oil against a selected clinical isolate of *A. baumannii* are shown in Figure 3.

Our study revealed very low level of activity of thyme oil against the tested clinical strains of *P. aeruginosa*. Thyme oil produced bacterial growth inhibition zones ranging from 7 to 12 mm for 11 *P. aeruginosa* strains, but it proved to be totally inactive against eighteen isolates of this pathogen (Table 2).

The next step of the study was to determine MIC values of thyme oil at different concentrations against *A. baumannii* strains using the serial dilution method. The obtained results confirmed that the antibacterial activity of thyme oil, expressed as MIC values, ranging from 0.25 to 2 µL/mL, was high. The MIC_{50} and MIC_{90} of these isolates were found to be 0.5 and 1 µL/mL, respectively. The lowest MIC of 0.25 µL/mL was observed against ten *A. baumannii* strains while the values of 0.5 µL/mL and 1 µL/mL were found against 31 and 17 isolates, respectively. Only one strain exhibited MIC of 2 µL/mL (Fig. 4).

DISCUSSION AND CONCLUSION

In the twenties of the last century, a French chemist René Gattefossé became interested in the healing properties of essential oils when he applied lavender oil to a burn on his hand. In 1937, Gattefossé coined the word "aromatherapy" to describe the use of essential oils in therapy (2). Recently, there has been a growing interest in the use of natural products in medicine. Despite bringing new antimicrobial drugs, mainly antibacterial, to market they are still inefficient. There is an urgent need to search for new agents, including the natural products showing high antimicrobial activity and no adverse effects. Research has proven that essential oils have a remarkable antimicrobial potential and are highly effective against both Gram-positive and Gram-negative bacteria. Several studies have documented that essential oils can increase the bacterial susceptibility to drugs, even of the most resistant strains (3, 4, 11), therefore therapeutic strategies of essential oils in combination with antibiotics not only could improve treatment outcomes but could also slow down the increase in antibiotic-resistant bacteria. The strongest antibacterial activity is exhibited by phenolic-rich essential oils; one of them is thyme oil which is derived from thyme herb

(*Thymus vulgaris*) (12). Thymol, the main component, accounts for 30-50% of thyme oil and carvacrol up to 5%. Thyme essential oil also contains a range of additional compounds, such as terpenes, tannins, flavonoids, saponins and minerals (2). Thyme acts as an expectorant so it is used in respiratory tract diseases. Due to its antimicrobial and antiseptic activity, thyme is used in skin disinfection, treatment of seborrheic dermatitis and bacterial eczema as well as to accelerate wound healing. Thyme has also many uses in aromatherapy and skin care: massage, baths, inhalation and facial cleansing (2, 13, 14).

The results of our studies confirmed the high activity of thyme oil against multidrug-resistant clinical strains of *A. baumannii* isolated from patients hospitalized in various wards. We obtained similar results using the disc diffusion technique (zones of bacterial growth inhibition ranged from 7 to 44 mm) and the serial dilution method (MICs from 0.25 to 2 µL/mL). Our results are consistent with that of Łysakowska et al., who studied multidrug-resistant bacilli of the genus *Acinetobacter* isolated from the hospital environment and hospitalized patients. They determined the MIC values in the range from 0.25 to 1 µL/mL (15).

Hersch-Martínez et al. studied susceptibility of various species of bacteria to thyme oil using disc diffusion method and they obtained an average diameter of the zones of inhibited bacterial growth ranging from 6.4 to 35.7 mm. For *P. aeruginosa* strains, this value was 6.4 mm (16). In our study, *P. aeruginosa* zone of inhibition did not exceed the diameter of 12 mm, which was regarded as very low antibacterial effect.

Hammer et al. determined the antimicrobial activity of 53 essential oils, including oil of thyme, against various bacterial species with the use of serial dilution technique, both agar and broth. The minimum inhibitory concentrations with thyme oil to the reference strain NTCT 7844 of *A. baumannii* was 0.12 µL/mL and *P. aeruginosa* NTCT 10662 - over 2 µL/mL (17).

Kędzia et al. examined the antimicrobial activity of thyme oil against 31 bacterial strains of various species, including three *A. baumannii* strains and one strain from each of the species: *P. aeruginosa* and *P. stutzerii*. They used serial dilutions of thyme oil in Mueller-Hinton agar. The MIC values for *A. baumannii* strains were found to range from 0.5 to 2 mg/mL, for *P. aeruginosa* over 4 mg/mL and for *P. stutzerii* 0.5 mg/mL (18).

Sienkiewicz and Wasiela tested 30 *P. aeruginosa* strains using thyme oil prediluted in ethanol and then serially diluted in agar. The MIC values were found to range from 1 to 2.5 µL/mL (19).

In agreement with these findings, we also demonstrated high antimicrobial activity of thyme oil against *A. baumannii* strains.

Although these findings are encouraging, it is necessary to continue research on antibacterial activity of essential oils, especially with regard to multidrug-resistant strains of clinically important bacterial species. It is worth mentioning that many products containing essential oils and recommended in treatment of various conditions, are protected by patents. Synergistic antimicrobial effect of essential oils and antibacterial agents not only could help to eradicate the etiological agent of infection but could also slow down the increase in antibiotic-resistant bacteria (20). Current treatments of serious bacterial infections caused by resistant strains include combined therapies of broad-spectrum antibiotics (21).

Particular attention should be given to colistin, a polymyxin antibiotic, which has been reintroduced in clinical practice for treatment of infections due to multidrug-resistant bacterial strains including *A. baumannii* and *P. aeruginosa* (MDR, XDR, PDR). Colistin, even though effective, exhibits adverse effects and may interact with other medicines. This is particularly of concern in patients with serious comorbidities. Unfortunately, among the colistin-only-susceptible hospital isolates, colistin-resistant strains have emerged recently (22). Prolonged antimicrobial therapy in hospitalized patients perturbs the normal flora, promoting worsening of the serious opportunistic infections.

In the recent years, multidrug-resistant strains have been increasingly isolated but also a small number of new antimicrobial drugs have been introduced for clinical use. Therefore, it is necessary to continue research on new products, including essential oils, because their use in combination therapy with antibiotics could be an alternative therapy for selected infections.

It is necessary to continue research on antibacterial activity of essential oils as bacterial drug-resistance to currently administered treatments is ever increasing. Our results show that the *in vitro* activity of thyme oil against multidrug-resistant strains of *A. baumannii* is high and this suggests that this oil can be used in the treatment of infectious diseases and to eradicate alert pathogens from the hospital environment.

Conflict of interest

The authors have declared no conflict of interest.

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