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Antibiotic resistance among *Escherichia coli* urinary isolates and their susceptibility to clove essential oil

SUMMARY

Escherichia coli is a Gram-negative, facultatively anaerobic, rod-shaped, coliform bacterium, which is a primary cause of urinary tract infections. Resistance to antibiotics has become a particular problem in recent decades. Consequently, there is an unmet need for new therapeutic options. It has been observed that essential oils have bactericidal effects. The antimicrobial susceptibility testing for *Escherichia coli* isolates obtained from urine of patients with urinary tract infections was determined via disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015). Essential oil from clove – *Syzygium aromaticum* (L.) Merill et L.M. (*Myrtaceae*) was analyzed by GC-FID-MS. Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were detected by using the micro-dilution broth method. *Escherichia coli* clinical isolates are characterized by high resistance to ampicillin, amoxicillin with clavulanic acid, norfloxacin, trimethoprim/sulfamethoxazole, tetracycline, tobramycin and ticarcillin. Clove oil possesses strong inhibiting and killing properties against *E. coli* isolates, among them the ones resistant to recommended antibiotics. The results of this study highlight the need for testing the efficacy of new agents to inactivate bacteria in clinical settings.

Keywords: *Escherichia coli*, clove oil, urinary tract infections

STRESZCZENIE

Escherichia coli jest Gram-ujemną, względnie beztlenową bakterią, która jest główną przyczyną infekcji dróg moczowych. Oporność na antybiotyki staje się coraz większym problemem ostatnich dekad, dlatego istnieje ogromna potrzeba poszukiwania nowych terapii lekowych. Badanie wrażliwości na leki przeciwbakteryjne izolatów *Escherichia coli* wyizolowanych z moczu pacjentów z zakażeniami dróg moczowych określono metodą dyfuzyjno-krążkową według European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015). Skład olejku eterycznego z goździkowca korzennego – *Syzygium aromaticum* (L.) Merr&Perry (*Myrtaceae*) został określony metodą GC-FID-MS. Minimalne stężenie hamujące (MIC) i minimalne stężenie bójcze (MBC) zbadano, stosując metodę mikrorozcieńczeń. Izolaty kliniczne *Escherichia coli* charakteryzowały się wysoką opornością na ampicylinę, amoksycylinę z kwasem klawulanowym, norfloksacynę, trimetoprim/sulfametoksazol, tetracyklinę, tobramycynę i tikarcylinę. Badanie wykazało silne właściwości hamujące i bójcze olejku goździkowego wobec izolatów *E. coli*, w tym opornych na rekomendowane antybiotyki. Wyniki wskazują na potrzebę badań nad skutecznością nowych środków do dezaktywacji bakterii w warunkach klinicznych.

Słowa kluczowe: *Escherichia coli*, olejek goździkowy, zakażenia układu moczowego

INTRODUCTION

Urinary tract infection (UTI) is one of the most common conditions observed in general practice, accounting for up to 6% of consultations. About 150 million people worldwide develop UTI each year (9). It affects more women than men (24). 20% of women at any time have asymptomatic bacteriuria and around 40% of them develop at least one UTI during their lifetime (19).

Urinary tract infection (UTI) is the presence of urinary tract microorganisms above the bladder – generally > 10^5 / ml (significant *bacteriuria*). Asymptomatic bacteriuria means that bacteria tend to multiply up to 10^5 colony-forming units (CFU) per ml without any clinical symptoms (17). Symptomatic UTIs are classified as cystitis, pyelonephritis, and urosepsis, considering that the urosepsis syndrome is the most severe form and that pyelonephritis is more severe than cystitis (26). Zacché and Giarenis classified UTIs as either uncomplicated or complicated depending on the presence of structural or neurological urinary tract abnormalities (28). The risk factors are phenotyped according to the ORENUC system: O indicates unknown risk factors; R – risk of recurrent UTIs but without risk of a more severe outcome; E – extraurogenital risk factors; N – relevant nephropathic diseases; U – urologic resolvable (transient) risk factors; C – permanent external urinary catheter and unresolved urologic risk factors (14).

Most UTIs are caused by the ascent of microorganisms through the urethra, although some microorganisms can reach the urinary tract by hematogenous or lymphatic spread (11). Most UTIs are monomicrobial. The most common uropathogen in uncomplicated upper and lower urinary tract infections is *Escherichia coli* (70–95% of cases) and *Staphylococcus saprophyticus* (5–10% of cases) (4, 26). Occasionally there are isolated bacteria from *Enterobacteriaceae* family, such as *Proteus mirabilis* and *Klebsiella* spp. (20). The microbial spectrum of complicated UTIs is broader and includes species of *Pseudomonas*, *Enterococcus*, *Staphylococcus*, *Serratia*, and *Providencia*, viruses and fungi (21).

Escherichia coli is a Gram-negative, facultatively anaerobic, rod-shaped, coliform bacteria found in the lower intestine (25). Most *E. coli* strains are harmless, but some serotypes can cause serious disease (6). The harmless strains are part of the normal flora of the gut, can benefit their hosts

by producing vitamin K2 (3), and have a symbiotic relationship (13). Uropathogenic *E. coli* (UPEC) is one of the main causes of urinary tract infections. They are equipped with pathogenic factors like: Fimbriae P, Fimbrial Adhesins Dr and Capillary Afa II Afa III, and Hly A cytolysin.

Bacterial infections of urinary tract are treated with antibiotics or chemotherapeutics, at the initial empirical treatment period, and then on the basis of antibiogram. Polish recommendations for treating UTI include amoxicillin (and other semisynthetic penicillins), cephalosporins (mostly II and III generation), carbapenems, aztreonam, trimethoprim-sulfamethoxazole, ciprofloxacin, nitrofurantoin and the aminoglycosides. According to the World Health Organization, antibiotic resistance is a growing problem which poses a serious threat to public health. The consequences of infection with resistant microorganisms can be severe: increased mortality, prolonged stays in hospital, loss of prophylaxis for patients undergoing surgery and other medical procedures, as well as increased costs of treatment (5). Therefore, new therapeutic options should be proposed. This proves the need to develop new therapeutic options.

Strong antiseptic activity of essential oils has been known for many centuries. A large number of essential oils and their constituents have been investigated for their microbial properties against some bacteria. Clove oil is an essential oil extracted from the clove plant. There are three types of clove oil: bud oil derived from the flower-buds of *S. aromaticum* contains 60–90% of eugenol, leaf oil derived from the leaves contains 82–88% of eugenol, and stem oil derived from the twigs contains 90–95% of this compound (18). The antimicrobial activities of clove was proved against several bacteria and fungi which were the case of food contamination (27). Pure clove oil or mixes with rosemary oil were tested against *E. coli* and other bacteria. The results showed the strong growth inhibiting activity in concentration between 0.062% and 0.500% (v/v) (10).

The aim of this study was to investigate the patterns of resistance among *E. coli* isolates obtained from urine of patients with UTIs and check their susceptibility to clove essential oil.

MATERIALS AND METHODS

Escherichia coli clinical isolates were obtained from urine of patients with UTIs. The examination of urine samples was based on the presence of $\geq 10^5$ CFU of microorganisms per ml in urine culture. The bacteria were cultured according to standard microbiological methods with the use of Columbia Agar (Graso, Poland) and Mac Conkey Agar (Graso, Poland). They were identified using API 20 E tests (bioMerieux, France). Identification was confirmed by using the automated instrument VITEK 2 (bioMerieux, France).

The antimicrobial susceptibility testing for *Escherichia coli* isolates was determined via disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015). The following discs were used: AM – ampicillin (10 µg), AMC – amoxicillin/clavulanic acid (20 µg /10 µg), CXM – cefuroxime (30 µg), CTX – cefotaxim (30 µg), CIP – ciprofloxacin (5 µg), NOR-norfloxacine (10 µg), SXT – trimethoprim/sulfamethoxazole (1.25 µg /23.75 µg), NT-nitrofurantoine (300 µg), CN-cefalexine (30 µg), TET – tetracycline (30 µg), ATM – aztreonam (30 µg), FOX – cefoxitin (30 µg), TGC – tigecycline (15 µg); CAZ – ceftazidime (30 µg), TOB – tobramycin (10 µg), FEP – cefepime (30 µg), TIC – tikarcillin (75 µg).

Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were detected by the use of the micro-dilution broth method. The tested essential oil was diluted in 96% ethanol PURE (POCH, Gliwice, Poland) yielding a concentration of 95% *v/v* of oils. This stock solution was mixed with a 100 µl Mueller-Hinton Broth to obtain concentrations from 1.5 µl/ml to 4.5 µl/ml and transferred to 96-well microtiter plates. An inoculum containing $1.5 \cdot 10^8$ CFU in the amount of 10 µl per well was added to medium with various clove oil concentrations. The MIC was determined as the lowest concentration of oil which inhibits the visible growth of bacteria after 24 hours of incubation at 37°C under aerobic conditions. The MBC was determined by transferring

cultures which were cultivated in higher than MIC concentrations of oil on Columbia Agar medium (Graso, Poland) and incubating them at 37ºC for 24 hours. The control media containing only alcohol did not inhibit the growth of bacterial isolates. The studies were repeated three times.

For the study purposes, essential oil from clove – *Syzygium aromaticum* (L.) (L.) Merr&Perry (*Myrtaceae*) was provided by POLLENA-AROMA, Poland. It was analyzed by GC-FID-MS in the Institute of General Food Chemistry, Łódź University of Technology. Identification of components was based on the comparison of their MS spectra with those of the laboratory-made MS library, commercial libraries (NIST 98.1, Wiley Registry of Mass Spectral Data, 8th Ed. and MassFinder 3.1) and with literature data (1, 15) along with the retention indices on apolar column (Rtx-1, MassFinder 3.1) associated with a series of alkanes with linear interpolation (C_8-C_{26}).

The composition of the clove oil was found to meet the requirements of the European Pharmacopoeia 8 (7) and of the Polish Pharmacopoeia IX (22) for the two main components: eugenol amounted to 86.0% (required 75.0–88.0%) and β-caryophyllene to 9.8% (required 5.0–14.0).

RESULTS AND DISCUSSION

The efficacy of antibiotic treatment depends on the identification and determination of the uropathogen pattern of resistance. In our study 96.7% of UPEC isolates showed resistance to ampicillin, 73.3% to amoxicillin with clavulanic acid, 86.7% to ciprofloxacin, 53.3% to norfloxacine and 56.7% to trimethoprim with sulfamethoxazole. It means that the tested *E. coli* isolates were characterized by high resistance to penicillin and other antibiotics recommended in UTI. UPEC isolates also showed high resistance to tetracycline (70.0%) and tobramycin (46.7%). Among the cephalosporins, the most effective was ceftazidime (3.3% resistance), then: cefotaxim (6.7%), cefoxitin (13.3%) cefuroxime (16.7%), cefepime (16.7%) and cefalexine (20.0%). 13.3% of *E. coli* isolates were resistant to nitrofurantoine. A number of sensitive and resistant strains of *E. coli* are presented in Table 1.

Table 1. Number of *E. coli* isolates resistant and sensitive to antibiotics

Number of *E. coli* isolates	**Antimicrobial agents**																
	AM	AMC	CXM	CTX	CIP	NOR	SXT	NT	CN	TET	ATM	FOX	TGC	CAZ	TOB	FEP	TIC
Resistant	29	22	5	4	4	16	17	4	6	22	1	4	2	1	15	5	26
Sensitive	1	8	25	26	26	14	13	26	24	8	29	26	28	29	15	25	4

The tree diagram, shown in Figure 1, groups the applied antibiotics in terms of their effect on the tested strains.

The cluster analysis shows that among the used antibiotics 3 groups can be distinguished: the first group includes antibiotics to which, most of *E. coli* isolates were resistant (AM, AMC, TET, TIC); the second group includes antibiotics for which the number of sensitive and resistant isolates is similar (TOB, SXT, NOR). The third group (CAZ, ATM, NT, FOX, FEB, CTX, CN, TGC, CIP, CXM) collects antibiotics for which the number of resistant isolates was the lowest.

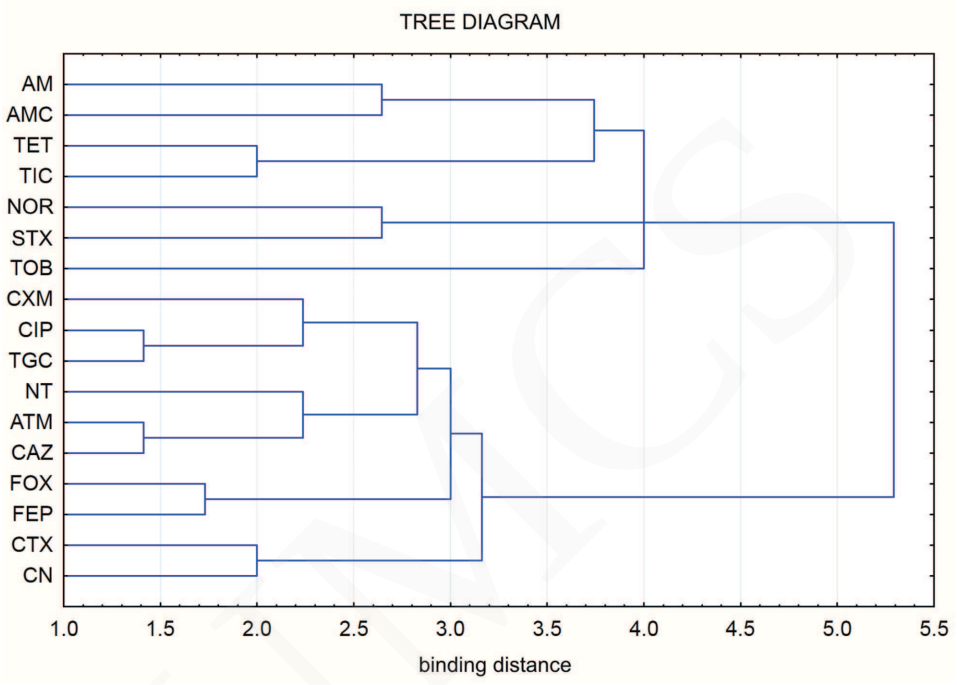

Fig. 1. Tree diagram of antibiotics sensitivity of *E. coli* isolates. The diagram was made using the full bindings and Euclidean distance method.

The effectiveness of clove oil against *E. coli* isolates suggests that it could potentially be used as a supplement to UTI therapy or as an alternative therapy – all tested isolates were sensitive to studied oil. Based on preliminary studies, it was found that the MICs and MBCs for clove oil fall into the following range: MIC from 2.1 to 3.1 mg/ml and MBC from 3.1 to 4.2 mg/ml (Fig. 2). For more than 70% of clinical strains of *E. coli*, MIC and MBC values were 2.6 and 3.7 mg/ml respectively (Tab. 2).

Table 2. Percentage of *E. coli* isolates in relation to MIC and MBC values determined for clove oil

Parameter	Value [mg/ml]	Percentage of isolates [%]
MIC	2.1	10.0
	2.6	**73.3**
	3.1	16.7
MBC	3.1	20.0
	3.7	**70.0**
	4.2	10.0

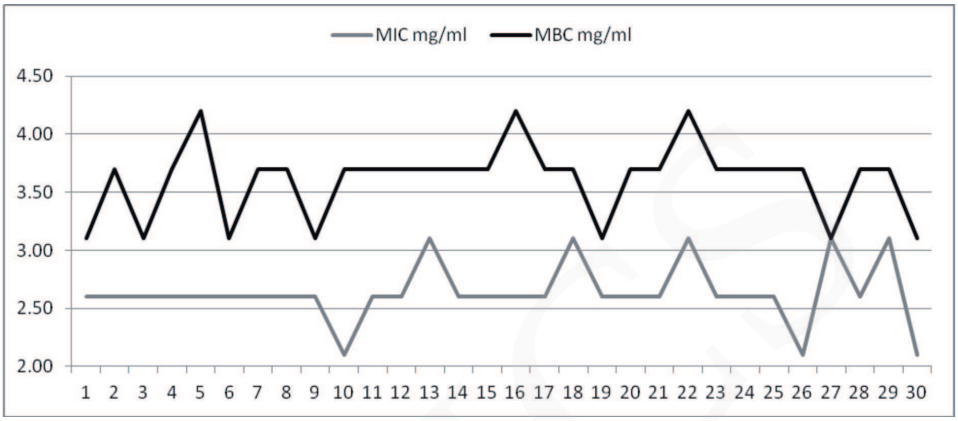

Fig. 2. Values of MIC and MBC *E. coli* isolates from urine determined for clove oil.

Saeed and Tariq (23) showed that the clove essential oil has a broad spectrum of antibacterial activity against Gram-negative bacteria. In this study clove oil inhibited the growth of all tested clinical isolates obtained from a range of clinical samples of stool, urine, blood and pus from wound. It was active against 100 isolates belonging to 10 different species of Gram-negative bacilli: *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Klebsiella ozaenae*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Salmonella typhi*, *Shigella dysenteriae* and *Vibrio cholera*. The inhibition zones were between 11.87 ± 3.22 for *E. coli* isolates and 23.75 ± 3.03 for *V. cholera* isolates, but no data on the composition of the tested commercial clove oil. Fagere and Al Magboul (8) investigations confirmed the very strong antibacterial properties of clove oil against Gram-negative and Gram-positive bacteria. According to their study conducted with the use of the cup plate agar diffusion method and the micro-dilution method, the MIC of the clove oil against reference strains of *Escherichia coli* and *Pseudomonas aeruginosa* was 1.5 mg/ml, and the MBC against *E. coli* was 3.125 mg/ml and against *P. aeruginosa* was 6.25 mg/ml. We obtained the similar results of the MIC and MBC values in relation to the *E. coli* coming from urine, including those against isolates resistant to antibiotics recommended in UTI. Our tested clove essential oil was consisistent with the requirements for medical use. Other authors reported on the antibacterial and antifungal activity of clove essential oil also in combination with antimicrobial drugs and its potential in the treatment of urogenital infections (2). The clove essential oil is safe for humans at low concentrations. The World Health Organization (WHO) established that the acceptable daily intake of clove oil per day is 2.5 mg/kg of weight in humans (16). The toxic effect has been proven for eugenol (as the main constituent), which is easily absorbed when administered orally, reaching rapidly plasma and blood with

mean half-lives of 14.0 h and 18.3 h, respectively. A cumulative effect has been hypothesized and associated to relieve of neuropathic pain after repeated daily administrations (12).

In conclusion, due to the fact that antibiotic resistant bacteria are a serious threat to public health worldwide, there is a need to search for new methods of controlling pathogens. A promising alternative to antibiotics are essential oils. Clove oil could be encapsulated and used as a supplement to conventional antibiotic treatment. The results of this study highlight the importance of testing the efficacy of new approaches to inactivate bacteria in clinically relevant settings.

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