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RESEARCH ARTICLE OPEN ACCESS

Synergy of Herbal Oil Extracts/Antibiotic Combinations in Drug- Resistant Uropathogenic *E. coli*

D.M. Hassan[1]*, S.M. Elnagdy[2], A.Abu-Taleb[2], B.A. Gomaa[2] and N.S. Soliman[1]

[1]Department of Clinical and Chemical Pathology, Faculty of Medicine, Cairo University, Cairo - 11562, Egypt.
[2]Department of Botany and Microbiology, Faculty of Science, Cairo University, Cairo - 12613, Egypt.

Abstract

Urinary tract infection (UTI) is the second most common infection worldwide and *Escherichia coli* (*E. coli)* is the chief causing pathogen. Antimicrobial resistance results into therapeutic failure. This study aimed to incrimination of *E. coli* as uropathogen harbouring *cnf1* gene (necrotoxin virulence)and to detect the *in vitro* antimicrobial efficacy of several selected essential oil/antibiotic combinations against the isolated uropathogenic *E. coli*. Well diffusion method was used to determine the minimal inhibitory concentrations (MICs) to essential oils (EOs) individually. Broth micro-dilution method was used to determine the minimal inhibitory concentrations (MICs) to antibiotics and in combinations, to figure out the interactive efficacy of tested combinations. Necrotoxin *cnf1* gene was detected by PCR assay. *E.coli* was detected at a rate of 40.6% of the isolated urinary pathogens and 47.8% showed ESBL production. *In-vitro* synergistic efficacy was only observed for cinnamon oil/gentamycin combination in 100% of selected 15 tested isolates (P-value <0.001). The *cnf1* virulence gene was detected in 40% of tested isolates showing no significant correlation the conferred pattern of resistance among the *E. coli* isolates. Cinnamon/gentamycin combination against drug-resistant *E-coli* is promising and can pave the way for further clinical trials to formulate pharmacological combinations.

Keywords: Antibiotics, essential oils, necrotoxin gene, synergistic effect

***Correspondence:** dinamhassan12@gmail.com; +201006911788

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INTRODUCTION

Urinary tract infection (UTI) is one of the most common causes of febrile diseases in children. In low-income countries, UTIs are ranked as the third most common bacterial infections in children[1,2]. *E.coli* is the chief uropathogen, accounting for 80% of UTIs[3]. The emergence of resistance among bacteria has worsened the therapeutic clinical outcome of bacterial infections[4]. Antimicrobial resistance in *E. coli* can be mediated by the production of extended-spectrum beta-lactamases (ESBLs) that confer resistance to all β-lactam antibiotics leading to the emergence of multidrug-resistance (MDR)[5,6]. Recently, essential oils (EOs) have been proven to serve as antimicrobials[7]. The synergistic effect of oil/antibiotic combinations exceeds the sum of the individual effects of the oil and antibiotic[8]. However, further studies are needed to elucidate mechanisms by which oils modulate bacterial drug resistance[8].

Necrotoxigenic *E. coli* (NTEC) produces *cnf1* toxin, which is considered highly lethal among bacterial toxins and is implicated in cystitis and pyelonephritis with remarkable rates[10].

The present study aimed to test the antimicrobial effects of different types of EOs and investigate the *in vitro* efficacy of selected oil/antibiotic combinations against *E. coli* uropathogen. Furthermore, we aimed to detect the *cnf1* virulence gene by a PCR assay.

EXPERIMENTAL

Collection of urine samples

The present study included 400 urine samples collected from children suspected of having UTIs at tertiary-care paediatric hospital in the period from March 2017 to September 2017. The study conformed to the ethical guidelines of the 2013 'Helsinki Declaration' and was approved by the research committee of the paediatric hospital at Cairo University.

Isolation of urinary pathogens

Urine samples were inoculated into CLED agar plates (Oxoid, UK) and incubated at 37°C for 24-48 h. Pink colonies were suspected to be *E. coli*. Isolates were further identified based on Gram staining and conventional biochemical reactions including (IMVC, TSI and urea reaction). Antimicrobial susceptibility testing of microorganisms was determined by the Kirby-Bauer disk diffusion method as per the recommendations of the Clinical and Laboratory Standards Institute. (CLSI)[11,12]. *E. coli* isolates were cultured on Muller-Hinton Agar plate with addition of Ampicillin (10 ug), amoxiciiin-clavulanate (20/10ug), Ampicillin-sulbactam (10/10ug), tetracycline (30ug), trimethoprim-sulfamethoxazole (1.25 ug + 23.75ug), cefazoline (30 ug), cefuroxime (30 µg), ceftazidime (30ug), ceftriaxone (10ug), cefepime (30 ug), cefoxitin (30 ug)gentamicin (10ug), nalidixin acid (30ug), nitrofurantoin (300ug), and cefotaxime (30ug), imipenem (10µg), meropenem (10µg), ciprofloxacin (5µg), levofloxacin (5µg). All disks were supplied from (Oxoid, UK) and were placed on the media in 20-30 mm with other disks. The boronic acid disk (Oxoid, UK)disk containing 30 µg of cefoxitin and 400 µg of boronic acid was added for AMPC detection also Ceftazidime- clavulanate (30\10 µg)was added for ESBL detection.

The plates were incubated for 18-24 h at 37°C. *E. coli* (ATCC25922; American Type Culture Collection (ATCC), Manassas, VA, USA) was used as the control bacterial strain.

ESBL screening

ESBL producers were 1st identified by reduced zone diameters of ceftazidime (30ug) ≤ 22 mm, ceftriaxone (10 ug)≤ 25 mm, or Cefotaxime (30ug) ≤ 27mm , while ESBL confirmation was done by the double disk diffusion method where a ≥ 5 mm increase in a zone diameter for Ceftazidime-clavulanate (30\10 µg) compared to ceftazidime (30ug) alone confirms ESBL production[13].

AMPC screening

The organism that is resistant to cefoxitin and or demonstrated 5 mm or greater zone around the disk containing cefoxitin and boronic acid compared to the disk containing cefoxitin alone was considered as AmpC producer[14].

Detection Minimal inhibitory concentration (MIC) for Essential oils (EOs) and antibiotics

Oils of cinnamon (*Cinnamom umverum*), thyme (*Thymus vulgaris*), clove (*Syzygium aromaticum*), peppermint (*Mentha piperita*), lavender (*Lavandula angustifolia*), anise (*Pimpinella anisum*), rosemary (*Rosmarinus officinalis L.*), lemongrass (*Cymbopogon citratus*), cumin (*Cuminum cyminum*) and castor bean (*Ricinus communis*) were obtained from the National

Research Center (Cairo, Egypt). Each oil was tested individually against *E. coli* by the well diffusion method[15] and the one with the widest inhibitory zone was selected to be tested in combination with each of 3 antibiotics (gentamycin, meropenem and cefotaxime) (Oxoid, UK). The individual MICs of EO and antibiotics against *E. coli* isolates were detected by the broth micro-dilution method in Mueller Hinton broth (Oxoid, UK) (with Tween 20 at a final concentration of 0.5%) into a 96-well micro-plate (2017)[11,16,17]. The MIC was defined as the minimal concentration of tested agent that inhibited bacterial growth[16,17].

Testing the efficacy of oil/antibiotic combinations

The efficacy of the selected three oil/antibiotic combinations was tested against 3 groups of *E. coli* isolates (ESBL *E. coli*, AMPC *E. coli* and non-ESBL-non-AmpC *E. coli*). The antimicrobial effect of the combinations was detected by the broth micro-dilution method in Mueller-Hinton broth (with Tween 20 at a final concentration of 0.5%). Ten-fold dilutions of antibiotics and EO were prepared in a 96-well micro-plate[9]. The MIC values of each antibiotic and EO alone and in combination were determined, and the fractional inhibitory concentration index (FICI) was calculated according to the following equations:

FIC of antibiotic= (MIC of antibiotic in combination)/ (MIC of antibiotic alone)

FIC of EO = (MIC of EO in combination)/ (MIC of EO alone)

FICI = FIC of EO + FIC of antibiotic

FICI ≤ 0.5, synergistic; FICI > 0.5–4.0, no interaction; FICI > 4.0, antagonism[9].

The bacteriolytic effect of individual antibiotics compared to oil/antibiotic combinations was detected by measuring the optical density (O.D.) at 600 nm.

Detection of the necrotoxin gene (*cnf1*) by multiplex PCR

As this study aimed at detection of (*cnf1*) gene and its correlation to antibiotic resistance pattern, considering the previously published study by Duzgun *et al*., 2019, who found that (*cnf1*) gene which is chromosomally encoded as the most common necrotoxin gene associated with antibiotic resistant *E. coli* in urinary tract infection[18], we chose the necrotoxin gene (*cnf1*) to be investigated in 100 *E. coli* isolates by PCR assay. Bacterial DNA was extracted by the boiling method, and PCR was carried out using a set of oligonucleotide primers for *cnf1* gene[19].

RESULTS

This study included 400 urine samples from patients clinically suspected of having UTIs, The *E. coli* was the chief isolate and accounted for 40.6% of the positive culture (Fig.1). ESBL and AmpC resistance patterns were found in *E. coli* with rates of 47.8% and 20.8%, respectively.

Table 1 summarizes the inhibitory zones of different oils tested by well diffusion against three representative isolates of the three recovered *E. coli* categories (ESBL, AmpC and non-ESBL-non-AmpC *E. coli*). The widest inhibition zone range (3.2-3.6 cm) was recorded for cinnamon oil. The efficacies of cinnamon oil/gentamycin, cinnamon oil /meropenem and cinnamon oil/ cefotaxime were tested against a total 15 isolates[5 isolates selected from each of the 3 *E. coli* groups (ESBL, AmpC and non-ESBL-AmpC)] and FICs are represented in Table 2. A synergistic effect was observed only for the cinnamon oil/gentamycin combination against 100% of the 15 tested. The calculated mean of FIC of the tested cinnamon oil/gentamycin combination showed statistically significant differences among the 3 *E. coli* groups (P-value <0.001).Cinnamon/gentamycin combination showed a 94% decrease in microbial growth, while gentamycin alone showed a 76% decrease (Table 3).

Table 1. Inhibitory zone diameters (in mm) of tested oils against the selected 3 groups of *E-coli* isolates

Oil/ bacteria	*Non ESBL Non-E.coli*	ESBL *E.coli*	AMPC *E.coli*
Cinnamon	36	34	32
Rosemary	19	17	17
Thyme	27	22	21
Clove	22	18	18
Peppermint	16	13	13
Lemon grasses	17	16	16
Castor	0	0	0
Lavender	0	0	0
Anise	0	0	0
Cumin	0	0	0

The *cnf1* necrotoxin gene was investigated

Table 2. Fractional inhibitory concentration indices (FICIs) of the 3 tested oil/antibiotic combinations

Isolates	cinnamon/meropenem cinmer			cinnamon/cefotaxime cincxm			cinnamon/gentamycin cen gent		
	A/B	A/B	FICI	A/B	A/B	FICI	A/B	A/B	FICI
ESBL *E. coli*									
1	1.75/3.5	0.0625/1.25	0.55	1.75/3.5	0.00625/1.25	0.505	0.175/3.5	0.00625/0.125	0.1
2	1.75/3.5	0.0625/1.25	0.55	1.75/3.5	0.625/12.5	0.55	0.175/3.5	0.00625/1.25	0.05
3	1.75/3.5	0.625/1.25	1	1.75/3.5	6.25/12.5	1	0.175/3.5	0.00625/1.25	0.05
4	0.175/0.35	0.0625/1.25	0.55	0.35/0.175	0.625/1.25	0.55	0.0175/0.35	0.00625/0.125	0.1
5	1.75/3.5	0.625/1.25	1	1.75/3.5	6.25/12.5	1	0.175/3.5	0.00625/1.25	0.05
AmpC *E. coli*									
1	1.75/3.5	0.625/12.5	0.55	0.175/3.5	0.625/1.25	0.55	0.175/3.5	0.0625/1.25	0.1
2	1.75/3.5	0.625/12.5	0.55	1.75/3.5	0.625/12.5	0.55	0.175/3.5	0.0625/1.25	0.1
3	1.75/3.5	6.25/12.5	1	1.75/3.5	6.25/12.5	1	0.175/3.5	0.0625/1.25	0.1
4	0.175/0.35	0.625/12.5	0.55	0.175/0.35	0.0625/1.25	0.55	0.0175/0.35	0.0625/1.25	0.1
5	1.75/3.5	6.25/12.5	1	1.75/3.5	6.25/12.5	1	0.175/3.5	0.0625/1.25	0.1
Non ESBL-non-AmpC*E. coli*									
1	0.175/0.35	0.00625/0.0125	1	0.175/0.35	0.0625/0.125	1	0.00175/0.35	0.0000625/0.125	0.005
2	0.175/3.5	0.0625/0.125	0.55	1.75/3.5	0.00625/1.25	0.505	0.00175/3.5	0.0000625/0.125	0.001
3	1.75/3.5	0.000625/0.0125	0.55	1.75/3.5	0.0625/1.25	0.55	0.00175/3.5	0.0000625/0.125	0.001
4	0.175/0.35	0.00625/0.0125	1	0.175/0.35	0.0625/0.125	1	0.00175/0.35	0.0000625/0.125	0.005
5	1.75/3.5	0.000625/0.0125	0.55	1.75/3.5	0.0625/1.25	0.55	0.00175/3.5	0.0000625/0.125	0.001

A: MIC of antibacterial agent in the most effective combination; B: MIC of antibacterial agent alone
FICI: fractional inhibitory concentration index. FICI ≤ 0.5 synergistic; FICI > 0.5–4.0 no interaction; FICI > 4.0 antagonism (Yap *et al*., 2013).

by PCR in 100 out of a total of 115 recovered *E. coli* isolates, which were divided according to their susceptibility profiles into 41 ESBL, 12 AmpC and 47 non-ESBL-AmpC isolates. The *cnf1* gene was detected in 40 out of 100 *E. coli* isolates and occurred among the 3 *E. coli* groups with the following frequencies: 23/41 ESBL (56.1%), 5/12 AmpC (41.6%), 12/47 non-ESBL-AmpC (25.5%) (Fig.2).

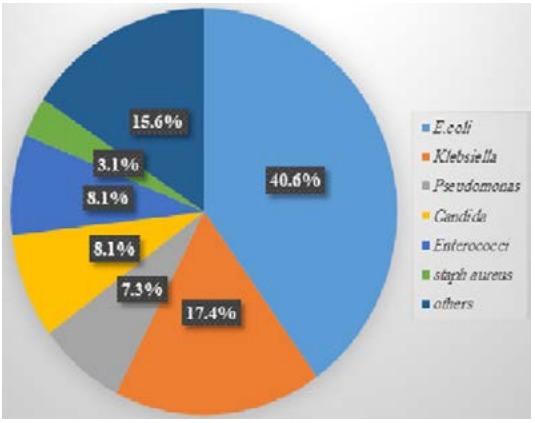

Fig. 1. Characterization of organisms isolated from urine culture.

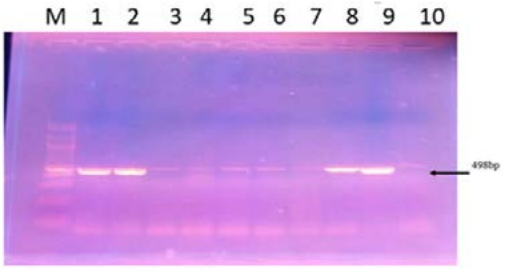

Fig. 2. Detection of cnf1 gene by PCR. Lane M: DNA ladder (100 bp); Lane 1: positive control (498 bp); Lanes (3&4&5&6&7 and 10) Negative. Lanes (2,8 and 9): Positive isolates.

DISCUSSION

UTIs are common infections in clinical practice. Approximately 150 million people are diagnosed with UTIs each year[20]. In the present study, 71.7% (287/400) of the urine samples were culture positive. This result was consistent with a study that reported positive cultures in 74.8% of urine samples; however was not complying with another study that reported a lower rate of 32.5%[21,22]. Variations in infection rates can be attributed to epidemiological variation among different geographical areas, as well as sex variation in the study population[23]. In the present study, *E. coli* accounted for 40.6% of the

Table 3. Bacteriolytic effect of oil/antibiotic combination compared to the individual effect of the antibiotic alone

Combinations	Growth decrease % by antibacterial agent in mix. with cinnamon	Growth decrease % by antibacterial agent alone	Chisquare	P-value
cinnamon/meropenem on AMPC *E.coli*	97.8%	89%	0.433	0.510
cinnamon/gentamycin on Non ESBL-Amp*CE.coli*	94%	76%	1.710	0.191
cinnamon/cefotaxime on non ESBL-Amp*CE.coli*	99.2%	85%	1.065	0.302
cinnamon/cefotaxime on AMPC *E.coli*	84%	75%	0.509	0.475
cinnamon/gentamycin on ESBL *E.coli*	97.8%	82.2%	1.780	0.182

isolated uropathogens, which was consistent with many studies that reported *E. coli* as the chief uropathogen in UTIs[20,21,24].

In the present study, ESBL production was demonstrated in 47.8% of the *E. coli* isolates, which was consistent with that of another study(41.9%). ESBL production was reported at higher rates of 63.6% and 66.78% by other studies, which could be due to the criteria used to select the study populations, who had complicated UTIs[25,26]. According to the Infectious Diseases Society of America, ESBL-producing *E. coli* are among the six drug-resistant microbes for which new therapies need to be developed[27].

Many studies have been published on the antimicrobial properties of plant extracts against different microbes[28-30] especially cinnamon oil, as a safe antimicrobial alternative. Cinnamon oil at least partially gets metabolized into cinnamic acid in stomach and small intestine and almost completely gets metabolized into cinnamic acid in liver before it is absorbed into blood[31]. As reported by several studies, cinnamon oil has a powerful antimicrobial effect due to deformation of the microbial cytoplasmic membrane[28-30]. In the present study, cinnamon oil showed the strongest antibacterial effect,with a significant difference (P-value <0.001) among the three *E. coli* groups (ESBL *E. coli*, AmpC *E. coli* and non-ESBL-AmpC *E. coli)*, which agreed with several previous reports [22,28,32,33], however opposed by a study that showed no synergy[34]. The present study had the advantage of testing oil/antibiotic combinations against drug-resistant *E- coli* isolates from patients, as most of published reports tested ATCC drug- susceptible strains[15].

The *cnf1* virulence gene was detected in 40% of isolates. Several studies reported variable prevalence rates of 61%, 22.9% and 37%, that could be attributed to different study population[35-37,39]. Previous studies showed that the NTEC isolates had variable degrees of resistance towards antimicrobial agents[19]. Moreover, it was assumed that *E- coli* serotypes involved in *cnf1* production differ from those of ESBL production[19,38].

CONCLUSIONS

ESBL production and *cnf1* gene were detected among *E. coli* urinary isolates with considerable rates. Synergy was only observed with Cinnamon oil/gentamycin combination. Further clinical researches shall be conducted to introduce promising oil/antibiotic combinations into clinical field.

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AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the Supplementary Files.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

None

ETHICS STATEMENT

This article does not contain any studies involving animals or human participants performed by any of the authors

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