Egyptian Journal of Medical Microbiology Volume 28 / No.1 / January 2019 55-63 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Studies on Fluoroquinolone resistance of *E. coli* isolates from patients admitted in Mansoura University Hospitals and its Control

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Key words:
Fluoroquinolone resistant;
E. coli;
MAS-PCR;
QRDRs;
Ethanolic Plant extracts

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ABSTRACT

Background: *Fluoroquinolones resistance usually occurs due to mutation in the quinolone resistance-determining regions (QRDRs) in the gyrA and parC genes that can be detected by multiplex allele-specific PCR (MAS-PCR). Herbal medicines are used as alternative treatment for disease caused by resistant bacteria.* ***Objectives:*** *This work aimed to detect resistance to fluoroquinolones in E. coli isolates from patient admitted in Mansoura University Hospitals; additionally, to identify certain natural plant extracts that could be used against fluoroquinolone resistant E. coli.* ***Methodology:*** *Fifty clinical E. coli isolates were collected from patients admitted in Mansoura University Hospitals. Fluoroquinolones susceptibility pattern was tested by disk diffusion method. Out of 50 isolates, 25 fluoroquinolone resistant E. coli were selected to detect mutations in gyrA and parC genes by MAS-PCR. Ethanolic plant extracts were tested against FQ resistant E. coli isolates by using agar well diffusion method.* ***Results:*** *E. coli isolates showed highest resistance to ciprofloxacin (76%) followed by norfloxacin (72%), levofloxacin (70%), and ofloxacin (68%). Double mutations at gyrA gene were detected at position 83 and 87 of QRDRs in 12 (48%) FQ resistant E. coli and at parC gene at position 80 and 84 of QRDRs in 3 (12%) FQ resistant E. coli. While single mutation at position 83 and 87 was found in QRDR of gyrA in 8 (32%) and 5 (20%) of FQ-resistant E. coli, respectively and single mutation at position 80 and 84 was found in QRDR of parC in 21 (84%) and 1 (4%) of FQ-resistant E. coli, respectively. Ethanolic extract of Clove had more antibacterial activity compared to other extracts.* ***Conclusion:*** *high rate of fluoroquinolone resistance among clinical E. coli isolates was detected and this necessitates monitoring the microbial trends and resistance patterns. Plants may be used as natural antibiotics in the treatments of antibiotic resistant E. coli infections.*

INTRODUCTION

The bacterial resistance to antibiotics is a threat to public health throughout the world. The bacteria which grow in presence of several drugs or carrying several resistances genes are called multi-drug resistant (MDR)[1]. The emergence of microbial resistance toward antibiotics increased in a terrible rate. The current shortage of effective drugs, lack of effective prevention measures and few new antibiotics underdevelopment will require the evolution of new options for therapy and alternative antibiotic treatments[2].The misuse of antimicrobial drugs accelerates the emergence of drug-resistant strains and raising antimicrobial resistance. Also, poor infection control practices, inadequate sanitary conditions and inappropriate food-handling participate in spreading resistance[3].

The consequences of resistance affect not only the ability to treat infection, but also the cost and duration of treatment[4]. *E. coli* are Gram-negative bacteria belonging to the family Enterobacteriaceae[5] and found in lower intestine as normal flora of the gut. Most *E. coli* strains are harmless, which can benefit their hosts by producing vitamin K2 and prevent the establishment of pathogenic bacteria in the intestine of the host while some *E. coli* serotypes cause severe food poisoning in humans[6]. *E. coli* has shown an increasing antimicrobial resistance to most antibiotics[7]. Fluoroquinolones are a relatively new class of synthetic antibiotics with potent bactericidal, broad-spectrum activity against clinically important pathogens[8].

The newer fluoroquinolones (Ciprofloxacin, Levofloxacin, Norfloxacin, and Ofloxacin) have shown broader spectrum of antibacterial activity including Gram negative and Gram-positive bacterial infections[9]. Fluoroquinolones have become prevalent in the treatment of urinary, respiratory, gastrointestinal, urogenital, intra-abdominal and skin infections[10] as it exists in oral and intravenous preparations[11]. Mutation in DNA gyrase of *gyrA* and topoisomerase IV of *parC* gene are the most common mechanisms of resistance to fluoroquinolones. Other mechanisms, including, efflux pump and several plasmid-mediated resistances[12].

The resistance to fluoroquinolones was correlated to mutations that lead to amino acid substitutions in *gyr*A and *parC* genes, in a region called quinolone resistance determining region (QRDR) that is located in the DNA-binding surface of the enzymes[13]. The scientists developed new drugs from natural sources such as plants, which have been extensively used as alternative treatment of diseases[14] with minimal side effects, being inexpensive and safe compared to the synthetic drug[15].

These plants contain phytochemicals active compounds such as flavonoids, tannis, saponins, alkaloids, terpenes[16], vitamins (A, C, E and K), carotenoids, polyphenols, pigments, enzymes and minerals[17] which are responsible for its antimicrobial activities. The extract of these herbal plants are used in treatment of acne, diarrhea, cold, cough, digestive disorders etc. this study aimed to detect resistance of fluoroquinolones in *E. coli* isolates from patients admitted in Mansoura University Hospitals; additionally, to identify certain natural plant extracts that could be used against fluoroquinolones resistant *E. coli*.

METHODOLOGY

Collection of samples and identification of *E. coli*:

Clinical samples (urine, blood, sputum, wound swabs and throat swabs) were collected from patients admitted in different diagnostic Departments of Mansoura University Hospitals (Specialized medical, Convalesce and critical care, Pediatric, and Emergency hospitals). These samples were cultured using the standard media (CLED agar for urine samples, Blood and MacConkey's agar for blood, sputum, wound swab and throat swab samples) and incubated aerobically at 37°C overnight. The identification of *E. coli* isolates was done by colony morphology, microscopic examination after Gram staining, and biochemical tests including Kligler Iron Agar (KIA), Lysine Iron Agar (LIA), Motility, Indole, Ornithine medium (MIO), Urease and citrate utilization tests[18].

Antimicrobial susceptibility test:

Antibiotic susceptibilities of *E. coli* isolates were done by Kirby Bauer disc diffusion method[19] using Muller-Hinton agar medium. The antibiotics tested were second and third generation fluoroquinolones including: Ciprofloxacin, CIP (5 µg); Levofloxacin, LEV (5 µg); Norfloxacin, NOR (10 µg) and Ofloxacin, OFX (5 µg). The clear zones were measured and compared with the standard recommendation of Clinical Laboratory Standard Institute (CLSI)[20].

Molecular study on fluoroquinolone-resistant *E. coli*:

Twenty-five of FQ-resistant *E. coli* were used to detect the presence of mutation in QRDRs of *gyrA* and *parC* genes using MAS-PCR.

Bacterial DNA Extraction:

Genomic DNA extracts of *E. coli* to be used as templates in this study was done by Thermo Scientific kits in accordance with the manufacturer's recommendations. Genomic DNA was stored at -20°C until used.

Primers and MAS-PCR:

The presence or absence of mutations in QRDRs of the *gyrA* and *parC* genes of FQ-resistant *E. coli* was detected by MAS-PCR reaction using gene-specific primers as summarized in Table (1). The *uspA* gene was used as an internal control. For *gyrA* gene, the reaction mixture (25µl) contained 1µl DNA template, 12.5 µl master mix (10× buffer, dNTPs (dGTP, dATP, dCAT, dTTP), and Taq DNA polymerase), 1µl of each Allele-specific primers (*gyr*A 83 F and *gyr*A 87 R) and 5.5µl water. For *parC* gene, the reaction mixture (25µl) contained 1µl DNA template, 12.5 µl master mix (10× buffer, dNTPs (dGTP, dATP, dCAT, dTTP), and Taq DNA polymerase), 0.5µl of each Allele-specific primers (*par*C 80 F and *parC* 84 R) and 8.5µl water. The samples were gently vortexed and the MAS-PCR was performed using Programmable Thermal Controller (MJ Research, INC., USA). Amplified products were visualized on 2% agarose gel stained with ethidium bromide under UV light.

Table 1: Primer Sequences for MAS-PCR Assays

Target gene	Primer	Sequence 5'–3'	Product size (bp)	Reference
***gyr*A**	gyrA F gyrA R	5'-TACACCGGTCAACATTGAGG-3' 5'-TTAATGATTGCCGCCGTCGG-3'	647	(39)
***gyr*A83** ***gyr*A87**	gyrA 83 F gyrA 87 R	5'-TAC-CAT-CCC-CAT-GGT-GAC-TC-3' 5'-GC-CAT-GCG-GAC-AAT-CGT-GTC-3'	440 255	(27)
***par*C**	parC F parC R	5'AAACCTGTTCAGCGCCGCATT-3' 5'-GTGGTGCCGTTAAGCAAA-3'	395	(39)
***par*C80** ***par*C84**	parC 80 F parC 84 R	5'-AAT-ACC-ATC-CGC-ACGGCG-ATA-G-3' 5'-CGC-CAT-CAG-GAC-CAT-CGG-TT-3'	289 153	(27)
***usp*A**	uspA F uspA R	5'- CCGATACGCTGCCAATCAGT-3' 5'-ACGCAGACCGTAGGCCAGAT -3'	884	(40)

Preparation of plant extracts:

Plant materials of six plant species were included in this study (Table 2) were collected from herbalists and markets in Mansoura, Egypt. The collected herbal plants were dried and pulverized into fine powder. The powdered material was stored in air tight sterile containers and protected from sunlight until required. 10 g of every dried powdered plant material were mixed with 100 ml of 95% ethanol solvent in sterile conical flask which was covered with foil paper and placed on a rotatory shaker for 24 hrs., then filtered through Whitman filter paper (No 1). The supernatant was collected and concentrated in vacuum for 15 min at 37°C using a Rotatory evaporator to make the final volume half of the original volume (stock solution). The concentration was then dissolved in 10 ml of 1% dimethylsulfoxide (DMSO). All extracts were sterilized by filtration through bacterial filter of pore size 0.45µm using positive pressure, then filtrate was kept at 4°C in refrigerator till use.

Antibacterial activity of herbal plant extracts:

Agar well diffusion method was used to evaluate antimicrobial activity of each plant extract. Muller Hinton Agar medium was prepared and inoculated with FQ resistant *E. coli* suspension by streaking the sterile nontoxic cotton swab in three directions over the entire surface of the agar plates to obtain a uniform inoculum. The density of the *E. coli* suspension was equivalent to that of 0.5 MacFarland standard (1.5 x 10^8 CFU/mL) .Sterile cork borer was used to make wells of 6mm in diameter in the agar plate. 150 µl of Plant extracts were introduced into each well using sterile Pasteur pipette and allowed to stand for 1 hour at room temperature to diffuse the plants extracts into medium. The DMSO was used in the same manner as negative control. The plates were then incubated at 37ºC for 18-24 hours. After incubation the entire diameter of the inhibition zone was measured in three different directions on all 3 replicates and the average value was tabulated then subtracting the diameter of the well.

Determination of the Minimum inhibitory concentrations (MICs) of selected herbal plant extracts:

The most effective plant extracts were used; *Syzygium aromaticum* (Clove) and *Foeniculum vulgare* (Fennel) which showed antibacterial activity against FQ resistant *E. coli*. The MIC was determined by using microtiter plate technique. Different concentrations of clove extracts range from 7.8 x 10^{-6} mg/ml to 4.25 mg/ml and for fennel range from 1 x 10^{-5} mg/ml to 5.3 mg/ml were prepared by serial dilution with nutrient broth. Sterile 96-well plates was filled with 100 µl of FQ-resistant *E. coli* suspended in nutrient broth in each well except last row. Then, 100 µl of first dilution of tested extracts (Clove 4.25 mg/ml and Fennel 5.3 mg/ml concentration) were added into the first row (12 wells) of the plate. Then, the rest of serial dilutions were added in successive rows by using a micropipette except the last two rows. In the row before the last one containing only FQ-resistant *E. coli* isolates which were used as a positive control. The last row containing uninoculated nutrient broth medium was used as a negative control. The test plate was incubated at 37°C for 18-24 hours. After incubation, the resulting turbidity was observed as an indicator of bacterial growth. Assessment of turbidity by optical density readings at 600nm was done with a Beckman DU-70 UV-V Spectrophotometer[21].

Table 2: Family, scientific, English, Arabic names and parts used from each plant in preparing extracts

Family	Scientific name	English name	Arabic name	Used part
Myrtaceae	*Syzygium aromaticum*	Clove	القرنفل	Flowers
Apiaceae	*Foeniculum vulgare*	Fennel	الشمر	Seeds
Umbelliferae	*Pimpinella anisum*	Anise	اليانسون	Seeds
Fabaceae	*Trigonella feonum-graecum*	Fenugreek	الحلبه	Seeds
Zingiberaceae	*Zingiber officinale*	Ginger	الزنجبيل	Rhizome
Labiate	*Mentha piperita*	Peppermint	النعناع	Leaves

Ethical approval

This study was approved by the local Medical Research Ethics Committee and written informed consents were obtained from patients.

This study was registered at IRB (institutional research board) and was given a code number R/17.12.298.

RESULTS

***E. coli* isolates:** Fifty *E. coli* isolates were identified by colony morphology, microscopic examination and biochemical tests.

Antimicrobial susceptibility:

Fifty *E. coli* isolates were tested for their resistance to second and third generation of fluoroquinolones.

The *E. coli* isolates showed high resistance to ciprofloxacin (76%) followed by norfloxacin (72%), levofloxacin (70%) and ofloxacin (68%) (Fig. 1).

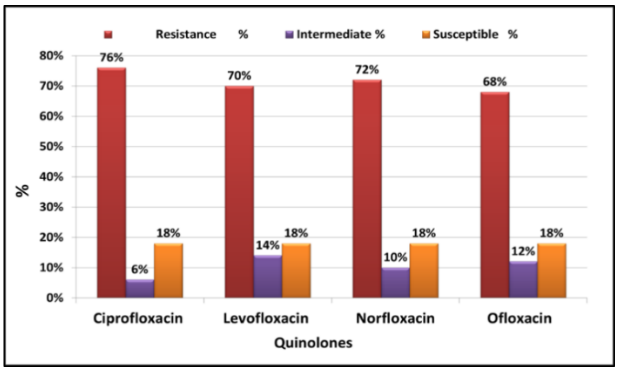

Fig. 1: Comparative susceptibility of *E. coli* isolates against quinolones

Detection of mutations in fluoroquinolones resistance genes:

Twenty-five *FQ resistant E. coli* were used to detect the presence of the expected resistance genes using MAS-PCR. The results in Fig. 2A showed that single mutations encoding Ser83 and Asp87 were observed in QRDR of *gyrA* in 8 (32%) and 5 (20%) respectively. Twelve (48%) FQ-resistant *E. coli* had double mutations in both Ser83 and Asp87 of *gyrA* encoding region. The results in Fig. 2B showed that single mutations encoding Ser80 and Glu84 were observed in QRDR of *parC* in 21 (84%) and 1 (4%) respectively, and three (12%) FQ-resistant *E. coli* isolates had double mutations in both Ser80 and Glu84 of *parC* encoding region. On the other hand, no mutation was observed in FQ-susceptible *E. coli* no. 8 (wild type).

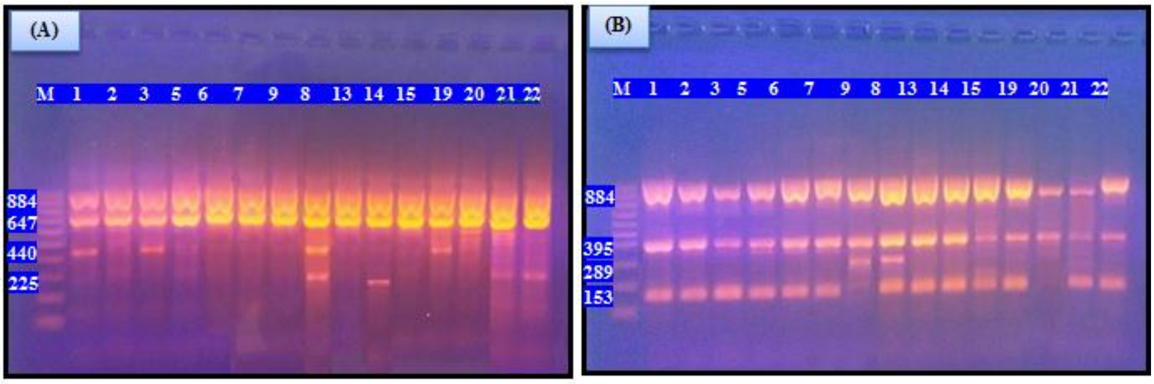

Fig. 2 (A-B): MAS-PCR products of *gyrA* (A) and *parC* (B) genes from susceptible (control) (8) and resistant *E. coli* isolates. (M) DNA marker on 2% agarose gel.

(A): Single mutation was detected in *E. coli* isolates no. (1, 3, 14, 21 and 22)
Double mutation was detected in *E. coli* isolates no. (2, 5, 6, 7, 9, 13, 15 and 20)
No mutation was detected in *E. coli* isolate no. (8)
(B): Single mutation was detected in *E. coli* isolates no. (1, 2, 3, 5, 6, 7, 9, 13, 14, 15, 19, 21 and 22)
Double mutation was detected in *E. coli* isolate no. (20)
No mutation was detected in *E. coli* isolate no. (8)

Antibacterial activity of herbal plant extracts:

Six plant species were investigated to evaluate their antibacterial activity against FQ-resistant *E. coli* using agar well diffusion method. Evaluation of antibacterial activity of these plant extracts was recorded in Table (3) and illustrated in Fig. 3. Of all extracts, the ethanolic one of Clove was the most active with inhibition zones diameter ranged between 12mm-22mm and Fennel caused inhibition zones diameter ranged between 10mm-18mm. Followed by Peppermint, Anise, Fenugreek and ginger respectively.

Table (3): Antimicrobial activity of ethanolic plant extracts against clinical *E. coli* isolates

Resistant *E. coli* isolates No.	Diameter of inhibition zone (mm) of different ethanolic plant extracts					
	Clove	Fennel	Anise	Fenugreek	Ginger	Peppermint
1	18	12	8	0	5	10
2	12	13	10	0	5	12
3	14	0	0	0	0	10
9	20	0	0	5	0	0
15	15	11	0	0	0	0
20	20	18	0	0	0	10
25	15	0	0	0	0	0
33	22	10	6	0	0	0
37	18	0	0	0	0	15
40	13	10	0	0	0	0

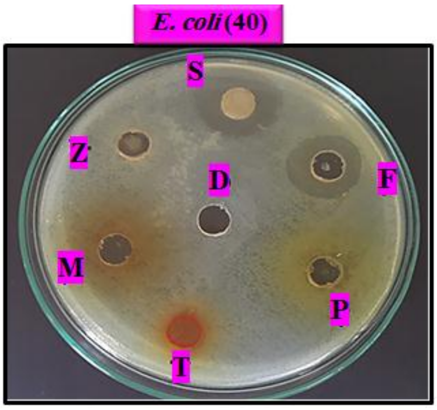

Fig. 3: Inhibition zones of different ethanolic plant extracts against clinical *E. coli* isolates where S= *Syzygium aromaticum*, F= *Foeniculum vulgare Miller*, P= *Pimpinella anisum*, T= *Trigonella feonum-graecum*, Z= *Zingiber officinale*, M= *Mentha piperita*, D= DMSO

The results in Tables (4) showed inhibition zone diameter of alcoholic extract of these plants compared with FQ against clinical *E. coli* isolates. The crude extracts of tested herbal plants showed good activity against clinical *E. coli* isolates whilst FQ therapy has limited effect as shown in Fig. 4.

Table 4: Comparison between activity of fluoroquinolones and different alcoholic plant extracts against clinical *E. coli* isolates.

E. coli isolates No.	Diameter of inhibition zone (mm)									
	CIP	LEV	NOR	OFX	Clove	Fennel	Anise	Fenugreek	Ginger	Peppermint
1	0	0	0	0	18	12	8	0	5	10
2	0	5	0	0	12	13	10	0	5	12
3	0	0	0	0	14	0	0	0	0	10
9	0	0	0	0	20	0	0	5	0	0
15	0	0	0	0	15	11	0	0	0	0
20	0	0	0	0	20	18	0	0	0	10
25	0	0	0	0	15	0	0	0	0	0
33	10	0	0	0	22	10	6	0	0	0
37	0	0	0	0	18	0	0	0	0	15
40	0	10	0	5	13	10	0	0	0	0

CIP: ciprofloxacin, LEV: levofloxacin, NOR: norfloxacin, OFX: ofloxacin.

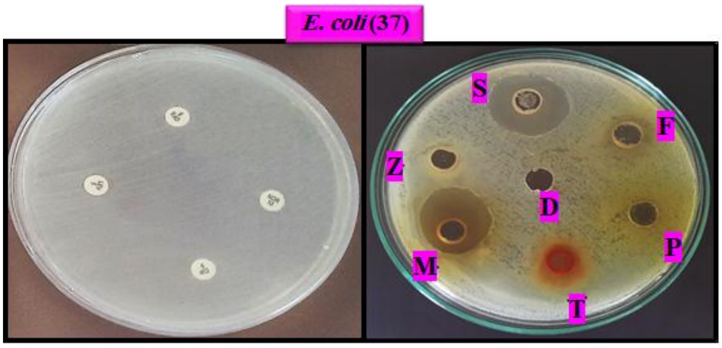

Fig. 4: Comparison between activity of fluoroquinolones and different alcoholic plant extracts against clinical *E. coli* isolates S= *Syzygium aromaticum*, F= *Foeniculum vulgare Miller*, P= *Pimpinella anisum*, T= *Trigonella feonum-graecum*, Z= *Zingiber officinale*, M= *Mentha piperita*, D= DMSO

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Minimum inhibitory concentrations (MIC's) of the effective plants extract:

The MIC value of the most effective plant extracts (*S. aromaticum* and *F. vulgare*) were $8.28x10^{-6}$ mg/ml and $1x10^{-5}$mg/ml respectively. The results in Fig. 5 indicated that decrease in growth of *E. coli* with increase of plant extracts concentration and vice versa.

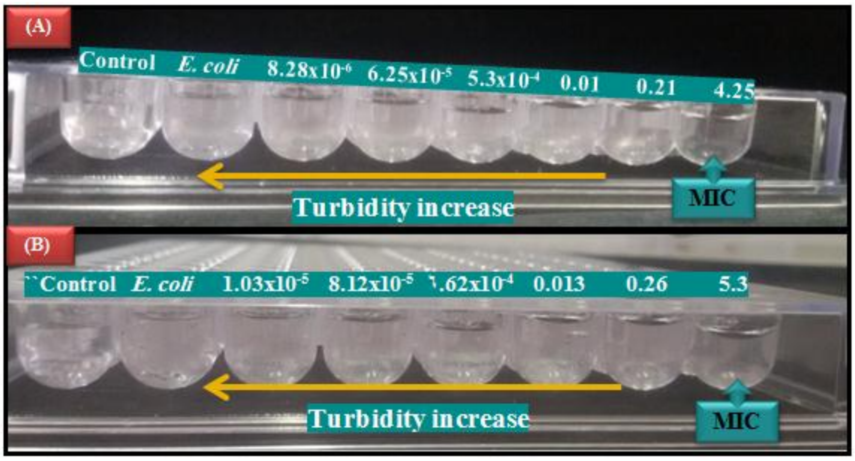

Fig. 5: MIC of clove (A) and fennel (B) extracts determined in Microtiter Plate

DISCUSSION

Resistance to antibacterial agents is highly prevalent in bacterial isolates worldwide, particularly in developing countries. Normal intestinal flora is a reservoir for resistance genes; the prevalence of resistance in *E. coli* is a useful indicator of antibiotic resistance in bacteria at the community. In this study, 42% of *E. coli* were isolated from urine samples. In other studies, 58% of *E. coli* were isolated from urine samples[22]. Also, Kudsi and Kelmani found that *E. coli* isolates from urine sample accounted for (46.6%) higher than from different clinical samples collected from hospitals and diagnostic centers in Kalaburagi region[23].

Of 21 *E. coli* isolates from urine samples, 15 (62%) isolates were from females and 6 (38%) were from males. This result indicated that the female patients had higher prevalence of UTI than in males. A number of factors are associated with high prevalence of infection in females such as shorter and wider urethra in females than in males, lack of antimicrobial properties of prostatic fluid that contain zinc which acts as bactericidal substance in males, hormonal changes which affect the mucosal adherence of bacteria and trauma of urethra during sexual intercourse[24].

The *E. coli* isolates were collected from different clinical specimens showed different degree of susceptibility to fluoroquinolone antibiotics. *E. coli* were highly resistant to ciprofloxacin (76%) followed by norfloxacin, levofloxacin and ofloxacin with 72%, 70% and 68% resistance, respectively. In another study the highest resistance of *E. coli* was detected with ciprofloxacin (89%), norfloxacin (75%), ofloxacin (74%) and levofloxacin (25%) in Indian adjoining communities[25]. The prevalence and rate of resistance among pathogenic bacteria differ vastly based on geographical location and hospital type, but it is raising enough to be considered a health threat[26].

In this study, the mutations in *gyrA* and *parC* genes were detected in all FQ resistant isolates. The distributions of mutations in *gyrA* gene showed that Ser83 was the most frequent mutation (32%) and Asp87 was the second common substitution (20%) while for *parC* gene, the most common mutation was Ser80 (84%), followed by Glu84 (4%) substitution. Similarly in another study, the Ser83 substitution was the most frequent mutation detected in *gyrA* gene in (89.19%) of isolates, while the Asp87 substitution was the second common mutation detected in the *gyrA* which was found in (79.28%) isolates. Additionally in the *parC* gene of *E. coli* isolates, the most common mutation was Ser80 (82.88%), followed by Glu84 substitution (31.53%)[27]. these results showed that mutation in both QRDR of *gyrA* and *parC* occurs concurrently. A single point *gyrA* mutation, at codon 83, is sufficient to generate FQ resistance but the additional mutation in *gyrA*, and/or *parC* mutation, is associated with an increased FQ resistance[28].

In the present study, the ethanolic extract of Clove was the most active one with inhibition zones diameter ranged between 12mm-22mm and Fennel caused inhibition zones diameter ranged between 10mm-18mm. Followed by Peppermint, Anise, Fenugreek and ginger respectively. In agreement with our results, Clove extracts had potent antimicrobial activity against *E. coli* with inhibition zones diameter ranged between 16mm-20mm[29].

On other hand, in this study no antimicrobial activity was detected in extracts of Fenugreek and dry ginger against *E. coli* isolates at the specific dose. In contradictory study, antibacterial activity of above ethanolic plants extract was detected against *E. coli*[30] and Fenugreek seed extract to treat sever skin inflammation[31]. This variation may be because of the dose used in this study, the method of extraction of medicinal plants, the method of antibacterial study, the genetic variation of plant, age of the plant or the environment[32]. However, the inability of the extracts to inhibit the growth of the tested organisms at lower concentrations may be due to the low levels of the active ingredient (the bioactive compounds) in the concentration of the extracts.

In the present study, the inhibition of growth of *E. coli* with the crude extracts of herbal plants was more pronounced with clove extracts as compared to fluoroquinolones antibiotics. The clove extract has greater antibacterial activity among all the extracts and the maximum value of the zone of the inhibition is noted against *E. coli* was 22mm approximately 2.1 times than ciprofloxacin (10mm).

These results are supported by other studies reported that Clove extracts had potent antimicrobial activity against *E. coli* with inhibition zones diameter ranged between 16mm-20mm[2, 33, 34]. Moreover, the maximum inhibition zone of clove in our results was higher than that reported by Kumar *et al.* who concluded that clove showed minimum effect on *E. coli* and maximum inhibition zone was (15mm)[35].

In this study, FQ-resistant *E. coli* strains were found sensitive to a lot of tested plant extracts. This has clearly indicated that these extracts might have different modes of action than that of antibiotics on test organisms. This observation agrees with hypothesis of Hasegawa *et al*. and Eloff they suggested that it is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens[36, 37]. Such activity of plant extracts may be a result of the presence of broad spectrum antimicrobial compounds (tannins, saponins, phenolic compounds, essential oils and flavonoids) or general metabolic toxins[38].

CONCLUSION

In conclusion, Fluoroquinolones antibiotic resistance becoming a global problem for public health which threatens the lives of hospitalized individuals as well as health care cost and long-time treatment. Therefore, it is important issue to be addressed by policy makers to formulate a strict fluoroquinolones antibiotic prescription policy for bacterial infections in our country. Scientists have realized an immense potential in natural products from medicinal herbal plants to serve as alternative source of combating infections in human being which may also be of lower cost and less toxicity. However, further studies are needed to better evaluate the potential effectiveness of the crude extracts as the antimicrobial agents.

Acknowledgments

We thank Head and members of Microbial genetics unit in Medical microbiology and Immunology department , Faculty of Medicine, Mansoura University for molecular analysis.

Financial support. None

Conflict of interest. No conflict of interest relevant to this article

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