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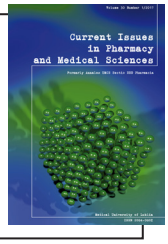

Molecular characterization of ESBL gene in *Citrobacter* spp. and antibacterial activity of omega-3 against resistant isolates

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ABSTRACT

The study aimed to investigate the prevalence and resistance pattern of different *Citrobacter* spp., phenotypically and genotypically, to β-lactam antibiotics, then to evaluate the antibacterial activity of omega-3 extracted from flaxseed against isolates that harboring resistance genes. Herein, 19 *Citrobacter* isolates were isolated from 100 stool and urine samples taken from patients attended to AL-Sadar Hospital during June-December 2016. Clinical samples were then cultured on specific media, thereafter, isolates were identified depending on morphological, biochemical characteristics and VITK-2. The results showed that the *Citrobacter* spp. comprise 19/78 (24%) of positive bacterial growth on macConky agar, from which 14.1% were *C. freundii*, *C. koseri* represented 6.1% and *C. farmeri* were 3.8% of the total. The results of antibiotic susceptibility showed that all *Citrobacter* 100% isolates were resistant to ampicillin and cefoxitin, but were sensitive to imipinim. Moreover, the isolates initally showed different degrees of resistant to β-lactam antibiotics. Furthermore, by confirmatory test, the results observed that 17/19 (89.4%) of the isolated were extended-spectrum β-lactamase (ESBL – producers). Finally, using the PCR technique to detect bla_{Genes}, the results revealed that 14/17 (82.3) of potential ESBL producing *Citrobacter* harbored one or more ESBL genes. These included 10 isolates of *C. freundii* and 4 isolates of *C. koseri*. In related work, extracts of essential fatty acid semicarbazide – omega3 (EFASC) from *Linum usitatissium* (Flaxseed) were tested to evaluate their activity against resistant isolates. The results demonstrate the broad spectrum antibacterial property of EFASC compounds against resistant bacteria. In conclusion, this study found increase prevalence of multi-drug resistance MDR *Citrobacter* spp. as causative agents in clinical cases. Considering the antibacterial activity of EFASC that displayed high activity against resistant pathogens, deservedly, attention must be paid to developing their use as alternative antibiotics.

INTRODUCTION

Citrobacter is a gram-negative rod motile bacteria, a *Enterobacteriaceae*. Its name is derived from its ability to use citrate as a sole carbon source [1]. Infections with *Citrobacter* spp. have seen increasing importance as a cause of serious nosocomial outbreaks that are difficult to treat using most common antibiotics [2,3]. Indeed, locally, several studies refer to the prevalence levels of *Citrobacter* infections [4,5,6].

Extended-Spectrum β-Lactamase (ESBL), a member of the β-lactamases enzymes family that hydrolyzes the β-lactams ring, leads to loss of bactericidal activity of a wide variety of antibiotics, including third generation cephalosporins and penicillins [7]. The increase prevalence of ESBL producing gram- negative bacteria is a significant problem in treating bacterial infection, in addition to the different induced side effects such as allergy to some antibiotics, nephrotoxicity, ototoxicity and alteration of normal gut flora. For this reason, seeking new alternative medicines which control pathogens while showing reduced side effects has become a crucial part of drug development research. Yet even now, 'green' medicine has been used for the medication of different bacterial disease [8].

Seeds from *Linum usitatissium* (Flaxseed), which contain about 36 to 40% of oil, are a rich source of the following unsaturated essential fatty acids (USFA): Omega 3 (linolenic acid), Omega 6 (linoleic acid) and oleic acid content [9]. Linolenic acid and other compound of essential fatty

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acids (EFAs) have been assessed as possible new agents for treating skin infections caused by *P. acnes* and *S. aureus* [10]. Of note, several studies have confirmed the successful use of USFA against *S. aureus, Pseudomonas* spp. and *L. monocytogenes* [11]. The semicarbazides found in the EFAs are the raw material of the semicarbazones that possess a wide spectrum of antibacterial activities [12].

MATERIALS AND METHODS

Bacterial Characterization

A total of 100 stool and urine specimens were collected under aseptic conditions from patients attending to Al-Sadar Medical City in AL-Najaf province. These were inoculated on MacConkey agar and XLD agar (Oxoid Cambridge, UK) and incubated at 37°C for 24 h. The morphological characteristics of the colonies including size, shape and color, were recorded, the suspected *Citrobacter* were made relevant by biochemical test [13], then finally confirmed by using a Vitek-2 Compact (Bio Mérieux, France).

Antibiogram test. Antibiotic susceptibility was carried out on all isolates using the Kirby Bauer disc diffusion method. The results were inter-operated by measuring the zone of inhibition in mm. Subsequently, using the results of testing with cefotaxime, ceftazidime, ceftriaxone, and aztreonam (30 µg of each), β-lactam resistant isolates (ESBL Production) were initially screened out according to [14]. Their presence were then confirmed by the disk approximation test according to [15]. Herein, any augmentation which appear to increase the diameter of the inhibition zone between the central amoxi-clav disk and any of the surrounding disks for the cefotaxime, ceftazidime, ceftriaxone, and aztreonam that was distributed around the amoxi-clav disk was considered as positive for the presence of ESBL Production. Disks that showing resistance or intermediate susceptibility were recorded.

Genomic DNA extraction. The cell pellets from all resistant isolates were used for extracting genomic DNA by way of the Genomic DNA Mini extraction kit (Geneaid, USA), following the manufacturer's instructions. The extracted DNA was kept in sterile eppendorf tubes and stored at -20°C prior to PCR.

Detection of resistance genes. PCR amplification for detecting the four bla_{Genes}, bla_{CTX-M}, bla_{TEM}, bla_{SHV} and bla_{OXA} was carried out according to Bioneer corporation, Korea practice, as shown in Table 1.

Table 1. The sequences of synthesized oligonucleatoide

Primer name	Sequences (5-3)		Molecular weight of amplicon (bp)	Ref.
CTX-M	F	CGCTGTTGTTAGGAAGTGTG	754	16
	R	GGCTGGGTGAAGTAAGTGAC		
SHV	F	AGGATTGACTGCCTTTTTG	392	17
	R	ATTTGCTGATTTCGCTCG		
OXA	F	ATATCTCTACTGTTGCATCTCC	619	18
	R	AAACCCTTCAAACCATCC		
TEM	C	ATCAGCAATAAACCAGC	516	18
	H	CCCCGAAGAACGTTTTC		

PCR mixtures (25 µL) containing 5 µL of DNA template, 12.5 µL master mix (Promega,USA) and 1.25 µL of each primer and 5 µL of sterilized distilled water were used. PCR amplifications were performed in an Agilent, USA Thermo Cycler according to manufacturer's procedures [16,17,18]. PCR products were then electrophoresized on 1.5% agarose gels, stained with ethidium bromide (Biobasic, Canada), visualized by UV illumination and were photographed by a Cleaver gel documentation system (Biometer/Germany).

Plant collection. Flaxseed were obtained from the local market in Al-Najaf City. The seeds were cleaned and the foreign materials were removed. The dried seeds were then powdered, and stored in a refrigerator at 4°C to await further processing [19].

Preparation of oil. The flaxseed oil was extracted using hexan solvent (1: 4 w: v) in a Soxhlet apparatus (Preciso, England) for 24 h. Then, EFA was isolated from the oil using a Cleavenger (Shepreth, England), according to [19]. Purity and identification of EFA-omega3 compounds by TLC was carried out according to [20].

Preparation of EFA – Semicarbazide (EFASC). One gram of EFA-omega-3 were dissolved in 4 ml of methanol and 1 : 1 H_2SO_4, then 4mg of thiosemicarbazide in methanol were added to this solution with constant stirring at room temperature for 4 h. Following this, NH_4OH was added till alkaline, then stirred for about 15 min and kept overnight. The resulting crystals was filtered, dried and recrystallized [21]. A stock solution was prepared by dissolving 500 mg of dried extracts with 1 ml DMSO to give a final concentration of 500 mg/ml, from which a serial concentration was prepared [19]. The agar well diffusion method was then applied to determine the EFASC antibacterial activity, according to [22].

Statistical analysis

Analysis of data was performed by using Statistical Package for Social Science (SPSS) system/ version 17 and Microsoft Office Excel 2007. Results were expressed as mean ± S.D. P-value was considered significant when it is less than 0.05. The analysis of variance (ANOVA) was also applied.

RESULTS

1. Identification of *Citrobacter*

Citrobacter spp. gram negative colonies appear small, pink and convex on MacConkey agar, and yellow, smooth, flat and round on XLD agar. All the 19 isolates of *Citrobacter* spp. were lactose fermenting, motile and gave positive test results for catalase, methyl-red and citrate, and negative results for Indole (except *C. koseri*), oxidase and Voges-Proskuaer. These also have the ability to ferment glucose on Kligler iron agar (A/A). The results demonstrate an ID message confidence level that is considered excellent by way of the VITEK-2 compact system.

Nineteen bacterial isolates were identified as *Citrobacter* spp. from the 78 positive bacterial growths seen on MacConkey agar and recovered from the 100 clinical specimens collected. This result showed a frequency 24%. The isolates were represented by 11 (14.1%) isolates of *C. freuendii*, and 5

(6.41%) *C. koseri,* while 3 isolates (3.8%) were identified as *C. farmeri* (Figure 1), and 59 isolates (75.65%) showed growth of other gram negative bacteria which included *Klebsiella* spp., *E.coli*, *Pseudomonas* spp. and *Proteus* spp. (Figure 1):

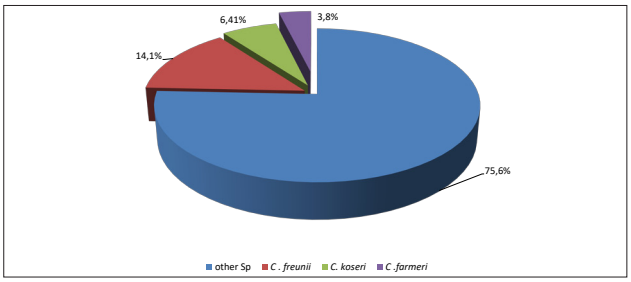

Figure 1. Pie chart showing the distribution of *Citrobacter* spp.

2. Antibiogram test

The results of the antibiogram tests for all *Citrobacter* spp. to 11 antibiotics were summarized in (Figure 2). This revealed that all isolates were multi-drug resistant and all were 100% resistant to ampicillin and cefoxitin, while 100% sensitive to imipenem antibiotic. Among the third-generation cephalosporins tested, *C. freundii* appear highly resistant against amoxi-clav and cefotaxime (90.1% of all isolates), while resistance to ceftazidime and cefriaxon was recorded in 81.8% of all isolates, and various levels of resistance were observed towards oxacillin (72.7%), ciprofloxacin (54.5%) and gentamycin (63.6%). The resistance pattern of *C. koseri* and *C. farmeri* are shown in (Figure 2).

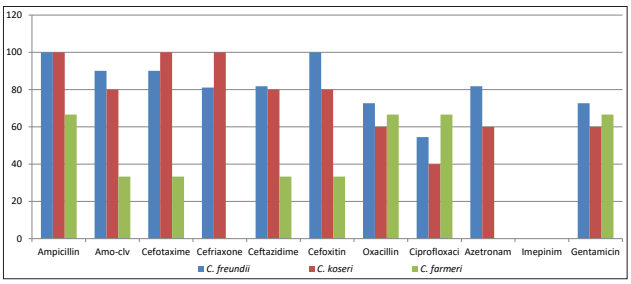

Figure 2. Antibiotic resistance pattern of *Citrobacter* spp.

3. Correlation in antibiotic resistance between phenotypic (initial, confirmatory) and genotypic

As shown in Table 2, the results of initially and confirmatory testing revealed that all *Citrobacter* isolates were considered potential ESBL-producers initially, while the confirmed results showed that only 17/19 (89.4%) of the isolates were actual ESBL producers. The results of detecting for bla_{Genes} ($bla_{CTX\text{-}M}$, bla_{TEM}, bla_{SHV} and bla_{OXA}) via PCR revealed that 14/17 of all potential ESBL producing *Citrobacter* carried at least one of the ESBL genes. These included 10 isolates of *C. freundii* and 4 isolates of *C. koseri*.

The results also revealed that 12 isolates contained only one ESBL gene type. These are: 5 $bla_{CTX\text{-}M}$, 3 bla_{TEM}, 2 bla_{SHV} and 2 bla_{OXA}. Moreover, 2 isolates of *C. freundii* had the combination of two genes: one of *bla*SHV genes in combination with $bla_{CTX\text{-}M}$ genes, and one of bla_{TEM} genes in combination with $bla_{CTX\text{-}M}$ genes (Table 2, Figure 3).

Table 2. The correlation of phenotypic, genotypic to antibiotic resistance in *Citrobacter* spp.

Name & No.of isolate	Phenotypic		Genotypic			
	Initial	Confirmatory	CTX-M	TEM	SHV	OXA
C. freundii 1	+	+	-	✚	-	-
C. freundii 2	+	+	-	✚	-	-
C. freundii 3	+	+	✚	-	✚	-
C. freundii 4	+	+	✚	✚	-	-
C. freundii 5	+	+	✚	-	-	-
C. freundii 6	+	+	-	-	-	✚
C. freundii 7	+	+	-	-	-	✚
C. freundii 8	+	+	-	-	✚	-
C. freundii 9	+	+	✚	-	-	-
C. freundii 10	+	+	✚	-	-	-
C. freundii 11	+	+	-	-	-	-
C. koseri 1	+	+	-	-	✚	-
C. koseri 2	+	+	-	✚	-	-
C. koseri 3	+	+	✚	-	-	-
C. koseri 4	+	+	✚	-	-	-
C. koseri 5	+	+	-	-	-	-
C. farmeri 1	+	+	-	-	-	-
C. farmeri 2	+	-	-	-	-	-
C. farmeri 3	+	-	-	-	-	-
Total	19	17	7	4	3	2

+ resistant, ✚ have resistant gene

4. Frequency of ESBL genes in *Citrobacter* spp.

The results of ESBL-gene distribution detection has revealed that CTX-M β-lactamase was the most prevalent (43.7%) among the ESBL producing isolates; followed by TEM -β-lactamase (25%) and SHV β-lactamases (18.75%), while OXA β-lactamase gave 12.5% (Figure 3, 4A,B,C,D):

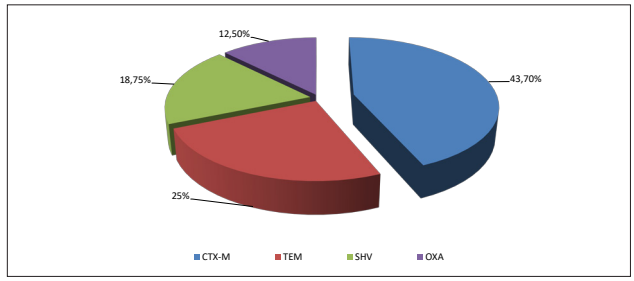

Figure 3. ESBL gene distribution *Citrobacter* spp.

5. Thin Layer Chromatography

The analysis of the TLC chromatography of EFASC – omega 3 as shown in (Table 3) has revealed the presence of light brown spots in day-light and light green spots by using UV light after iodine spray. Such spots have R*f* equal to 0.37, similar to the standard (EFASC-Omega3) which has R*f* value 0.37, and which appear dark brown in day-light and dark green under UV-light.

Table 3. Thin Layer Chromatography Essential Fatty Acid Semicarbazide of flaxseed (oil) compounds

Properties	EFA (Omega 3)	EFASC
Rf	0.37	0.37
Color by day-light	Dark Brown	Light Brown
Color under UV-Light	Dark Green	Light Green

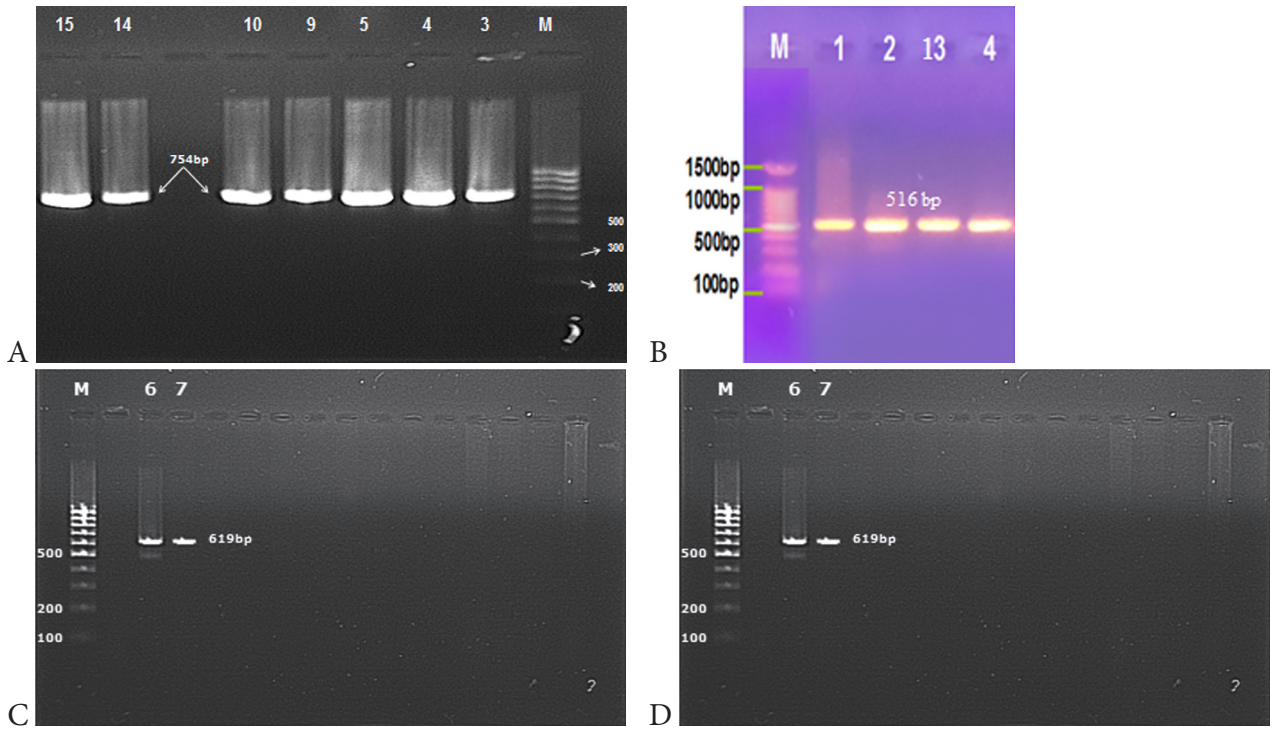

Figure 4. Ethidium bromide stained agarose gel (1.5% agrose gel, 75 V, 1.25 hours) showing PCR amplification products with (**A**) CTX-M gene. Lane L: Ladder (100 - 1517bp). Lane (3,4,5,9,10) C. f. No. 3,4,5,9,10, respectively. Lane (14,15) *C. koseri* No. 3,4. (**B**) TEM gene. Lane (1,2,4) C. f. No. 1,2,4. Lane (13) C. koseri No. 2. (**C**) OXA gene. Lane (6,7) C. f. No. 6,7 (**D**) SHV gene. Lane (3,8) C. f. No. 3,8, respectively. Lane (12) C. koseri No. 1

6. Evaluation of the antibacterial activity of EFASC against resistant isolates

The antibacterial activity assessment showed that the highest extract inhibition zone in a 500 mg/ml concentration was that generated by the growth of *C. koseri* – **31 ± 0.93** and *C. freundii* – **29 ± 0.93**. Such results also revealed high (significant) correlation with EFASC concentration (62.5, 125, 250 and 500 mg/ml) against the isolates – as explained in (Table 4). Furthermore, the technique revealed that even low conc. of extract were more active than cefreaxone against these pathogens:

Table 4. Antibacterial activity of EFA SC against *Citrobacter* spp. isolates

Conc. of EFASC	*C. freundii*	*C. koseri*
500 mg/ml	29 ± 0.93	31 ± 0.93
250 mg/ml	27 ± 1.20	28 ± 1.20
125 mg/ml	22 ± 0.79	24 ± 0.99
62.5 mg/ml	20 ± 0.75	22 ± 0.75
Cefreaxone	19 ± 0.5	18 ± 0.48

DISCUSSION

We found that, over all, *C. freundii* was the most common infection agent recovered from the different clinical specimens. This was not in conflict with the results of [23], who found that *C. freundii* is the most common pathogen in 6.6% of all diarreatic patients, 2% of all UTI patients and 2% from wounds. In our study, the second most common pathogen was *C. farmeri*. This result is similar too, with that of [24], who noted the commonality of 6% for *Citrobacter* spp. isolated from UTIs. Moreover, according to [25], *C. koseri* was the causative agent of UTIs, while [26] isolated *C. farmeri* from UTI and wound infections.

World-wide, antimicrobial resistance is a major clinical problem in treating bacterial infection. In our work, most of the isolates can be considered multi-drug resistant. This result is in agreement with a study done locally by [6], who found *C. freundii* isolates were 100% resistant to cefoxitin and had varying degree of resistance to ceftazidime, aztreonam, ciprofloxacin and gentamicin. Our results were not different from the results of [27], who isolated *C. freundii* from UTIs and found that all *Citrobacter* isolates were resistant to cefotaxime and ceftriaxone, and can be considered as being MDR-bacteria. Moreover, [23] also observed that *C. freundii* had 100% resistance to β-lactam antibiotics. In addition, [28] observed increased prevalence of multidrug resistance in *Enterobacteriaceae*, making nearly all members of this family showing resistance to B-lactam and cephalosporin. This they saw even in rarely pathogenic members such as *Pantoea* spp. They put forward that this came about as a result of horizontal gene transfer. The work of [29] also found that *C. koseri* were the predominant urine pathogen and recorded high rates of resistance to cefexime, amox-cla and cephaloxin. In addition, according to [30], some *Citrobacter* isolates contain chromosomally mediated β-lactamases such as Cephalosporinase and Penicillinase that have led to the emergence of drug resistance and treatment failure despite initial susceptibility [30].

In our study, 17 (89.4%) of the isolates were ESBL producers. The rates of resistance to cephalosporins and monobactam might be considered, therefore, as markers for the production of ESBL by these isolates. This may

have come about by their producing the common group of class A β-lactamases, consisting of TEM, SHV and CTX-M β-lactamases that have extended hydrolytic spectrum activity to cephalosporins [31].

We hold that the proportion of ESBL-producing *Enterobacteriaceae* are increasing worldwide. This finding is in accordance with that of [27], who reported that *C. freundii* were ESBL-producers and that isolates possess both CTX_M 1 and 2 genes. What is more, [4] revealed that *C. freundii* were ESBL-producers and hold to 100%, the CTX-M gene. The prevalence of bacteria which produced more than one type of ESBL enzyme is considered most dangerous [32].

Our study shows that CTX-M β-lactamase was the most prevalent (43.75%) among the ESBL producing isolates (Figure 3). This is in agreement with several other studies such as [33] in Najaf, who found that CTX-M β-lactamase was the most prevalent (38.7%) among the ESBL producing G-ve isolates, followed by SHV (33.9%); while TEM and OXA β-lactamases were the less (27.4% for each). Of note, [34] noticed that all the *Citrobacter* spp. harbor *bla* genes, the prevalence of these genes being $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV} and bla_{AmpC}, respectively. Finally, [35] revealed that *C. koseri* isolated from UTI patients were multi-drug resistant and harbored the TEM, SHV-ESBL genes. According to [36], however, SHV β-lactamases enzymes are mainly found in G-ve bacteria.

Our TLC analysis revealed the presence of brown spots in the same locale as the EFA omega 3 standard. Both of these gave Rf = 0.37. Such results are in accordance with [19] who found that the Rf value = 0.36, and with [37], who explained that EFA – omega 3, when using hexan solvent, gave an Rf value of 0.34.

In the second part of our experiment, fourteen isolates that revealed β-lactam antibiotic resistance were chosen so as to examine the impact of EFASC extracts. The result of this part of the experiment illustrated the susceptibility of the resistant isolates to EFASC even in low concentrations. Indeed, generally, 12 of the 14 (85.7%) resistant bacteria showed growth inhibition despite different concentrations of EFASC. The results of [19] also show that EFASC seed oil extracts possess good antibacterial activity against nosocomial infection bacteria. Furthermore, our results are in agreement with those of [21], who indicated the strong effect of EFASC against *E.coli* and *S.aureus* at varied levels. Moreover, our results are compatible with the study of [38], who found that the antibacterial action of fatty acids is usually attributed as being a property of the long-chain unsaturated fatty acids, including oleic acid, linoleic acid and linolenic acid. Indeed, [39] noted that polyunsaturated essential fatty acids play a role in inhibiting the growth of bacteria that contain a penicillinase plasmid. In this regard, [40] explained that fatty acids modulate the fluid permeability of cell membranes – which can greatly affect membrane properties.

CONCLUSIONS

EFASC can be considered useful alternative therapeutic agents in treating a wide range of antibiotic-resistant bacteria because they are safe, highly active and dependable, with less harmful side effect than antibiotics. The last cost more, have sometimes dangerous and often uncomfortable side effects, and to which, most bacteria now show resistance.

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