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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

This study aimed to investigated the prevalence and resistance pattern of different *Citrobacter* species phenotypically and genotypically to β-lactam and some most common antibiotics then evaluate the antibacterial activity of omega-3 extracted from flaxseed against isolates that harboring resistance genes. 19 *Citrobacter* isolates were isolated from100 stool and urine samples taken from patients attended to AL-Sadar Hospital during June-December 2016. Clinical samples were cultured on specific media, thereafter isolates were identified depending on morphological, biochemical characteristics and VITK-2. The results showed that the *Citrobacter* comprise 24% of isolated bacteria which divided into 11 (14.1%) were *C. freundii*, 5 (6.41%) *C. kosaeri* and *C. farmeri* were 3 (3.8%). The antagonistic activity was evaluated by observing a clear zone of inhibition growth, the results showed that all *Citrobacter* (100%) isolates were resistant to Ampicillin, cefoxitin and sensitive to Imipinim, also the isolates showed different degrees of resistant to β-lactam antibiotics initially. By confirmatory test the results observed 17/19 (89.4%) isolated were ESBL producers finally using PCR technique to detect bla– genes (blaCTX-M, OXA, SHV and Z). The results revealed that 14/17 (82.3) of potential ESBL producing *Citrobacter* were harbor one or more of ESBL genes they included 10 of *C. freundii* and 4 of *C. koseri*. The extraction of essential fatty acid semicarbazide (omega-3) from *Linum usitatissium* (Flax seed) were tested to evaluate their activity against resistant isolates, results explained broad spectrum antibacterial property of EFASC compounds against resistant bacteria. In conclusion, this study found increase prevalence of MDR *Citrobacter* spp as causative agents in clinical cases. Considering antibacterial activity of EFASC, it was observed highly activity against resistant pathogens deservedly, therefore attention must be paid to development their used as alternative antibiotics.

Key words: Citrobacter infection, ESBL-genes, omega-3 antibacterial activity

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INTRODUCTION

Citrobacter is a gram negative rods motile bacteria, one members of Enterobacteriaceae. It name derived from its ability to use citrate as a sole carbon source (JANDA et al. 1994). Infections with

Citrobacter spp. have been increasing importancy as a cause of serious nosocomial outbreaks and difficult to treated by most common antibiotic (NADA et al. 2004; SHIH et al. 1996). Locally, several studies referred to the prevalence levels of *Citrobacter* infections (AL-HASNAWI 2014; AL-HISSNAWY et al. 2012; TUWAIJ 2016). Extended spectrum B-lactam (ESBL) a members of β-lactamases enzymes hydrolyzes the β-lactams ring lead to loss of bactericidal activity of wide variety of antibiotics including third generation cephalosporins, penicillins and monobactam (HARVEY & CHAMPE 2012). The increase prevalence of ESBL producing gram negative bacteria is a significant problem in treating bacterial infection, in addition to different side effects like allergy to some antibiotic, nephrotoxicity, ototoxicity, and alteration of normal gut flora. For this reason seeking to a new alternative medicine to control pathogens with reduced side effects has become a crucial part of drug development research. On the other hand, green medicine has been used for the medication of different bacterial disease (FUAD et al. 2012).

Linum usitatissimum L. (Flaxseed) the annual plant. Its' seeds containing about 36 to 40% of oil is a rich source of the following unsaturated essential fatty acids: Omega 3 (linolenic acid), Omega 6 (linoleic acid) and oleic acid content (WANG et al. 2017). Linolenic acid and other compound of (EFAs) used as possible new agents to treat skin infections caused by *P. acnes* and *S. aureus* (DESBOIS & LAWLOR 2013). Several study confirmed the successful treatment of USFA against *S. aureus, P. aerogenosa, L. monocytogenes* (SHIN et al. 2007) semicarbazides are the raw material of semicarbazones that possess a wide spectrum of antibacterial activities (SINGHAL & PAUL 2011).

METHODOLOGICALLY BACTERIAL CHARACTERIZATION

A total of 100 clinical specimens from Stool and Urine were collected under aseptic condition. These specimens were collected from patients attending to Al-Sadar Medical City in AL-Najaf province 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, color, were recorded, the suspected Citrobacter were relevant by biochemical test (MACFADDIN 2000), then finally confirmed by using Vitek-2 Compact (Bio Mérieux, France).

Antibiogram test: Antibiotics susceptibility was carried out on all isolate using Kirby Bauer disc diffusion method. Results were inter operated by measuring the zone of inhibition in mm. Then using results of cefotaxime, ceftazidime, ceftriaxone, and aztreonam (30 µg of each one) as initially screened to detect β-lactam resistant isolates (ESBL Production) according to (CLSI 2014). Then confirmed by the disk approximation test according to (BATCHOUN et al. 2009). Any augmentation (increase in diameter of inhibition zone) between the central Amoxiclav disk and any of the cefotaxime, ceftazidime, ceftriaxone, and aztreonam (surrounded the plate around the Amoxiclav). Disks that showing resistance or intermediate susceptibility was recorded.

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

Detection of resistance genes: PCR amplification for detection the four bla genes, bla-CTX-M, bla-TEM, bla-SHV and bla-OXA were carried out according to information of Bioneer corporation, Korea) as shown in Table 1. PCR mixtures (25 µL) contained 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 was used. PCR amplifications were performed in Agilent, USA Thermo Cycler according to condition (HASSAN et al. 2013; COLOM et al. 2003; SVÄRD 2007). PCR products were electrophoresized on 1.5% agarose gels, stained with ethidium bromide (Biobasic, Canada) and visualized by UV illumination and were photographed by a Cleaver gel documentation system (Biometer/Germany).

Tab. 1: The sequnces of synthesized oligonucleatoide

Primer name		-)	Molecular weight of mplicon (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		

Plant collection: Flaxseed were obtained from seller of herbal and medicinal plants in Al-Najaf City. The plants were washed with distilled water, then air dried, powdered, and stored in refrigerator at 4 °C for further processes (AHMAD et al. 2017).

Preparation of oil: The oil of Flaxseed were extracted with hexan solvent (1:4 w:v) by continous extraction in a soxhlet apparatus (Preciso, England) for 12 hours. Then isolation of EFA from oil using cleavenger (Shepreth, England) according to (AHMAD et al. 2017). Purity and Identification of EFA-omega3 compounds by TLC was carried out according to (HARBORNE 1984).

Preparation of EFA – Semicarbazide (EFASC): One gram of EFA (omega-3) were dissolved in 4 ml of methanol and 1:1 H2SO4, then 4gm of thiosemicarbazide in methanol were added to this solution with constant stirring at room temperature for 4 hr and then added NH4OH till alkaline stir for about 15 min and kept it overnight. Crystals was filtered, dried and recrystallized (BORHADE 2014). Determination of antibacterial activity Agar well diffusion method was used to determine the antibacterial activity of EFASC of Flaxseed compounds according to (EGHAREVBA et al. 2010).

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

RESULTS

Identification of Citrobacter

Nineteen of bacterial isolated were identified as *Citrobacter* spp from 78 positive bacterial growth on MacConkey agar recovered from the 100 clinical specimens collected with a frequency (24.3%). The isolates were represented by 11 (14.1%) isolates of *C. freuendii*, 5 (6.41%) *C. kosori* and 3 isolates (3.8%) were identify as *C. farmeri* (Fig. 1), while 59 isolates (75.65%) showed growth of other gram negative bacteria which included *Klebsiella* species, *E. coli*, *Pseudomonase* and *Proteus* species.

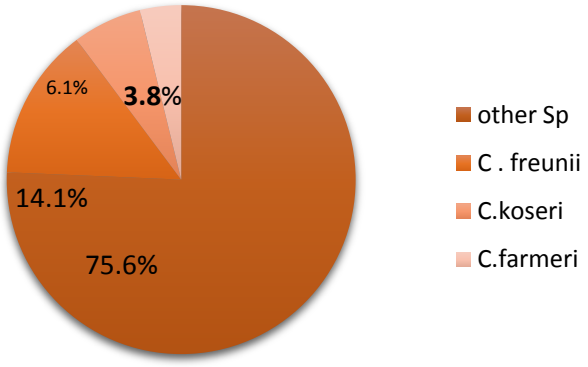

Fig. 1: Pie chart showing the distribution of *Citrobacter* spp.

Antibiogram test

The results of antibiogram tests for all *Citrobacter* species to 11 antibiotics were summarized in Figure 2. The results revealed that all isolates were multidrug resistant and all of them were 100% resistance to Ampicillin and Cefoxitin, while 100% sensitive to Imipenem antibiotic. Among the third-generation cephalosporins tested, *C. freundii* appear highly resistance against amox-clave and cefotaxime in 90.1% of isolates, while resistance to ceftazidime and cefriaxon was recorded in 81.8% of isolates, and various levels of resistance was observed towards oxacillin 72.7%, Ciprofloxacin 54.5% and Gentamycin 63.6%. The resistance pattern of *C. koseri* and *C. farmeri* were shown in Figure 2.

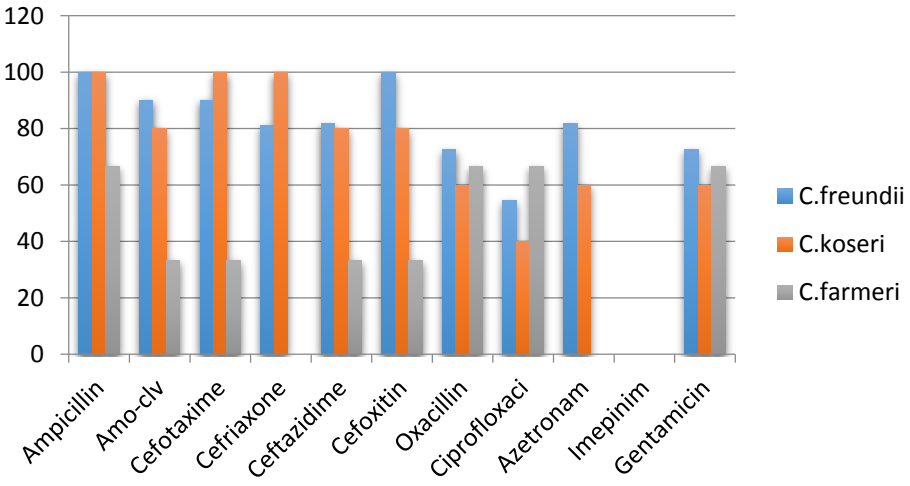

Fig. 2: Antibiotic resistance pattern of *Citrobacter* spp.

Initially, Confirmatory and Detection of resistance genes

As shown in Table 2 the results of initially and confirmatory test revealed that all *Citrobacter* isolates were gave a potential ESBL-producers initially, while the confirmed results showed that only 17/19 (89.4%) of the isolates were ESBL producers. The results for detection bla – genes (blaCTX-M , bla-TEM, bla-SHV and bla-OXA) by PCR revealed that 14/17 of potential ESBL producing *Citrobacter* were carried at least one of ESBL genes they included 10 of *C. freundii* and 4 of *C. koseri*. The results illustrated that 12 isolates were contained only one type of ESBL-genes as following 5 bla-CTX-M, 3 bla-TEM, 2 bla-SHV and 2 bla-OXA genes. While, 2 isolates of *C. freundii* had the combination of two genes: one blaSHV genes combination with bla-CTX-M genes and one bla-TEM genes combination with blaCTX-M genes Table 2 and Figure 3.

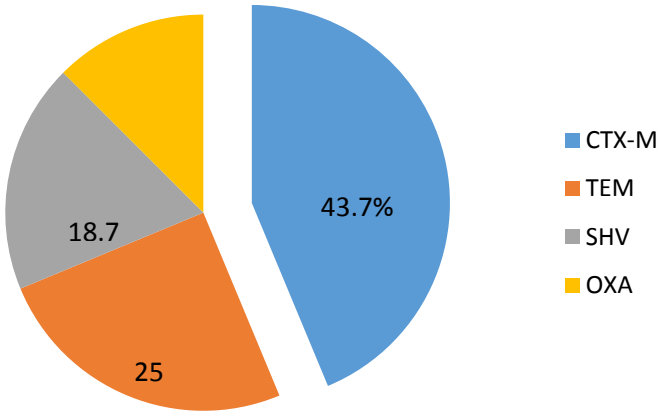

Fig. 3: ESBL-gene distribution in *Citrobacter* spp

Tab. 2: The frequency of phenotypic, genotypic to antibiotic resistance in *Citrobacter* spp.

Name & No. of isolate	Phenotypic		Genotypic			
	Initial	Confirmatory	CTX-M	TEM	SHV	OXA
C. f. 1			-		-	-
C. f. 2			-		-	-
C. f. 3				-		-
C. f. 4					-	-
C. f. 5				-	-	-
C. f. 6			-	-	-	
C. f. 7			-	-	-	
C. f. 8			-	-		-
C. f. 9				-	-	-
C. f. 10				-	-	-
C. f. 11			-	-	-	-
C. k. 1			-	-		-
C. k. 2			-		-	-
C. k. 3				-	-	-
C. k. 4				-	-	-
C. k. 5			-	-	-	-
C. far. 1			-	-	-	-
C. far. 2		-	-	-	-	-
C. far. 3		-	-	-	-	-
Total	**19**	**17**	**7**	**4**	**3**	**2**

Frequency of ESBL-genes in *Citrobacter* spp

The results of detection ESBL-gene distribution revealed that CTX-M β-lactamase was the most prevalent (43.75%) among the ESBL producing isolates; followed by TEM -β-lactamase (25%) and SHV β-lactamases were (18.75%) while, OXA β-lactamase gave (12.5%) Figure (3, 4 A, B, C, D).

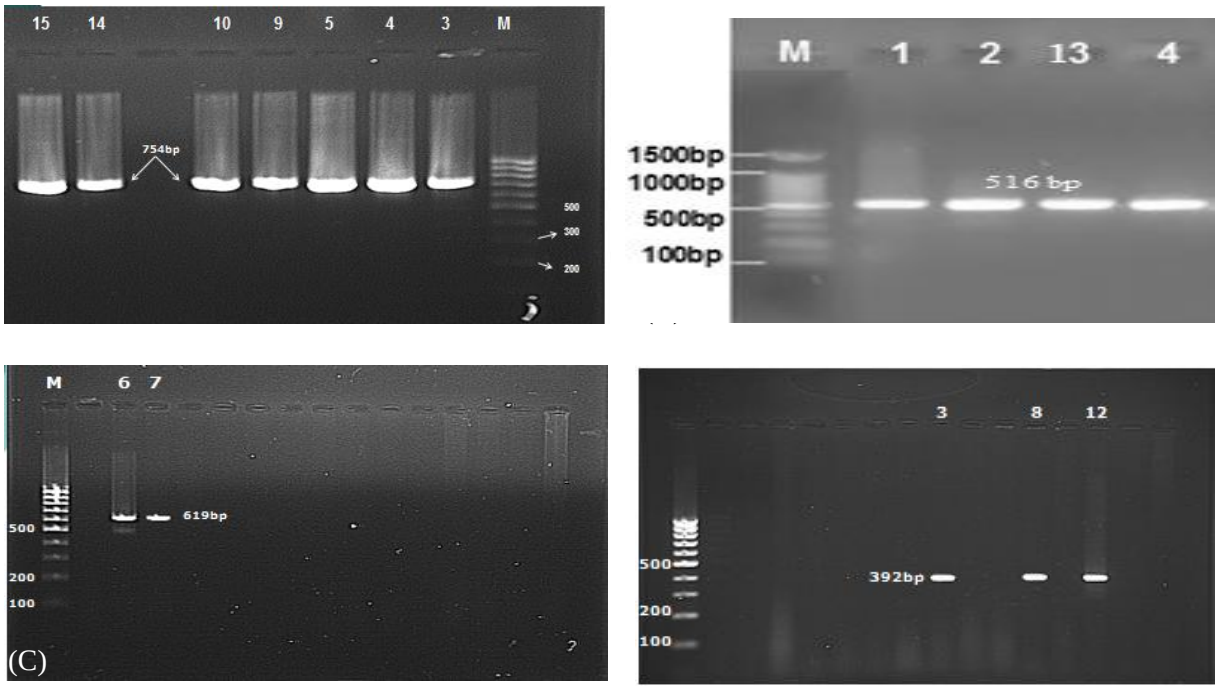

Fig. 4: Ethidium bromide stained agarose gel (1.5% agrose gel, 75 V, 1.25 hours) showing PCR amplification products with (A) CTX-M gene. Lane L: Ladder (100-1517 bp). Lane (3, 4, 5, 9, 10) C.f. No. 3, 4, 5, 9, 10 respectively. Lane (14, 15) C. koseri No. 3, 4. (B) TEM gene. Lane (1, 2, 4) C. f. No. 1,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

Thin Layer Chromatography

The analysis of TLC chromatography of EFASC –omega3 as show in Table 3 revealed the presence of light brown spot on day light and light green by using UV light deeper after spraying with iodine spray, has R*f* equal to 0.37 that similar to the stander (EFASC-Omega3) which has R*f* value 0.37 appear as dark brown at day light and dark green under UV-light.

Tab. 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

Evaluation the antibacterial activity of EFASC against resistant isolates

The result of antibacterial activity illustrated that highest inhibition zone of extracts in 500mg/ml concentration was demonstrated on the growth of *C. koseri* 31 ± 0.93 and *C. freundii* 29 ± 0.93. The results revealed highly effect with significant different in concentrations (62.5, 125, 250 and 500 mg/ml) of EFASC against isolates as explain in Table 4.

Tab. 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

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

This study revealed that *C. freundii* was the most infectional agents recovered from different clinical specimens. In the same line, (AL-MUSLEMAWI TH. A. 2007) founded that *C. freundii* is the most common pathogen in frequency 6.6% of diarreatic patients, 2% of UTI patient , and 2% from wound followed by *C. farmeri*. This result online with study done by (SALIH et al.2016) state that *Citrobacter* spp isolated from UTI with 6% percent. According to (STEWART et al. 2017) they recognized that *C. koseri* were causative agents of UTI while (WARREN et al. 2000) isolated *C. farmeri* from UTI and wound infection.

Antimicrobial resistance is a major clinical problem on treating bacterial infection worldwide. However, Most of these isolates are considered multidrug resistant this results agree with study done locally by (TUWAIJ 2016) who found *C. freundii* isolates were 100% resistance to cefoxitin and revealed varying degree of resistance to ceftazidime, aztreonam, ciprofloxacin and gentamicin. The results were not different widely from the results of (HASSAN et al. 2014) they isolate *C. freundii* from UTI and founded that all *Citrobacter* isolates were resistance to cefotaxime and ceftriaxone and considered as MDR-bacteria. AL-MUSLEMAWI (2007) who observed that *C. freundii* were 100%

resistance to β-lactam antibiotics while (METRI et al. 2013) They founded that *C. koseri* were predominant urine pathogen and recorded high rate of resistant to Cefexime, Amox-cla and Cephaloxin. Some *Citrobacter* isolate contain chromosomally mediated β–lactamases like Cephalosporinase and Penicillinase that lead to emergence of drug resistance and treatment failure despite initial susceptibility (SAMI et al. 2017). The resistance to Cephalosporins may be due to the phenomena minimizing membrane permeability based on membrane proteins purine (Porin-mediated permeability) this processes have a great impact of resistance to Cephalosporins (DANCER 2001).

In this study only 17 (89.4%) of the isolates were ESBL producers (Table 2). The rates of resistance to Cephalosporins and monobactam might be as markers for the production of ESBL by these isolates which may be by producing the common group of class A β-lactamases, consisting of TEM, SHV and CTX-M β-lactamases that has extended hydrolytic spectrum activity on Cephalosporins (BUSH et al. 1995).

Ten of *C. freundii* and 4 of *C. koseri* were carried at least one of ESBL genes they included 12 isolates were contained only one type of ESBL-genes. While, 2 isolates had the combination of two genes. This finding in accordance with 27 they reported that *C. freundii* were ESBL-producers and they founded that isolates possess CTX_M 1 and 2 genes. Also, AL-HASNAWI (2014) who revealed that *C. freundii* were ESBL-producers and possess 100 % CTX-M gene. The prevalence of bacteria which produced more than one type of ESBL enzymes is considered more dangerous for human hygiene (ERLANDSSON 2007).

This study show CTX-M β-lactamase was the most prevalent (43.75%) among the ESBL producing isolates Figure 3. Several studies improved this results such as (AL-MUHANNAK 2010) 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). Also, SHAHID (2010) who noticed in all the *Citrobacter spp* harboring *bla* genes and the prevalence of these genes as the following blaCTX-M, blaTEM, blaSHV, and bla ampC, respectively. Finally, PERILLI et al. (2005) revealed that *C.koseri* isolated from UTI patients were multidrug resistance and harporing TEM, SHV–ESBL genes. SHV β-lactamases enzymes are mainly found in G-ve bacteria (HUANG et al. 2004).

The analysis of TLC revealed the presence of brown spot on thin layer chromatography in the same local with standard (EFA omega 3) and both of them gave Rf = 0.37 these results in accordance with PANDYA et al. (2013) who explained that EFA – omega 3 by use hexan solvent gave Rf value 0.34 and with AHMAD et al. (2017) who founded that Rf value = 0.36.

The fourteen isolates that revealed β-lactam resistance antibiotics were choosen to examine the impact of EFASC extracts. The result of antibacterial activity illustrated that highly effect of different concentrations (62.5, 125, 250 and 500 mg/ml) of EFASC which illustrated the susceptibility of resistance isolate to EFASC.

The inhibition zone of EFASC of flaxseed was **29 ± 0.93** mm against *C. freundii* and **31 ± 0.93** mm against *C. koseri* in concentration 500 mg/ml, Table 4. In general 12 of 14 (85.7%) resistant bacteria were inhibit their growth by using different concentration of EFASC. Results of AHMAD et al. (2017) also show that EFASC of seed oil possess good antibacterial activity against nosocomial infection bacteria. In the same line (BORHADE 2014) indicated the strong effect of EFASC against *E. coli* and *S. aureus* at varied level and compatible with study by(SEIDEL & TAYLOR (2004) they 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. MOGENSEN (2009) mention that polyunsaturated essential fatty acid play role in inhibition the growth of bacteria that containing a penicillinase plasmid. In this regard (IBARGUREN et al.2014) explain that fatty acid modulate the fluid

permeability of cell membranes which can greatly affect membrane property.

Therefore EFASC consider useful approach in treatment wide range of antibiotic resistant bacteria because they are safe and dependable with less harmful than antibiotic which more cost, have side effect and most bacteria became resist against it.

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