Arab Univ. J. Agric. Sci., Ain Shams Univ., Cairo, Egypt
28(4), 1089-1103, 2020
Website: http://ajs.journals.ekb.eg

Reduction of Gentamicin Standard Dose Combined with Phytochemicals Against *Enterobacter Cloacae* DSM 3264 BRB Using Response Surface Methodology

[77]

Rawia F Gamal[1*], Rasha A Ebrahim[2], Samah H Abu-Hussien[1*]

1- Agric Microbiology Dept, Fac of Agric, Ain Shams Univ, P.O. Box 68, Hadayek Shoubra 11241, Cairo, Egypt
2- Medical Analysis Central Laboratories for Medical Analysis and Blood Bank of Armed Forces, Al-Khalifa Al-Ma'mun, Cairo, Egypt

*Corresponding author: samah_hashem1@agr.asu.edu.eg

Received 18 March, 2020 Accepted 16 October, 2020

Abstract

Antibiotic-phytochemicals combinations are used for enhancing the antibacterial efficiency against pathogens aiming to prevent microbial resistance development. This study was conducted at the Faculty of Agriculture, Ain Shams University in Cairo, Egypt, to study the in-vitro inhibitory activities of gentamicin antibiotic and phytochemical combinations of *Cinnamomum zeylanicum* (cinnamon), *Syzygium aromaticum* (clove) and *Mentha piperita* (mint) against *Enterobacter cloacae* DSM 3264 BRB by Minimal inhibitory concentrations (MICs) and Bactericidal inhibitory concentrations (MBCs) of the agents. Inhibitory activity of four recommended antibiotics (ciproflaxacin, cefepime, meropenem and gentamicin) and three oil plant extracts of the selected plants (cinnamon, clove, mint) were tested against *E. cloacae* DSM 3264 using well diffusion method on Muller-Hinton agar plates by studying MIC and MBC tests. Gentamicin was selected among the four tested antibiotics based on its high potential against *Enterobacter cloacae* DSM 3264 BRB. For the reduction of gentamicin standard dose, a synergism experiment between gentamicin and the three phytochemicals under investigation (cinnamon, mint and clove) was carried out using a new statistical approach of mixture drug design of response surface methodology against *E. cloacae* DSM 3264 growth. A mixture design of twenty mixture combination runs using different concentration levels of gentamicin and the three oil plants extracts was performed against *Enterobacter cloacae* DSM 3264 growth. The software "Design Expert® 12" Stat-Ease was used to analyze the experimental mixture design. Results showed that the standard recommended gentamicin dose 10 µg/100ml may be substituted by oil extract of *Cinnamomum zeylanicum* 4.75 % (v/v), *Syzygium aromaticum* 5.0% (v/v), *Mentha piperita* 5.0%(v/v) and gentamicin 0.25%(w/v) as an antibacterial agents. Analysis of clove, cinnamon and mint oils by GC-MS proved that the major components were 3-allyl-6-methoxyphenol (46.4%) followed by propylene glycol (53.59%), whereas cinnamon contains cinnamic acid and phenethyl ester being 41.79% while Mint contains menthol (32.9%) followed by menthone (27.7 %).

Keywords: Inhibitory activity, Gentamicin antibiotic, Cinnamon, Phytochemical combinations, *Enterobacter cloacae*, RSM

1 Introduction

Water-borne diseases cause more than about 2.2 million deaths per a year all over the world. Because of increasing enteric pathogens resistance against current antibiotics all over the world which reported by (WHO 2008), it's indeed needed to search for alternative antibacterial agents. *Enterobacter cloacae* is a member of Enterobacteriaceae family. As detected by MacNair et al (2018), it produces beta-lactamase enzyme, which is responsible for antibiotic resistance during healing treatments. It is sensitive to polymyxin B, ciproflpxacin, levofloxacin, doripenem, imipenem / cilastatin, meropenem, ertapenem, cefepime, trimethoprim-

sulfamethoxazole and gentamicin.. Egyptian's natural flora was used as a source of medicine & herbal medicine. Cinnamon oils have potential action against both of Gram-negative and Gram-positive bacteria (Baker and Grant 2018). Cinnamon has many components including: cinnamaldehyde, cinnamic acid, cinnamate and essential fatty acids such as Eugenol, L-borneol, L-bornyl acetate –Enerolidol. Cloves are the dried flower buds of *Syzygium aromaticum* tree. It belongs to family Myrtaceae. The most effect components in *Syzygium* sp. were eugenol (88.58%) which is used as an antimicrobial agent. Mint is an aromatic herbal plant member of the Lamiaceae family. The effective compounds found in mint were mainly known as monotorpenes such as menthol and menthone which have therapeutic uses as anti-fungal agents, (Mohammadinejad et al 2016). Different antibiotics were used to reduce diarrhea against infectious microbes such as: ciprofloxacin, levofloxacin which has a good activity against *Pseudomonas* and most Gram-negative. (Yanat et al 2017). Meropenem, (carbapenem antibiotic) is an alternative drug for severe *Enterobacter* infections. It is effective against most Gram-positive and Gram-negative bacteria. Cefepime, one of the ephalosporin fourth-generation inhibiting Gram-negative bacteria. Also, Gentamicin is an effective antibiotic against Gram-negative bacteria (Abd El-Mongy et al 2017, Serio et al 2017). Mixture Design is a statistical approach of RSM consisting of all combinations of each factor at its high and low levels. It is applied for calculating the dependence of response on ingredients proportionality. Theses designs are working on how the proportions of a mixture affect the response not the amounts (Delgado-Sánchez et al 2018). So, the main objective of this work was to study the in-vitro inhibitory activities (bactericidal and bacteriostatic) of gentamicin antibiotic and phytochemical combinations of *Cinnamomum zeylanicum* (cinnamon), *Syzygium aromaticum* (clove) and *Mentha piperita* (mint) against *Enterobacter cloacae* DSM 3264 BRB by calculating their Minimal inhibitory concentrations (MICs) and Bactericidal inhibitory concentrations (MBCs).

2 Materials and Methods

2.1 Bacteria used

A novel strain of *Enterobacter cloacae* DSM 3264 BRB, was tested for using phytochemicals as alternative antibiotics against its growth. This strain was isolated from river Nile in Cairo and identified using MALDI-TOFF with a score value of 2.17 (Ebrahim et al 2018). For *E. cloacae* DSM 3264 BRB culture maintenance, nutrient agar slants were inoculated then incubated at 4°C and sub-cultured at monthly intervals. Standard inoculum was prepared by inoculating 50 ml of the enrichment tryptone soy broth medium (TSB) (Oxoid 2006) in 250 ml Erlenmeyer flasks with a 3-5 single colonies of the sub-cultured strain. The inoculated flasks were incubated on a rotary shaking incubator (Lab-line Ltd.) at 120 rpm for 24 h at 37°C. After that, optical density of grown culture was adjusted on spectrophotometer at 625 nm for reading 0.06 – 0.8 which is equivalent to 10^6 CFU / ml considered as the standard inoculum (Saeed and Tariq, 2005) for further studies. For estimation of bacterial cell densities, standard growth curve was done to measure bacterial cell number (CFU/ml) against cell optical densities (OD) for completion of this experiment.

2.2 Collection and Preparation of plant essential oils' extracts

Soxhlet extraction method was used for preparing and extracting all plant oils in National Research Center (NRC) as described by (Redfern, et al 2014) in which, the selected plants were collected from local markets in Cairo. All collected plants were washed using tap water for 2-3 times. All leaves were shade dried for 10 to 15 days then crushed by grinding in the absence of any solvents. Defatting of plant leaves was done using petroleum ether followed by 100 ml of hydro-alcohol using a Soxhlet extractor for 24 hours and drying in desiccators. After drying, the hydro-alcohol was evaporated using a rotary evaporator leaving a small portion of extracted plant leaves (about 2 to 3 ml) in the flask's glass bottom. The hydro-alcoholic extract yielded a dark greenish residual mass which kept in sterile bottles at 10oC for further experiments.

2.3 Antibiotics drugs

Four antibiotic drugs (gentamicin, ciproflxacin, cefepime and meropenem) were used to test their inhibitory efficiacy against *E. cloacae* These antibiotics were purched from Abico, Novartes, Pharco and Amoun companies respectively. Drugs were diluted to reach a final concentration ranged between 0.25 to 10 µg/100µl for gentamicin and meropenem while reached 0.25 to 5.0 µg/100µl for ciprofloxacin, whereas ranged between 0.25 to 30.0 µg/100µl for cefepime (Haja et al 2014).

2.4 Inhibitory effect of different plant oils against tested pathogenic strain using well diffusion method

Anti-bacterial activity of different plant oils were tested separately using well diffusion method as described by NCCLS (1993) using Muller-Hinton agar medium (Oxoid, 2006). Its composition is beef extract (2.0 g/L), casein hydrolysate (17.5 g/L), starch (1.5 g/L), agar (17.0 g/L) with pH adjusted to 7.3 ±0.1 at 25°C. Twenty ml of Muller- Hinton agar medium was poured into Petri dishes. Poured dishes were inoculated with 1 ml of the tested pathogenic bacteria ($14X10^6$ CFU/ml) against distilled water as control treatment. After adsorption of the inoculum, Agar wells were made using a cork borer with 7 mm in diameter then, filled by 100 µl of the tested essential oil for each. After filling the wells, all inoculated Petri dishes were incubated at 37 °C for 24 h. All experiments were triplicated and the inhibitory activity was expressed as the mean of inhibition zone diameters (cm).

2.5 Minimum inhibitory concentration (MIC) of different plant oils against tested pathogenic strain

MIC was done using Tryptone soy broth (Oxoid, 2006). Its composition is tryptone (15.0), soybean (5.0), Sodium Chloride (5.0), Dipotassium Hydrogen Phosphate (2.5), glucose (2.5) g/L, with pH adjusted to 7.0±0.2 at 25°C. For preparing oil emulsions, oil concentrations were prepared (0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 40.0 % (v/v) in DMSO) then, 2% (v/v) of tween 80 were added. All concentrations of different plant oils were tested for their inhibitory activity against the tested strain. Inoculum was prepared by growing the tested strain in tryptone soy broth (TSB) for 24 h. at 37°C. Muller- Hinton agar medium was poured into Petri dishes, then inoculated with 1 ml of *E. cloacae* (10^6 CFU/ml) (Motlagh et al 2013).

2.6 Minimum inhibitory concentration (MIC) of used antibiotics

As described above, different concentration of gentamicin, ciprofloxacin, cefepime and meropenem were prepared then tested separately against the selected pathogenic bacterial strain according to (Jennifer 2001).

2.7 Minimum Bactericidal Concentration (MBC) for oil plant extracts and antibiotics used

Based on the results of MIC assay, the most efficient antibiotic and plant oil extracts were used for MBC determination using the modified broth dilution method as described by Davidson and Parish (1989) as follows: Muller- Hinton agar plates were prepared and TSB broth medium was prepared supplemented with 2% (v/v) tween 80 as reported by Lambert et al (2001). The experiment was performed by preparing different concentrations of selected antibiotic ranged from the standard concentration dose to 0.0625µg/100 µl and different plant oil concentrations ranged from 0. 5 – to 40% (µl/100µl). All prepared media were inoculated with 1 ml of bacterial standard inoculum ($14x10^6$ CFU/ml) and incubated at 37°C for 24 hrs. Bacterial count on Muller- Hinton agar and was calculated and optical density of TSB cultures were measured at 625 nm.

2.8 Gas chromatography-mass spectrometry analysis (GC/MS) of cinnammon, clove and mint essential oil

A Hewlett-Packard Gas Chromatographer (HP6890), coupled to VG Analytical 70-250S mass spectrometer was used for GC-MS analysis of the essential oils. This apparatus system is equipped with an HP-5MS (30 m x 0.25 mm, film thickness 0.25 µm) capillary column. Helium was used as carrier gas at flow rate of 1 mL/min. The oven program was set at a temperature range from 50 °C for 5 minutes to 280°C as oven-temperature increasing was programmed at 40°C/ 5 minutes and finally held isothermally for 5 minutes. A sample of 1µl was injected by split mode. For GC-MS detection, an election ionization system, with ionization energy of 70 eV was used. A scan rate of 0.6 s (cycle time: 0.2 s) was applied, covering a mass range from 35 to 600 amu. The identification of the essential oil compounds was based on the comparison of retention indices and mass spectra of the homolog's series of (C4-C28) with data generated under identical experimental conditions (Adams 2017).

2.9 Synergistic effects of gentamicin and selected plant oil extracts against *Enterobacter sp.* using mixture design of RSM

Mixture design of RSM was used to calculate all mixture constitutions' levels. The Sum of all mixture constitutes was expressed mathematically as $0 \leq X1 \leq 1$ in which this relationship is called fundamental constraint of mixtures. Optimal design with a full experiment of 20 runs was chosen to be used in the optimization process as presented in **Table 1**. The 3D triangle was designed to have the three diluted essential oils located the mixtures of essential oils / gentamicin, the equal portions mixture of the four components at the vertices of the triangle. Run trial No. (4) was replicated for 3 times to detect the pure error and to compare it with the lack of fit. Linear regression model (incomplete cubic) was used to express the responses as a function of in-dependent variables based on mixture design method in which, Y: The response (inhibition zone diameter) in cm. α1, α2, α3 represent the linear term coefficients. α12, α22, α23 represent the binary term coefficients. α123 was for the ternary term coefficient and ε represents the error term.

Table 1. Mixture design for gentamicin combined with essential plant oils

Run	(A) Cinnamon % (v/v)	(B) Clove % (v/v)	(C) Mint % (v/v)	(D) Gentamicin % (w/v)
1	2.58	5.00	5.04	2.38
2	2.62	7.04	5.08	0.25
3	1.07	7.71	5.53	0.68
4	4.75	5.00	5.00	0.25
5	2.58	5.00	5.04	2.38
6	0.50	7.11	7.07	0.31
7	0.59	7.06	5.00	2.35
8	0.50	9.25	5.00	0.25
9	0.50	5.00	9.25	0.25
10	1.49	6.00	6.05	1.46
11	2.58	5.00	7.10	0.31
12	0.98	5.52	7.71	0.79
13	0.50	7.11	7.07	0.31
14	1.17	5.00	5.71	3.11
15	2.58	5.00	7.10	0.31
16	3.15	5.52	5.48	0.84
17	0.50	5.00	5.00	4.50
18	0.50	5.04	7.12	2.34
19	0.59	7.06	5.00	2.35
20	2.62	7.04	5.08	0.25

2.10 Statistical analysis

Least significant different (LSD) was calculated using two ways ANOVA pack of SAS software package as described by SAS (2002). Determination coefficient (R^2) test was calculated using SPSS software package as described by Excel software package 2007**.** One-way ANOVA for synergic and model equation was calculated using mixture design using design expert 12 package.

3 Results and Discussion

3.1 Inhibitory activity of different plant oil extracts against *E. cloacae* DSM 3264 BRB

As described by (NCCLS 1993), well diffusion method was carried out to test the inhibitory effect of oil plant extracts against *E. cloacae* DSM 3264 BRB. **Fig 1** indicated that the tested strain was susceptible to clove, cinnamon and mint to reach an inhibition zone of 2.60, 1.75 and 1.45 cm, respectively.

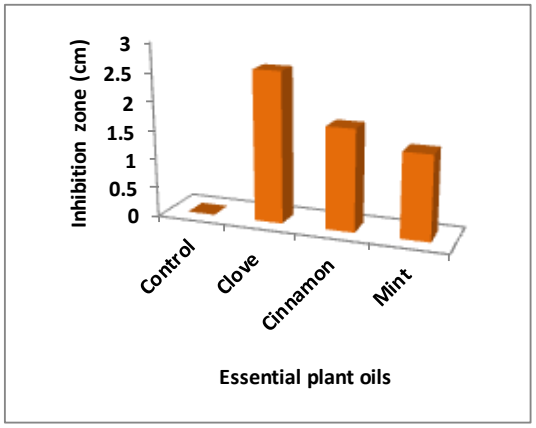


Fig 1. Inhibitory effect of different plant oil extracts against *Enterobacter cloacae* DSM 3264 BRB using well diffusion method

3.2 Minimum inhibitory concentration (MIC) of different plant oils against *E. cloacae* DSM 3264 BRB

MIC of the three plant oils were studied against the tested strain using macrodillution method according to Saeed and Tariq (2005). Data presented in **Fig 2** clearly show that *Enterobacter cloacae* had high sensitivity to all oil plant extracts at concentration of 5-40% emulsified in 2% tween 80 (v/v). Out of the three plant oil extrcts, cinnamon

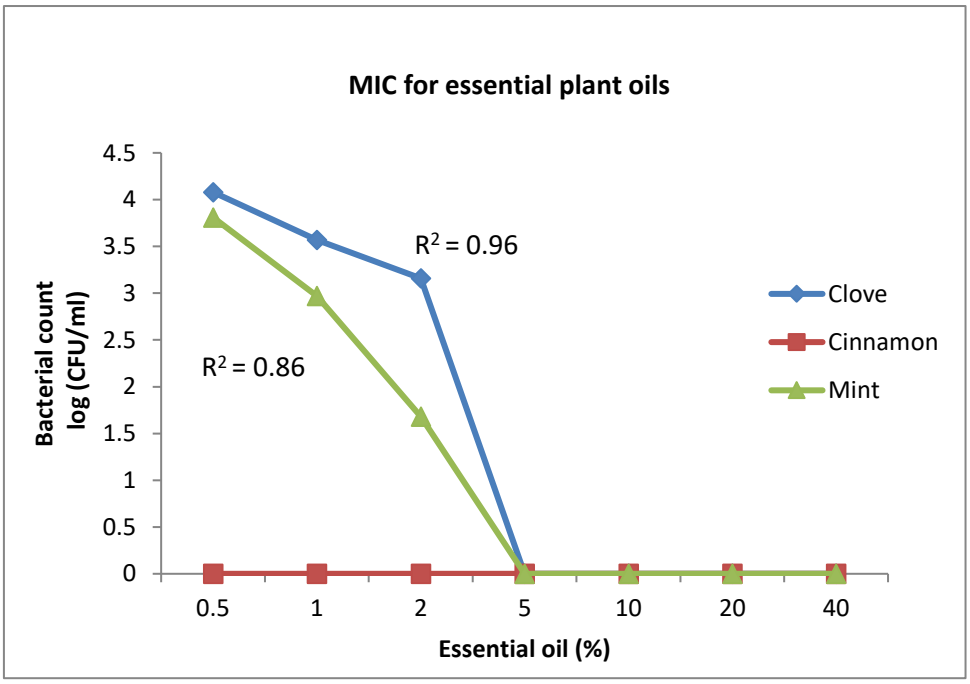


Fig 2. Spectrum activity of mint, clove and cinnamon oil plant extracts against *E. cloacae* and determination coefficient (R^2) between oil extracts and spectrum activity

oil had the ability to inhibit 100% of *E.cloacae* DSM 3264 BRB growth at all concentration ranged from 0.5- 40%. According to Liaqat et al (2017), cinnamon acts as anti-inflammatory agent against wide Gram-negative and Gram-positive bacteria to reach 12.17 – 29.5 mm due to the presence of flavonoid compounds such as βcaryophyllene, linalool and other terpenes (Paranagama 1991) also it contains a high percentage of eugenol, which has been identified as a compound exhibiting antimicrobial properties (Garg and Siddigui 1992).

3.3 Impact of different antibiotic concentrations against *E. cloacae* DSM 3264

Antibiotics were subjected to inhibitory test against *E. cloacae* DSM 3264 using well diffusion method. **Fig 3** illustrated that the four selected antibiotic drugs of gentamicin (GM), ciprofloxacin (CIP), meropenem (MEM) and cefepime (CPM) were calculated against *E. cloacae*strain after 24hrs at 37°C. Gentamicin showed a zone diameter of 1.05 - 2.2 cm with average (1.517 cm), ciprofloxacin 1.4 - 2.6 cm with average (2.070 cm), meropenem 1.55-2.850 cm with average (2.217 cm) and cefepime (CPM) were 1.25 - 2.9 cm with average (2.056 cm). Comparing the obtained data with standard zone of inhibition against *E. cloacae* using standard concentrations of different antibiotics according to CLSI (2015). Data revealed that the tested strain was susceptible (S) to antibiotics gentamicin, ciprofloxacin, meropenem and cefepime within range 2-10, 1-5, 2-10 and 10-30 (µg/100µl) respectively. The statistical analysis of the data refers that the most efficient antibiotics was arranged according to the highest zone of inhibition were cefepime 2.9, meropenem 2.85, ciprofloxacin 2.6 to gentamicin 2.2 cm.

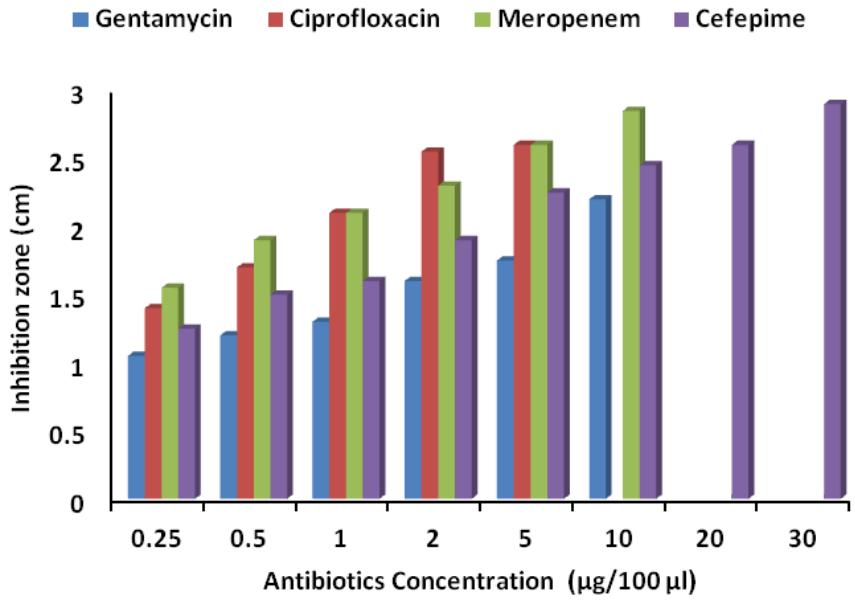


Fig 3. Impact of different antibiotic concentrations against *E. cloacae* DSM 3264 using well diffusion method expressed as inhibition zone (cm)

3.4 Minimum inhibitory concentrations (MIC) of different antibiotics against *E. Cloacae* DSM 3264

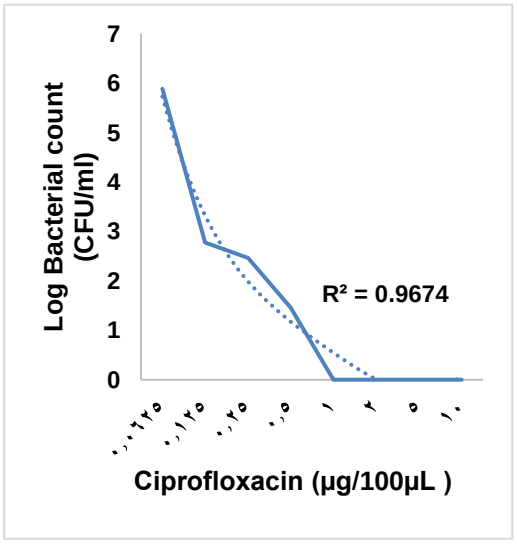
The minimum inhibitory concentration of gentamicin, ciprofloxacin, meropenem and cefepime against *E. cloacae* were recorded 0.25, 1.0, 5.0 and 5.0 µg/100 µl, respectively with determination coefficient (R^2) ranged between 0.9032 to 0.9833 for all the tested antibiotics as recorded in **Fig 4**. The obtained result of gentamicin was in disagreement with those obtained by Huang et al (2012), who mentioned that MIC value of gentamicin against *Enterobacter cloacae* was 16 µg/ ml. As a result, gentamicin at 0.25 µg/100µl was selected for carrying out the synergistic effect in addition to the three oil plant extracts (ginnamon, clove and mint), using mixture design Expert 12.

3.5 Bacteriostatic and bactericidal effect of selected plant oil extracts

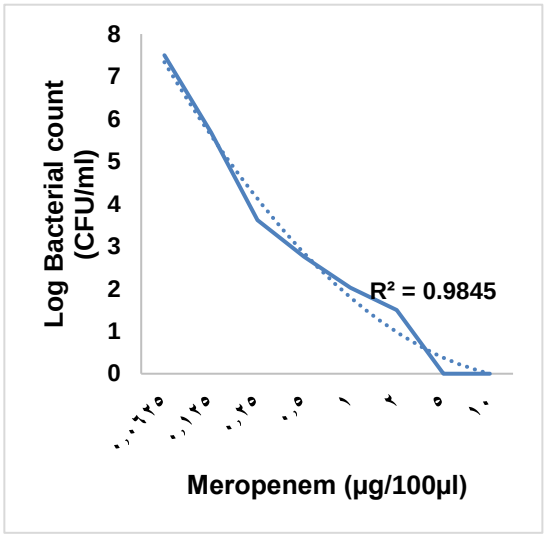
MBC/MIC ratio was calculated to express the role of plant oils extract as antibacterial agents against *E. cloacae.* **Fig 5** stated that all the tested plant oils extracts considered as bactericidal affect against in which their ratio was equal to 1. As the ratio of MBC/MIC equals to value 1 or 2, the oil is known as bactericidal agent, oppositely, if the value is greater than or equal to 4 – 16, the oil is known to be bacteriostatic agent. (Hashim 2015 and Elshikh et al 2016).

3.6 Synergistic effects of gentamicin and selected plant oil extracts against *E. cloacae* DSM 3264 BRB using Mixture design

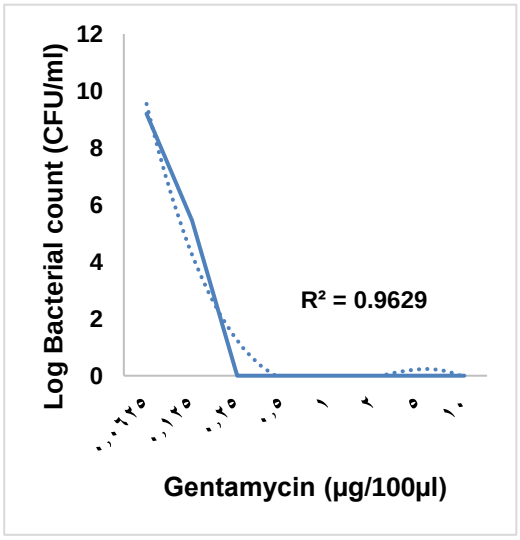
Because of the increasing of pathogens resistance against antibiotic drugs, alternative compounds of phytochemicals should be discovered to treat infectious diseases. The further experiment has been designed by select the 1/40 recommended dose of gentamicin (0.25 µg/100µl) against *E. cloacae* which resulted to intermediate response and added to mixture of the previous effective plant oil extract as a trial to increase its sensitivity (synergistic effect). Data presented in **Table 2** illustrated the 20 mixture design runs which showed that run number four of the four components of cinnamon, clove, mint and gentamicin with 4.75, 5.00, 5.00 (v/v) and 0.25 (w/v), respectively had the highest inhibitory effect against *Enterobacter cloacae* with predictive zone of 2.79 which is in agreement with the actual zone of inhibition value of 2.70. So this point was selected for test point validation test. Statistically, the selected model was well adjusted to observation.



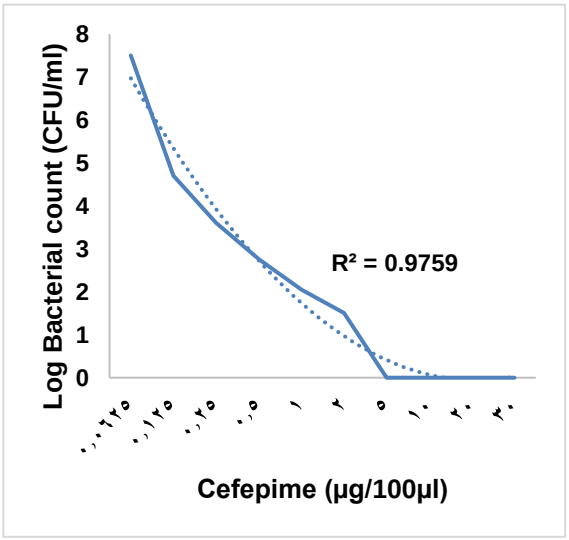
A) MBC of ciprofloxacin against *E.cloacae*



B) MBC of Meropenem against E.cloacae



C) MBC of gentamycin against E.cloacae



D) MBC of cefepime against E.cloacae

Fig 4. Determination coefficient (R^2) of the tested antibiotics concentration expressed as MIC against *E.cloacae* DSM 3264 BRB

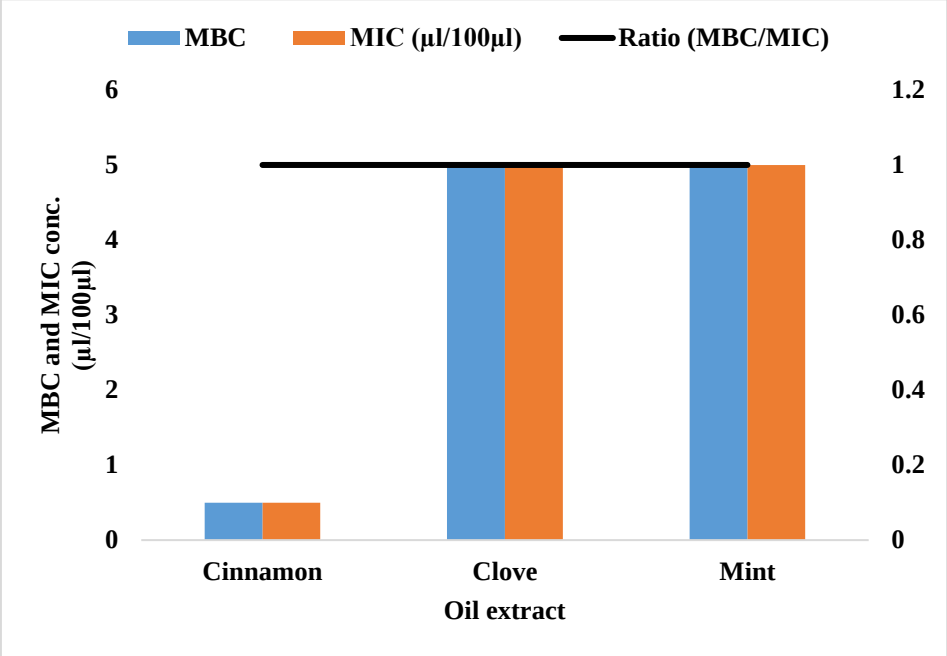


Fig 5. Antibacterial activity of different plant oil extracts against *Enterobacter cloacae* DSM 3264 BRB growth

Table 2. Actual and predicted inhibition zone diameters (cm.) of the mixture design of RSM for the synergistic inhibitory activities of (cinnamon, clove, mint) and gentamicin against *E. cloacae* DSM 3264 BRB growth

Run	(A) Cinnamon % (v/v)	(B) Clove % (v/v)	(C) Mint % (v/v)	(D) Gentamicin % (w/v)	zone of inhibition (cm)	Actual Value (cm)	Predicted Value (cm)
1	2.58	5.00	5.04	2.38	1.90	1.90	1.74
2	2.62	7.04	5.08	0.25	1.20	1.20	1.69
3	1.07	7.71	5.53	0.68	0.90	0.90	0.94
4	4.75	5.00	5.00	0.25	2.70	2.70	2.79
5	2.58	5.00	5.04	2.38	1.90	1.90	1.74
6	0.50	7.11	7.07	0.31	1.20	1.20	0.77
7	0.59	7.06	5.00	2.35	0.50	0.50	0.70
8	0.50	9.25	5.00	0.25	0.60	0.60	0.58
9	0.50	5.00	9.25	0.25	0.90	0.90	0.96
10	1.49	6.00	6.05	1.46	1.10	1.10	1.23
11	2.58	5.00	7.10	0.31	1.90	1.90	1.86
12	0.98	5.52	7.71	0.79	1.00	1.00	1.09
13	0.50	7.11	7.07	0.31	0.60	0.60	0.77
14	1.17	5.00	5.71	3.11	0.90	0.90	1.09
15	2.58	5.00	7.10	0.31	1.90	1.90	1.86
16	3.15	5.52	5.48	0.84	2.50	2.50	2.02
17	0.50	5.00	5.00	4.50	0.50	0.50	0.73
18	0.50	5.04	7.12	2.34	0.80	0.80	0.84
19	0.59	7.06	5.00	2.35	1.30	1.30	0.70
20	2.62	7.04	5.08	0.25	1.50	1.50	1.69

The Model F-value of 27.40 implies the model is significant. The model predicted R^2 of 0.7620 is in reasonable agreement with the adjusted R^2 of 0.8065 as the difference is less than 0.2. One-way ANOVA gives the following equation: (Zone of inhibition = 0.510568 cinnamon - 0.008980 clove + 0.080725 mint + 0.024921 gentamicin) which represents the mathematical model of the response in terms of the four constitue which are cinnamon, clove, mint and gentamicin with concentration 0.5% (v/v), 5.0 % (v/v), 5.0 %(v/v) and 0.25 % (w/v) respectively. This formulation was conducted in order to obtain a very sensitvite strain response against gentamicin with an inhbition zone diameter rafed from 0.5 to 2.7cm.

Data illustrated in **Fig 6** shows the desired inhibition diameter of 2.7cm against *Enterobacter cloacae* DSM 3264 BRB obtained by fixing cinnamon, clove, mint and gentamicin. After fixing the proportion of cinnamon, clove, mint and gentamicin at a value lower than or equal 15% according to this graph, proportion of cinnamon ranging between 0.5-4.75%, proportion of clove 5.0-9.25% and proportion of mint ranging between 5.0-9.25% and essential proportion of oil extract oil testing to 10.75 and not exceeding 15%. These results are clearer on the mixture 3D, showing the desired zone of interaction between cinnamon, clove, mint and gentamicin.

It is clear that the desigend inhibition zone more than 0.5 to 2.7cm was obtained by using 0.25% (w/v) Gentamicin which is more lower by 40 times of the standard concentraion which was (10%) and was successfully substituted by the synergy between cinnamon, clove and mint with 0.5, 5.0 and 5.0%, respectivaly. Therefore the reduction of gentamicin to a percentage of 0.25% was recommended. Absolutely, the increase in the proportion of essential oil for cinnamon automatically a reduction of the percentage of gentamicin having more inhibitory effect on *E. cloacae*. As described by Wendakoon and Sakaguchi (1995), cinamonaldehyd in cinnamon plays an important role in inhibiting the activity of amino acid decarboxylase enzyme leading to protein synthesis inhibition. Therefore, stops the microbial growth.

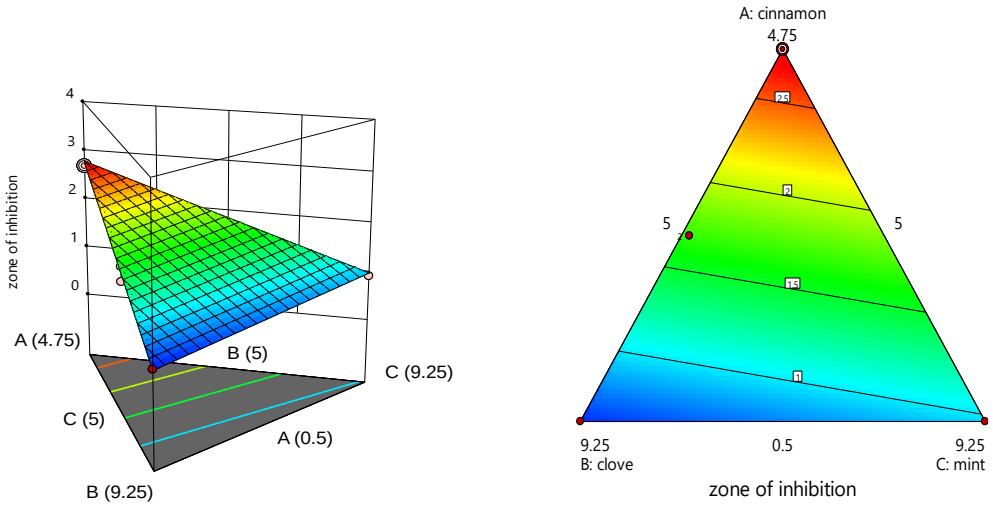


Fig 6. The desired inhibition diameter (2.7cm) against *E.cloacae* obtained by fixing cinnamon, clove, mint and gentamici

3.7 Gas chromatography-mass spectrometry analysis (GC/MS) of cinnammon, clove and mint essential oil

The chemical compounds of the three plant essential oils of *Mentha piperita*, *Cinnamomum zeylanicum* and *Syzygium aromaticum* were determined using the quantitative analysis of hydro distilled essential oils of the three selected plant oils using GC/ MS.

3.8 GC/MS analysis of mint (*Mentha piperita*) Essential oil

Figs 7, 8 and 9 showed the chemical compounds of the *Mentha piperita* essential oil. Mint is composed of 27 chemical compounds. Out of them, four major compounds of menthone and menthol are the largest components with ratio of 32.92% followed by menthone 27.75%, pulegone 4.06% and m-Mentha-1,8-diene 1.3528%.

Similar results were obtained by Yang et al (2018) who found that menthol and menthone were the major compounds of mint with a ratio of 50.14% and 24.43%, respectively. de Oliveira et al (2017) also analyzed mint using GC/MS and found that menthol compound had the highest chemical composition ratio among menthone.

3.9 GC/MS analysis of cinnamon *(Cinnamomum zeylanicum)* essential oil.

Data presented in **Figs 10 and 11** show the chemical component of cinnamon essential oil. cinnamon has eight main components, cinnamic acid, phenethyl ester is found to have the highest component (41.97%) and followd by cinnamaldehyde (24.42 %) and 3-Allyl-6-methoxypheno (m-eugenol) (18.55%).
A similar results were found by Wong et al (2014) who stated that cinnamic acid, phenethyl ester is found to have the highest component of cinnamon with ratio of 45%.

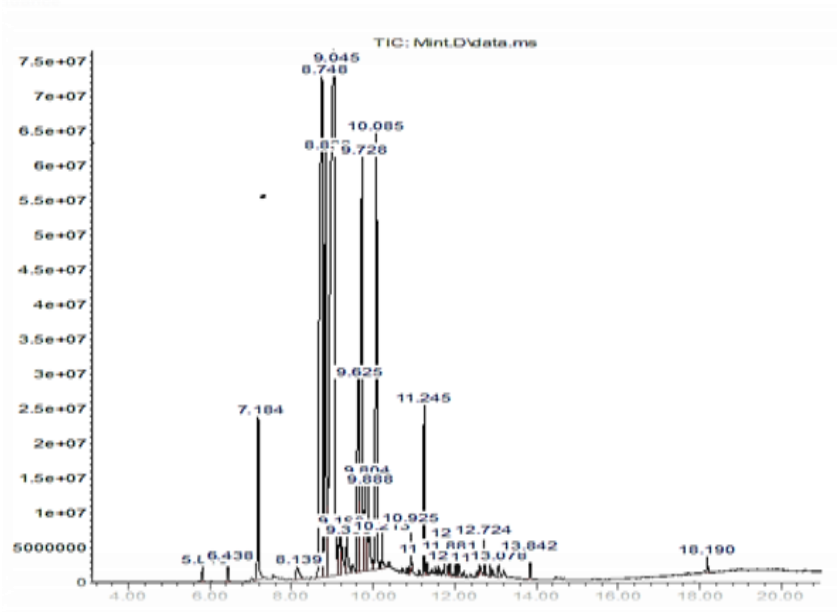


Fig 7. GC/MS analysis of mint (*Mentha piperita*) essential oil

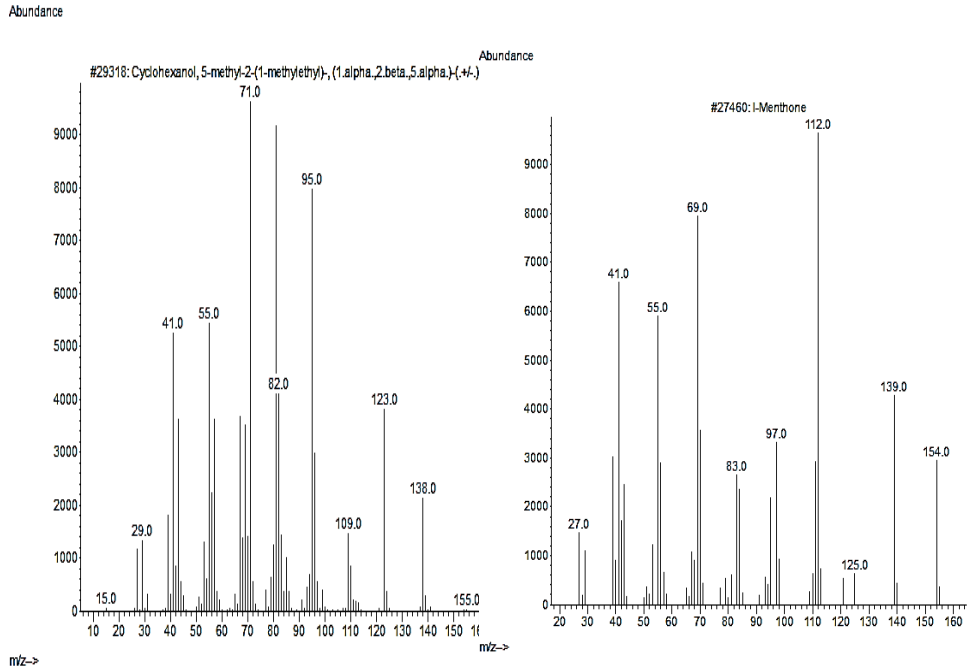


Fig 8. GC/MS Spectrum of Cyclohexanol, 5-methyl-2-(1-methylethyl)- (1alpha.,2 beta., 5 alpha.) - (+/-) (menthol) 32.92% (Left) and l menthone, 27.75 % (Right)

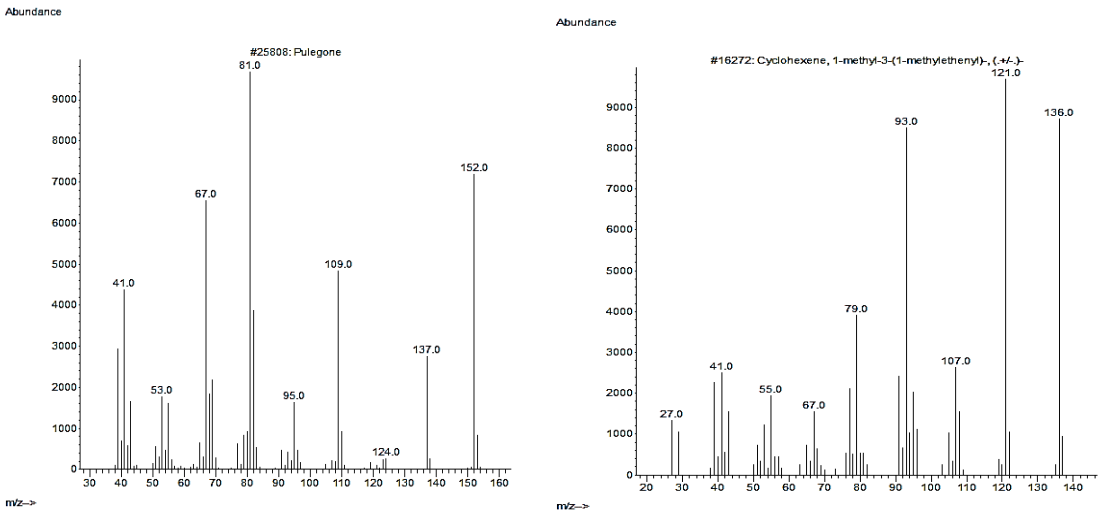


Fig 9. GC/MS Spectrum of pulegone 4.06% (left) and cyclohexene, 1 methyl, l-3- (1-methylethenyl) - (+/-) 1.35% (Right).

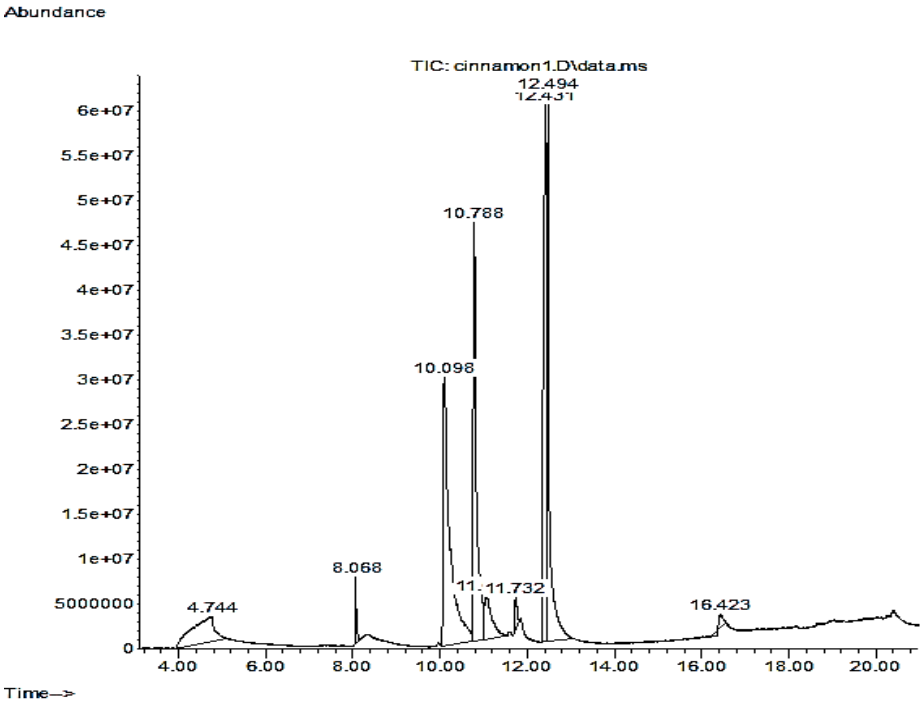


Fig 10. GC/MS analysis of cinnamon (*Cinnamomum zeylanicum*) essential oil

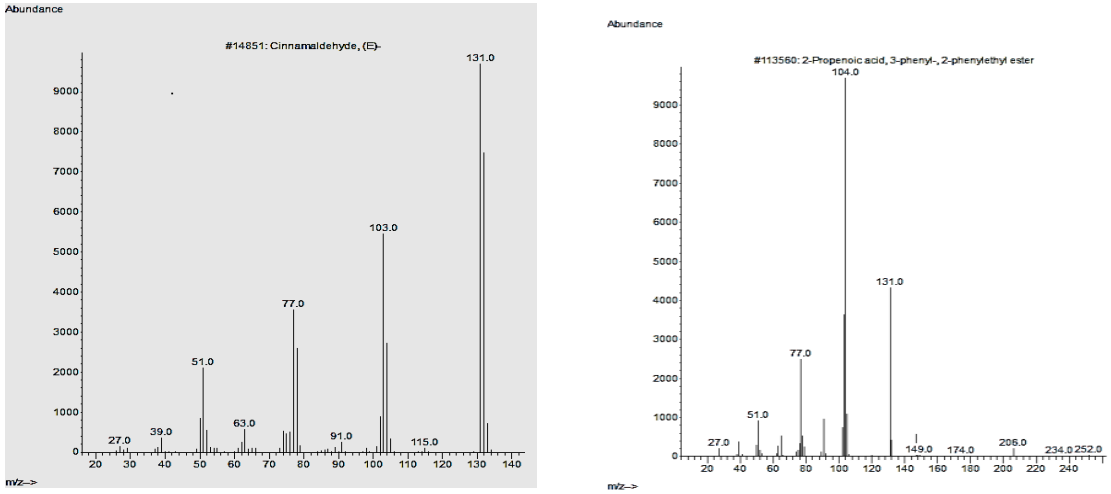


Fig 11. GC/MS Spectrum of cinnamaldehyde, (E)-24.4% (left) Propenoic acid, 3-phenyl-, 2-phenylethyl ester (cinnamic acid,phenethyl ester) 41.97% (Right).

4 Conclusion

A new statistical approach of mixture drug design was designed against *E.cloacae* DSM 3264 BRB strain growth showing that the standard recommended Gentamicin dose may be substituted by oil extract of *Cinnamomum zeylanicum* 4.75 % (v/v) , *Syzygium aromaticum* 5.0% (v/v), *Mentha piperita* 5.0%(v/v) and gentamicin 0.25%(w/v) as an antibacterial agents.

References

Abd El-Mongy, M; Mohamed, SET; Mabrouk, MI; Mohammed, AM; Hamza, HA (2017) Role of Foeniculum vulgare oil on the antimicrobial activity of some antibiotics against resistant pathogenic Gram-negative bacteria. *J of Biochem Microbiol Biotechnol* 5, 1-6.

Adams, RP (2017) Identification of essential oil components by gas chromatography/mass spectrometry. 5th ed. Carol Stream, IL: Allured Publ. Corp pp. 2 – 7.

Baker, BP; Grant, JA (2018) Cinnamon & Cinnamon Oil Profile. *New York State Integrated Pest Management (IPM Program), Cornell University Library, Geneva NY.* 2-16.

CLSI (Clinical and Laboratory Standards Institute) (2015) Antimicrobial Susceptibility Challenges**.** versions of M100 and M 60, Wayne, PA 19087, USA.

Davidson, PM; Parish, ME (1989) Methods for testing the efficacy of food antimicrobials. *Food Technol* 43, 148-155.

De Oliveira, KÁR; Berger, LRR; De Araújo, SA; Câmara, MPS; de Souza, EL (2017) Synergistic mixtures of chitosan and Mentha piperita L. essential oil to inhibit Colletotrichum species and anthracnose development in mango cultivar Tommy Atkins. *Food Microbial* 66, 96-103.

Delgado-Sánchez, C; Santiago-Medina, F; Fierro, V; Pizzi, A; Celzard, A (2018) Optimisation of “green” tannin-furanic foams for thermal insulation by experimental design. *Materials and Design* 139, 7-15.

Elshikh, M; Ahmed, S; Funston, S; Dunlop, P; Mcgaw, M; Marchant, R; Banat, IM (2016) Resayurin-based 96-well plate microdillution method for the determination of minimum inhibitorz concentration of biosurfactants. *Biotechnol Lett J* 38, 1015-1019.

Flury, BB; Ellington, MJ; Hopkins, KL; Turton, JF; Doumith, M; Woodford, N (2016) The differential importance of mutations within AmpD in cephalosporin resistance of *Enterobacter aerogenes* and Enterobacter cloacae. *Int J Antimicrobial Agents* 48, 555-558.

Garg, SC; Siddigui, N (1992) Antifungal activity of some essential oil isolates. *Pharmazie* 47, 467-468.

Haja, A; Khaja, MS; Bhanu, PK (2014) Aerobic bacteriology of wound infections with special reference to MRSA. *J. Clinic Experim Res* 2, 74-79.

Hashim, AI (2015) *The antimicrobial activity of Sudanese honeys alone and in combination with plant extracts and ethylenediamineteraacetic acid (edta)* (Doctoral dissertation, Cardiff Metropolitan University).

Huang, S; Dai, W; Sun, S; Zhang, X; Zhang, L (2012) Prevalence of plasmid-mediated quinolone resistance and aminoglycoside resistance determinants among carbapeneme non-susceptible Enterobacter cloacae. *PLoS One* 7, 47636 - 47643.

Jennifer, MA (2001) Determination of minimum inhibitory concentration. *J Antimicrobial Chemotherapy* 48, 5-16.

Lambert, RJW; Skandamis, PN; Coote, PJ; Nychas, GJ (2001) Astudy of minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacol. *J Appl Microbiol* 91, 453-462.

Liaqat, A; Khan, FH; Ali, L; Rashid, A (2017) Anti-Bio gram of the most common dietary additives (Spices) against common problematic organisms. *APMC* 11, 276-282.

MacNair, CR; Stokes, JM; Carfrae, LA; Fiebig-Comyn, AA; Coombes, BK; Mulvey, MR; Brown, ED (2018) Overcoming mcr-1 mediated colistin resistance with colistin in combination with other antibiotics. *Nature Communications* 9, 458-465.

Mohammadinejad, R; Karimi, S; Iravani, S; Varma, RS (2016) Plant-derived nanostructures: types and applications. *Green Chemist* 18, 20-52.

Motlagh, MK; Yahyaei, M; Rezaei, M; Ghorbanpour, M (2013) Study on antibacterial effect of thyme and peppermint aqueous extracts on *Staphylococcus aureus* and *Escherichia coli* strains causing mastitis in Camels. *Int J Tradition Herb Med* 1, 112-115.

NCCLS [National Committee for Clinical Laboratory Standards] (1993) Performance Standards for Antimicrobial Disc Susceptibility Testing. Approved Standard NCCLS Publication M2-A5, Villanova, PA, USA.

Paranagama, PA (1991) Analysis of Sri Lankan essential oils by gas chromatography and mass spectroscopy Colombo, Sri Lanka: ITI.

Redfern, J; Kinninmonth, M; Burdass, D; Verran, J (2014) Using soxhlet ethanol extraction to produce and test plant material (essential oils) for their antimicrobial properties. *J Microbial Biology Edu* 15, 45-46.

Ebrahim A Rasha; Rawia F Gamal; Samah HA Mohamed; Abdel-Rahman RZ (2018) Impact of Allium sativum against Enterobacter sp. as water borne pathogenic bacteria isolated from River Nile. AUJAS 26, 2525-2531.

Saeed, S; Tariq, P (2005) Antibacterial activities of *Mentha piperita*, *Pisum sativum* and *Momordica charantia*. *Pakistan J Botany* 37, 997-1001.

SAS Institute (2002) SAS The Statistical Analysis System. SAS Institute, Cary, NC, USA. URL: http://www.sas.com

Serio, AW; Magalhães, ML; Blanchard, JS; Connolly, LE (2017) Aminoglycosides: Mechanisms of action and resistance. In *Antimicrobial Drug Resistance*, Springer, Cham, pp. 213-229.

Wendakoon, CN; Sakaguchi, M (1995) Inhibition of amino acid decarboxylase activity of Enterobacter aerogenes by active components in spices. *J.Food Protect* 58, 280-283.

WHO [World Health organiyation] (2008) Guidelines for Drinking-water Quality. Second Addendum to 3rd Edition, Genva, Switzerland. http://www.who.int.water_sanitation_health.

Wong, YC; Ahmad-Mudzaqqirand, MY; Wan-Nurdiyana, WA (2014). Extraction of essential oil from cinnamon (*Cinnamomum zeylanicum)*. *Orient J Chem* 30, 37-47.

Yanat, B; Rodríguez-Martínez, JM; Touati, A (2017) Plasmid-mediated quinolone resistance in Enterobacteriaceae: a systematic review with a focus on Mediterranean countries. *Europ J Clinical Microbiol Infect Diseases* 36, 421-435.

Yang, SK; Yap, PSX; Krishnan, T; Yusoff, K; Kok-Gan, C; WS Yap K Lai; Swee-Hua, EL (2018) Mode of Action: Synergistic Interaction of Peppermint (Mentha x piperita L. Carl) Essential Oil and Meropenem Against Plasmid-Mediated Resistant *E. coli*. *Shun-Kai Yang, Polly Soo-Xi Yap, Thiba Krishnan, Khatijah Yusoff, Kok-Gan Chan, Wai-Sum Yap, Kok-Song Lai and Swee-Hua Erin Lim. Records of Natural Products* 12, 582-594.

مجلة اتحاد الجامعات العربية للعلوم الزراعية، جامعة عين شمس، القاهرة، مصر
مجلد(28)، عدد(4)، 1089-1103، 2020
Website: http://ajs.journals.ekb.eg

تقليل الجرعة القياسية للجنتاميسين مخلوطاً ببعض المستخلصات النباتية لمقاومة الانتيروباكتر كلوكاي بواسطة طريقة نهج الاستجابة السطحي

[77]

راوية فتحي جمال[1]– رشا عبد الله ابراهيم[2] – سماح هاشم أبوحسين[1*]

1– قسم الميكروبيولوجيا الزراعية – كلية الزراعة – جامعة عين شمس– ص.ب 68 – حدائق شبرا 11241– القاهرة – مصر

2– المعامل المركزية التحاليل الطبية وبنك الدم بالقوات المسلحة الخليفة المأمون – القاهرة – مصر

*Corresponding author: samah_hashem1@agr.asu.edu.eg

Received 18 March, 2020 Accepted 16 October, 2020

الموجـــــــز

تم الحصول في عمل سابق على سلالة *Enterobacter cloacae* DSM 3264 BRB من مياه الغسيل الكلوي في مستشفى متخصص في القاهرة وتم تحديدها بإستخدام MALDI-TOFF. تم اختبار التأثير المثبط لكل من المضادات الحيوية من (Ciproflaxacin، Cefepime، Meropenem و Gentamicin) كذلك تم دراسة تأثير مستخلصات زيت النباتات المختارة (القرفة، القرنفل، النعناع) ضد سلالة *E. cloacae* DSM 3264 BRB. وكانت النتائج لتثبت ان المضاد الحيوي جنتاميسين يعتبر اعلي مضاد حيوي مؤثر علي نمو ميكروبات الانتيروباكتر. وبعد استخدام طريقة mixture design الاحصائية وجد انه يمكن استبدال الجرعة Gentamicin القياسية الموصى بها بكل من تركيز قرفة 4.75٪ (v/v)، قرنفل 5.0٪ (v/v)، نعناع 5.0٪ (v/v) وجنتاميسين 0.25 ميكروجرام/100مل.