

# Synergistic Effect between *Zingiber Officinale* Volatile Oil and Meropenem against *Acinetobacter Baumannii* Producing-Carbapenemase Isolated from Neurosurgery in Iraq

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## ABSTRACT

The existence of bacterial pathogens that are carbapenem-resistant is a big and growing health problem around the world. Carbapenem resistance (CR) is generally considered to be one of the most important resistance mechanisms and is found predominantly in gram-negative bacteria. *bla*<sub>OXA-48</sub> and *bla*<sub>VIM</sub> were documented for the first time in this study among *Acinetobacter baumannii* in hospitalized neurosurgery patients in Anbar, Iraq. The VITEK-2 system tested the susceptibility to various antibiotics, given to notice that from 22 clinical isolates of *A. baumannii*, 60 % and 55 % were resistant to both meropenem and imipenem, respectively. Phenotypic carbapenemase identification by Blue-carba test, and mCIM that all isolates produced carbapenemases, while (40%) gave a positive result with modified Hodg test. Both of *bla*<sub>OXA</sub> and *bla*<sub>VIM</sub> genes were detected by multiplex PCR, our results showed 4/7 (57.14%) strains positive for *bla*<sub>OXA</sub> gene and 3/7 (42.85%) strains harbored *bla*<sub>VIM</sub>. Our results revealed the coexistence of the genes *VIM* and *OXA-48* in three isolates (42.85%) of *A. baumannii*. The results have shown that there are wide variations in bacterial virulence factors productivity among all *A. baumannii* isolates such as biofilm and protease activity. All of isolates 10/10(100%) exhibited a biofilm-positive phenotype after 24 h of incubation. In the present study, the percentage of biofilm was 70%(strong), 20%(Intermediate), and 10% (weak). MIC of Ginger, meropenem, and cefepem were 50, 250, and 500 mg/ml, respectively. 10 isolates of *Acinetobacter baumannii* were investigated on their ability to produce carbapenemase by using iodometry assay. Ginger volatile oil effects on carbapenemase production and ginger gave a positive result against carbapenemase activity. Ginger volatile oil exhibited synergistic effect when combined with the checkerboard technique with meropenem against *A. baumannii*, while ganoderma had no effect. Furthermore, appropriate infection prevention procedures must be practiced, and doctors should be mindful of patients with certain risk factors.

**Keywords:** Zingiber officinale, volatile oil, carbapenem resistance, meropenem

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## INTRODUCTION

*Acinetobacter baumannii*, associated with nosocomial infections such as bacteraemia, pneumonia, meningitis, urinary tract infections and wound infections, is a significant opportunistic pathogen. (1). A cause for concern is the recent rise in outbreaks of multidrug resistant *A. baumannii* (MDRAB) worldwide. (2). In addition, ESKAPE that acquire multidrug resistance and virulence(3). *A. baumannii*, especially the multi-drug *A. baumannii* (MDRAB), has therefore gained more significance in the hospital environment as a human pathogen.(4)

The source of great economic benefit is medicinal plants. In synthesizing medicinal compounds, plant herbs are naturally talented. The invention of novel high therapeutic value drugs has led to the isolation and characterization of medicinal plant bioactive compounds. Due to rising concern about potentially harmful synthetic additives, treatment using medicines of natural origin is gaining popularity today (5). Significant antibacterial, antioxidant and antiviral activities have been reported in volatile oils (6). *Zingiber officinale* commonly known as ginger is one of the most commonly used spice worldwide with medicinal value. The ginger oil as a very good antibacterial, antifungal property and prevents food borne diseases

when used in food preparation(7). As a herbal medicinal plant, *Zingiber officinale* is used against various microbial pathogens because of its possible antimicrobial activity. The multi-drug resistant *A. baumannii* is responsible for a wide range of serious human infections as a significant nosocomial pathogen, especially in intensive care units(8). In fact, the emergence of new carbapenem-resistant *A. baumannii* is a serious problem that threatens communities due to the misuse of antibiotics and neglect in public hospitals without using modern sterilization methods. This study aims to evaluate a group of phenotypic methods for detection of these carbapenemase, investigate the ability of ginger to inhibit production of carbapenemase, evaluate the antibacterial effects of ginger in combination with meropenem against bacteria developing carbapenemase and investigate the effects of ginger.

## MATERIALS AND METHODS

### Isolation and identification of *A. baumannii*

All samples were collected from January 2019 to October 2019, it was about twenty-two of *A. baumannii* clinical isolates were isolated from sputum and wound of neurosurgery hospitalized patients in Iraq. The specimens collected were directly streaked on blood agar and

MacConkey agar, incubated for twenty-four hours at 37 ° C. In the laboratory under aseptic conditions. MacConkey agar was subcultured and incubated for another 24 h. at 37°C (9). Gram stain ability, growth at 44°C according to (9) and traditional biochemical tests were examined for all bacterial isolates. Identification of bacteria using automated VITEK-2 device (bioMérieux, France) methods using ID-GNB cards has been checked according to the manufacturer's instructions.

#### Antibiotic susceptibility test

The method of Kirby-Bauer was followed as mentioned by (10) checking for disk diffusion and AST cards for 10 different antibiotics: ticarcillin, ticacillin/clavulanic acid, piperacillin, piperacillin/tazobactam, ceftazidime, cefipeme, aztreonam, imipenem, meropenem, and minocycline (Mast group-England). The zones of inhibition formed around the discs were measured using a metric ruler by millimeter (mm) depended on (CLSI) (11). In susceptibility determination, *Escherichia coli* (ATCC 25922) as a control, it was used.

#### Phenotyping detection of carbapenemase enzymes

##### Blue-Carba Test

The Blue-Carba detection of carbapenemase was performed and interpreted as previously mentioned by Pires et al (12). Briefly, in a solution containing bromothymol blue and imipenem, overnight cultured colonies grown in Mueller Hinton agar were incubated for 2 h. Bacterial colonies were also incubated without imipenem in a bromothymol blue solution. The result was deemed positive when the imipenem-containing solution turned green or yellow and its color varied from that found in the negative control.

##### Modified Hodge test

The MHT was performed as described previously (13). A 0.5 McFarland dilution of 5 ml of broth of *Escherichia coli* ATCC 25922 was prepared. A dilution of 1:10 was streaked to a Mueller Hinton agar plate. At the center of the test area, a 10 µg meropenem susceptibility disc was placed. Test organism was streaked in a straight line from the edge of the disk to the edge of the plate. For 24 h., the plate was incubated overnight in ambient air at 37 ° C.

##### mCIM and eCIM test

According to the CLSI guideline, carbapenemase detection was performed with the mCIM and eCIM (13). For each strain, two tubes containing 2 ml of trypticase soy broth (TSB) were used in parallel: one tube was added with 0.5 M EDTA (Sigma) 20 µl; the other tube did not have EDTA. A fresh colony of tested strain was taken into each tube with 1 µl of inoculating loop. The 10 mg meropenem disk (oxid) was incubated for 4 h. at 35 ° C during suspension of the tested strain. Meropenem discs were then placed on the same Mueller Hinton agar plates from two tubes, followed by inoculation with the *E. coli* ATCC 25922. The mCIM was positive when the diameter of the inhibition zone was 6-15 mm or 16-18 mm for small colonies in the inhibitory zone. The eCIM findings can only be interpreted if the existence of carbapenemase is confirmed by the mCIM outcome. Compared to mCIM, a 5 mm rise in the zone diameter for eCIM indicates the possibility of developing metallo-carbapenemase.

##### Qualitative and quantitative biofilm assay

Biofilm production was measured using qualitative and quantitative assays, described by Marques et al. All the 10 *A. baumannii* isolates were transferred to blood agar for 24 h. at 35 ° C. The grown colonies were inoculated into tryptic

soy broth (TSB) and study activity of extracts against biofilm production (14).

##### Activity and production of protease

Examination of *A. baumannii* ability to protease production was performed according to (15). Protease activity and the effect of ginger volatile oil on protease activity was measured according to (16).

##### Molecular detection using multiplex PCR technique

DNA was isolated from seven *A. baumannii* carbapenem-resistant isolates using DNA isolation kit (company of bio-basic, Korea). Both of *bla*<sub>VIM</sub> and *bla*<sub>OXA-48</sub> primers in lyophilized form (company of Alpha DNA, Canada) were afforded and dissolved in sterile deionized distilled water. *bla*<sub>VIM</sub> (247 bp) sequences was 5'-CGCGGAGATTGARAAGCAAA- 3' (Forward), and 5'-CGCAGCACCRGGATAGAARA- 3' (Reverse), while *bla*<sub>OXA48</sub> (597 bp) sequences was 5'- AACGGGCGAACCAAGCATTTT - 3' (Forward), and 5'- TGAGCTTCTTTTGTGATGGCT - 3' (Reverse) (17).

In PCR assay, the initial denaturation was 94 ° C for 5 minutes with different primers. For 1 min, the cycle denaturation was 94 ° C. For both *bla*<sub>VIM</sub> and *bla*<sub>OXA-48</sub> primers, the time of annealing was 1 min, and the degree of temperature was 50 ° C. At 72 ° C, the extension time was 45 seconds. The final extension was performed at 72 ° C for 7 minutes for all genes.

Components of PCR reaction were 2 µl of 25 master mix (company of Bioneer, USA), 2 µl of each reverse and forward primer. 3 µl of DNA template and 9 µl of water-grade PCR to a final volume of 20 µl. With the assistance of Red-Safe staining and UV trans-illuminator documentation method, PCR products were electrophoresed for 1.5 h. and visualized.

##### Plants uses in this study

The *Zingiber officinale* rhizome was obtained from the Ramadi local market, while *Ganoderma lucidum* powder was obtained from Kuala Lumpur, Malaysia. The medicinal herbs were described at the Herbarium Center of Desert Studies, Anbar University, by Prof. Dr. Mohammed Othman. *Zingiber officinale* (Ginger) volatile oils were extracted using a clevenger-type apparatus by steam distillation according to (18), while *ganoderma* methanolic extract was prepared according to (19).

##### Estimation of carbapenemase activity

This method described by Sargent for determining carbapenemase by using meropenem as substrate for carbapenemase enzymes (20). Carbapenemase activity of *A. baumannii* were estimated before and after treatment with volatile oil of ginger and methanolic extract of *ganoderma* according to (21).

##### Minimum Inhibitor Concentration (MIC) determination:

Resazurin Microtitre-plate Assay (REMA) evaluated the minimum inhibitory concentration (MIC) of volatile ginger oil, *ganoderma* methanol extract and meropenem solution in compliance with Resazurin Microtitre-plate Assay (REMA) according to (22).

##### Synergism between plant extracts and antibiotics

The possible presence of synergy interaction between the volatile oil and antibiotics (meropenem, cefepem) was tested by the checkerboard method in 96 well microplates according to (23).

RESULTS AND DISCUSSION

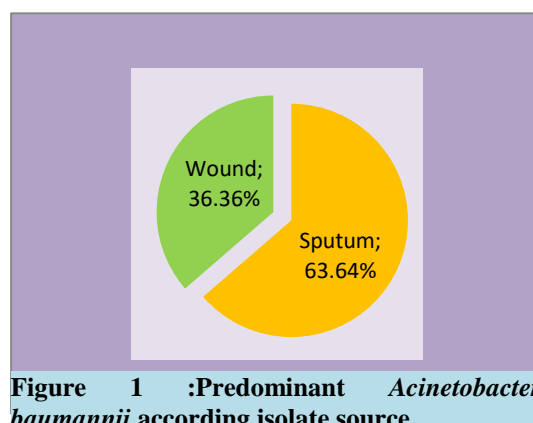
A. baumannii isolation and diagnosis

Figure 1 shows 14/22(63.64%) sputum specimens, and 8/22(36.36%) wound were identified as A. baumannii. The isolates were described as gram-negative coccobacilli and often arranged in diplococci. All biochemical tests results were described in table 1. All isolates appeared as small, pale and lactose non-fermenting colonies when

isolates were cultivated on MacConkey agar, while isolates appeared as opaque, creamy and non-hemolytic colonies on blood agar. For all A. baumannii isolates, growth at 44C° was positive, demonstrating the ability to grow at this temperature range. This test was used to differentiate A. baumannii (which could grow at this degree of temperature) from other species of Acinetobacter that could not grow at that degree of temperature.

Table 1- Biochemical test results for A.baumannii.

Biochemical Test	Result
Production of Catalase	+
Production of Oxidase	-
Citrate utilization	+
Production of Indole	-
fermentation of Lactose	-
Motility Test	-
Hemolysin production	(γ hemolysis)
Urease production	-
Kliglar iron agar (KIA)	Alkaline slant No change bottom, No gas No H2S

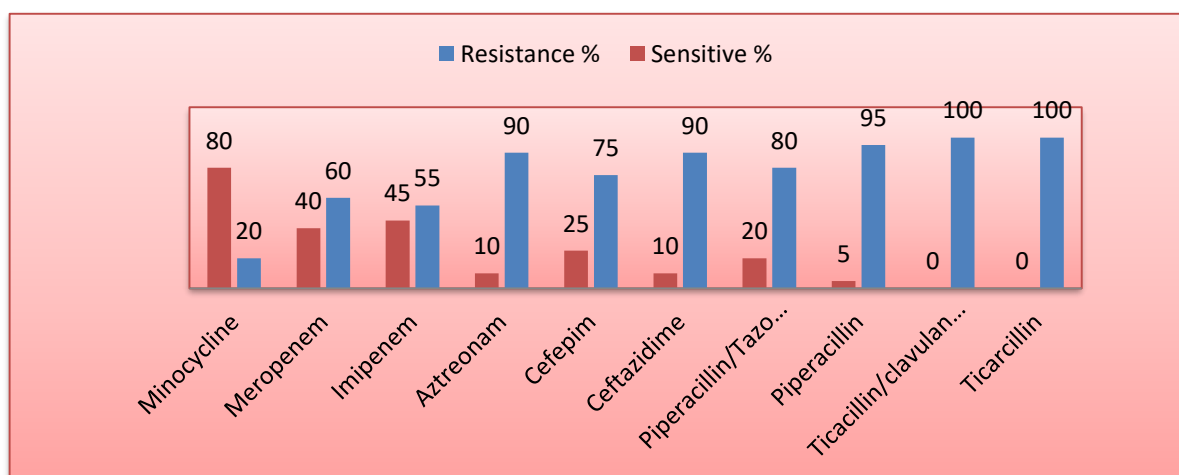


Susceptibility Test

Data presented in figure 2 shows a high-level resistance of A. baumannii clinical isolates to most of the antibiotics under test. The present study revealed that all A. baumannii clinical isolates had 100% resistance to ticarcillin and ticarcillin-clavulanic acid. This study also showed a highest resistance to piperacillin (95%), ceftazidime=aztreonam (90%), piperacillin-Tazobactam (80%), cefepem (75%). Given to notice that from 22

clinical isolates of A. baumannii, 60 % and 55 % were resistant to both meropenem and imipenem, respectively. The emergence of A. baumannii carbapenem-resistant organisms in Iraq has become a major therapeutic challenge. The percentage of Acinetobacter carbapenem-resistant in the past five years increased from 23% to 60%. In Ramadi city, Muteea et al reported A.baumannii resistant- carbapenem in percentage of 23% (24).

Figure 2. Antibiotic resistance of Acinetobacter baumannii clinical isolates.



Phenotyping detection of carbapenemase enzymes

Phenotypic carbapenemase detection using the following methods: blue-carba, modified hodge, modified CIM for carbapenemase production, and EDTA -CIM for differentiation between serine and metallo carbapenemase. The presence of carbapenemase was seen in all resistance isolates using Blue-CARBA test and mCIM. Tinguely et al., had been documented that all gram-negative bacteria isolates gave a positive result in this test

in percentage (100%) (25). On the other hand, modified hodge test gave 40% a positive result for this test. Sadiq and Sehlawi were reported in Najaf city that all isolates gave positive results for this test (24). In order to differentiation between serine (OXA-48) and metallo-β-lactamases production(VIM), eCIM was used as a phenotypic confirmatory tool for producing β-lactamases, where the results revealed (40%) was serine beta

lactamase while (60%) was metallo beta lactamase. Results are shown on table 3.

Table 2. phenotypic detection results

No.	Test	Result
1.	Blue-Carba Test	+(100%)
2.	Modified Hodge Test	+(40%), - (60%)
3.	mCIM	+(100%),
4.	eCIM	(40% serine carb.), 60% Metallo- carb.

#### Determination of carbapenemase enzymes

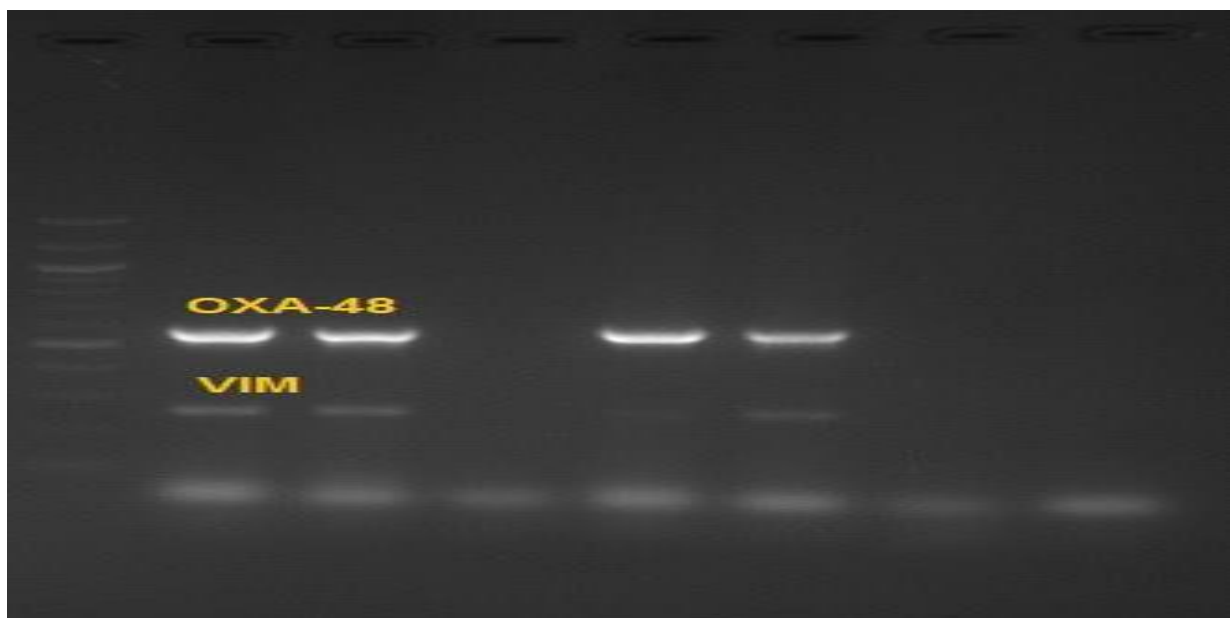
10 isolates of *A. baumannii* were investigated on their ability to produce carbapenemase by using iodometry assay. It was found that 10 isolates produced carbapenemase in percentage 100%. The ability of isolates to produce carbapenemase was various, some isolates were high activity, others were moderate, and some of them were low.

#### Molecular detection of *bla<sub>VIM</sub>* and *bla<sub>OXA-48</sub>*

The results revealed the existence of expected product size (597bp) for a *bla<sub>OXA-48</sub>* in 4/7 (57.14%) carbapenem-resistant *A. baumannii* strains. On the other hand, 3/7(42.85%) strains of carbapenem-resistant *A. baumannii* harbor *bla<sub>VIM</sub>*. The results revealed that *bla<sub>VIM</sub>* (247bp) and *bla<sub>OXA-48</sub>*(597) were present in 4/7(83.33 %) carbapenem-resistant *A. baumannii* strains and

3/7(42.85%) of non-harboring strains (*bla<sub>VIM</sub>* and *bla<sub>OXA-48</sub>*).

In this study, our findings revealed that the phenotypic tests were similar to the methods that were almost molecular. Various genes are involved in the resistance of Enterobacteriaceae to carbapenem, which may differ from area to area. In four strains of *A. baumannii*, the results also revealed the coexistence of both *bla<sub>OXA-48</sub>* and *bla<sub>VIM</sub>* genes under study. In comparison to other studies from Baghdad and Najaf, our report shows that *bla<sub>VIM</sub>* and *bla<sub>OXA-48</sub>* genes have not been identified in Iraq. (19,20). Figure 3. The presence of *bla<sub>VIM</sub>* and *bla<sub>OXA-48</sub>* in 5/6(83.33%) *Klebsiella pneumoniae* and 1(16.67%) non-harboring strains (*bla<sub>VIM</sub>*, *bla<sub>OXA-48</sub>*) was reported by Ahmed and Al Meani(27).



**Figure 3.** Multiplex PCR amplification fragments for *bla<sub>OXA-48</sub>* (597 bp) and *bla<sub>VIM</sub>* (247bp) gene detection among carbapenem-resistant strains of *A. baumannii*. Amplicons were electrophoresed on agarose gel (1 percent) for 1.5 h. at 70 V / cm, stained with Red-Safe (iNtRON, Korea) and visualized using a documentation system for UV trans-illumination.

Our research showed that the spread of new genes in Anbar province, such as *bla<sub>VIM</sub>* and *bla<sub>OXA-48</sub>*, was not previously documented as a result of international terrorist organizations of various nationalities that devastated our city in events that took place in 2014. In the emergence and distribution of various variants of carbapenemase encoding genes people's migration to local and global cities, medical tourism and cross-border patient transfers, in particular involving employees may play an important role.

#### Effect of ginger volatile oil on carbapenemase among *A. baumannii* isolates

The ratio of isolates which carbapenemases in current study was closely with (28). This variation in the activity of *A. baumannii* isolates in the production of  $\beta$ -lactamases may be due to the diversity of genes and isolates sources. Ginger volatile oil effects on carbapenemase production and ginger gave a positive result against carbapenemase activity. Essential oils are complex combinations of several different compounds, each of which contributes to the biological effects of these oils(7). Volatile oil increases the

cell membrane 's permeability and variations in their strength and differences in their oligomeric state and capacity to dissociate and insert into the cytoplasmic membrane are the product of target specificity. (29)

#### Biofilm formation

Biofilm formation was tested for 10 clinical isolates by microtiter plate (MTP). According to MTP, all of the isolates 10/10(100%) exhibited a biofilm-positive phenotype after 24 h. of incubation. In the present study, the percentage of biofilm was 70%(strong), 20%(Intermediate), and 10% (weak). On the other hand, the results showed a decrease in biofilm production of bacteria after treatment with ginger volatile oil. The treatment of bacterial isolates with ginger volatile oils lead to blocking their biofilm for all under-study isolates. Site-specific levels of biofilm formation and antibiotic resistance can vary, and the main factors responsible for this resistance can vary. As regard to resistance, the primary evidence shows that the high tolerance to antibacterial agents associated with biofilms cannot be explained by traditional mechanisms. Vary mechanisms assumed to be main factors in high biofilm resistance have been developed: (a) restricted diffusion, (b) neutralization

caused by enzymes, (c) diverse function, (d) slow growth rate, (e) persistent (non-dividing) cells, and (f) adaptive mechanisms of the biofilm phenotype(30).

#### Protease production and activity

Protease production test on skim milk agar showed that 6 /10 (60 %) of *A.baumannii* isolates gave a positive result to protease production, by estimation of area zone lysis about the bacterial colony. On the other hand, six isolates of *A.baumannii* were examined on their ability to produce protease in Brain-heart infusion broth. Our results showed there is variability in enzyme activity, where it ranged among high to low activity. After adding the sub MICs, the ginger plant extracts have been shown to have an effect on the production of these virulence factors that by reducing or block it.

#### Evaluation the effect of the combination of antibiotics and volatile oils

The increase in antibiotic bacterial resistance and the shortage of new antibiotics introduced on the market have resulted in the need to find alternative ways to deal with drug-resistant bacteria infections (31). According to REMA method, MIC of Ginger, MEM, and Cefepem were 50, 250, 500 mg/ml, respectively. Result on table 3.

Table 3. MIC of natural product and antibiotics.

No.	MIC of <i>Ginger</i>	MIC of <i>Ganoderma</i>	MIC of <i>Meropenem</i>	MIC of <i>Cefepem</i>
A. b	50	No effect	250	500

The results were revealed that *Zingiber officinale* that effected on *A. baumannii*, while *ganoderma* had no effect. *A. baumannii* checkerboard assays gave synergistic profiles only when ginger volatile oil was combined at tested concentrations with selected antibiotics and no antagonistic results were observed and additive results were observed. Synergistic effects of ginger with meropenem combination against *A. baumannii* were observed. The MIC values for the ginger volatile oil and antibiotics and the FIC index values for their combinations

are shown in table 4. The selection of *A. baumannii* isolate in combination between MEM and ginger volatile oil experiences because it carried carbapenemase genes, the most productivity of beta lactamase activity and PDR isolate. Another study, clove volatile oil exhibited markedly antibacterial activity and have inhibitory effected on AmpC  $\beta$ -lactamase. Clove volatile oil exhibited synergistic effect when combined with (Ceftazidimim, Cefepime and Ceftriaxone)(32).

Table 4. synergism between plant and Antibiotic.

NO	FIC of MEM.	FIC of ginger	FICI ( $\Sigma$ FIC)	Interpretation
A. <i>baumannii</i>	0.115	0.125	0.24	Synergistic

#### CONCLUSION

The present study showed that ginger extract could be active against MDRAB strains. Different concentrations and their use at appropriate concentrations can help to treat many microbial diseases better, also help to use the ginger as an alternative option for treatment of many microbial diseases. High coexistence of *bla*<sub>OXA-48</sub> and *bla*<sub>VIM</sub> encoding genes were produced by studied isolates of *A. baumannii*. On the other hand, combination of ginger volatile oil with antibiotic against bacteria could improve the susceptibility of bacteria toward these antibiotics to treat an infections resulted from drug - resistant bacteria. The mCIM test is a good method for detecting carbapenemase genes.

#### REFERENCES

- Nie D, Hu Y, Chen Z, Li M, Hou Z, Luo X, et al. Outer membrane protein A (OmpA) as a potential therapeutic target for *Acinetobacter baumannii*

- infection. *J Biomed Sci.* 2020;27(1):26.
- Al-Anazi KA, Al-Jasser AM. Infections caused by *Acinetobacter baumannii* in recipients of hematopoietic stem cell transplantation. *Front Oncol.* 2014;4:186.
- Santajit S, Indrawattana N. Mechanisms of antimicrobial resistance in ESKAPE pathogens. *Biomed Res Int.* 2016;2016.
- Quainoo S, Coolen JPM, van Hijum SAFT, Huynen MA, Melchers WJG, van Schaik W, et al. Whole-genome sequencing of bacterial pathogens: the future of nosocomial outbreak analysis. *Clin Microbiol Rev.* 2017;30(4):1015–63.
- Mustafa G, Arif R, Atta A, Sharif S, Jamil A. Bioactive compounds from medicinal plants and their importance in drug discovery in Pakistan. 2017;
- Park CH, Park YE, Yeo HJ, Chun SW, Baskar TB, Lim SS, et al. Chemical Compositions of the Volatile Oils and Antibacterial Screening of Solvent Extract from Downy

- Lavender. Foods. 2019;8(4):132.
7. Jiang Y, Liao Q, Zou Y, Liu Y, Lan J. Transcriptome analysis reveals the genetic basis underlying the biosynthesis of volatile oil, gingerols, and diarylheptanoids in ginger (*Zingiber officinale* Rosc.). Bot Stud. 2017;58(1):1-12.
  8. Aghazadeh M, Bialvaei AZ, Aghazadeh M, Kabiri F, Saliani N, Yousefi M, et al. Survey of the antibiofilm and antimicrobial effects of *Zingiber officinale* (in vitro study). Jundishapur J Microbiol. 2016;9(2).
  9. Forbes BA, Sahm DF, Weissfeld AS. Bailey & Scotts. Diagnostic Microbiol 11th ed St Louis, Missouri Mosby. 2002;
  10. Vandepitte J, Verhaegen J, Engbaek K, Rohner P, Piot P, Heuck CC, et al. Basic laboratory procedures in clinical bacteriology. World Health Organization; 2003.
  11. Institute C and LS. Performance standards for antimicrobial susceptibility testing. CLSI supplement M100. Clinical and Laboratory Standards Institute Wayne, PA; 2017.
  12. Pires J, Novais A, Peixe L. Blue-carba, an easy biochemical test for detection of diverse carbapenemase producers directly from bacterial cultures. J Clin Microbiol. 2013;51(12):4281-3.
  13. Kim H-K, Park JS, Sung H, Kim M-N. Further modification of the modified Hodge test for detecting metallo- $\beta$ -lactamase-producing carbapenem-resistant Enterobacteriaceae. Ann Lab Med. 2015;35(3):298-305.
  14. Marques VF, Motta CC da, Soares B da S, Melo DA de, Coelho S de M de O, Coelho I da S, et al. Biofilm production and beta-lactamase resistance in Brazilian *Staphylococcus aureus* isolates from bovine mastitis. Brazilian J Microbiol. 2017;48(1):118-24.
  15. Asha B, Palaniswamy M. Optimization of alkaline protease production by *Bacillus cereus* FT 1 isolated from soil. J Appl Pharm Sci. 2018;8(02):119-27.
  16. Harth L, Krah U, Linke D, Dunkel A, Hofmann T, Berger RG. Salt taste enhancing L-arginyl dipeptides from casein and lysozyme released by peptidases of Basidiomycota. J Agric Food Chem. 2016;66(10):2344-53.
  17. Patrik M, Magdalena R, Milan K. Research Article Primer Evaluation for PCR and its Application for Detection of Carbapenemases in Enterobacteriaceae Jundishapur. J Microbiol. 2016;9(1):e29314.
  18. Hathazi F-I, Arion M-N, Şoproni VD, Molnar CO, Pantea MD. The extraction of volatile oils from parsley leaves in microwave field. In: 2017 14th International conference on engineering of modern electric systems (EMES). IEEE; 2017. p. 67-70.
  19. Zhu M, Chang Q, Wong LK, Chong FS, Li RC. Triterpene antioxidants from *Ganoderma lucidum*. Phyther Res An Int J Devoted to Pharmacol Toxicol Eval Nat Prod Deriv. 1999;13(6):529-31.
  20. Sargent MG. Rapid fixed-time assay for penicillinase. J Bacteriol. 1968;95(4):1493.
  21. Sawai T, Takahashi I, Yamagishi S. Iodometric assay method for beta-lactamase with various beta-lactam antibiotics as substrates. Antimicrob Agents Chemother. 1978;13(6):910-3.
  22. Kolarević S, Milovanović D, Avdović M, Oalde M, Kostić J, Sunjog K, et al. Optimisation of the microdilution method for detection of minimum inhibitory concentration values in selected bacteria. Bot Serbica. 2016;40:29-36.
  23. Mutambuze JW. In Vitro Antimicrobial Activity of BTZ043 and PNU-100480 Against *Mycobacterium ulcerans*. Kent State University; 2014.
  24. Sadiq Z, Sehlawi A. Occurrence and Detection of Carbapenemase-Producing *Klebsiella pneumoniae* Clinical Isolates in Najaf Hospitals. Mag Al-Kufa Univ Biol. 2013;5(2).
  25. Hussein NH. Genotypic Detection of Carbapenem-Resistant *Escherichia Coli* Producing NDM-1 Gene for the First Time in Baghdad/Iraq. J Glob Pharma Technol. 2017;
  26. Leylabadlo HE, Asgharzadeh M, Aghazadeh M. Dissemination of carbapenemases producing Gram negative bacteria in the Middle East. Iran J Microbiol. 2015;7(5):226.
  27. Ahmed MM, Al Meani SL. Occurrence of *Klebsiella pneumoniae* carbapenemase KPC gene in *Klebsiella pneumoniae* isolated from patients in Anbar city of Iraq. Ann Trop Med Heal. 2019;22:108-16.
  28. Kiliç E, Çirak MY. Comparison of staphylococcal beta-lactamase detection methods. Fabad J Pharm Sci. 2006;31(2):79.
  29. Idalina A, Oliveira T De, Cabral JB, Suleiman T, Nobre G, Nascimento L, et al. In vitro antimicrobial activity and fatty acid composition through gas chromatography-mass spectrometry ( GC-MS ) of ethanol extracts of *Mauritia flexuosa* ( Buriti ) fruits. 2017;11(40):635-41.
  30. Stowe SD, Richards JJ, Tucker AT, Thompson R, Melander C, Cavanagh J. Anti-biofilm compounds derived from marine sponges. Mar Drugs. 2011;9(10):2010-35.
  31. Berkhout J, Melchers MJ, van Mil AC, Nichols WW, Mouton JW. In vitro activity of ceftazidime-avibactam combination in in vitro checkerboard assays. Antimicrob Agents Chemother. 2015;59(2):1138-44.
  32. Ibrahim MO, Al Meani SAL. DETECTION OF PLASMID-MEDIATED AMPC  $\beta$ -LACTAMASE GENES AND EVALUATION THE SYNERGISTIC EFFECT OF CLOVE VOLATILE OIL AND ANTIBIOTICS IN CLINICAL ISOLATES OF *KLEBSIELLA PNEUMONIA* IN IRAQ.