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The effect of *Nigella sativa* (black cumin seeds) and *Zingiber officinale* (Ginger rhizome) Essential Oils on Multidrug-Resistant Bacteria

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Abstract

Antibiotic resistance is growing and has limited the ability of physician's treatment decisions. Discovery of new, effective and safe substances that prevent troublesome infections is greatly needed to provide alternative therapeutic options. This study evaluated the antibacterial and synergistic effect of Hydro extracts of two plants essential oils of black seed (*Nigella sativa*) and ginger (*Zingiber officinale*) rhizome each alone and in combination, against selected different strains of gram negative and gram positive multidrug-resistant bacteria (*Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa , Escherichia coli* and Methicillin Resistant *Staphylococcus aureus*) isolated from burn patients. The isolated were identified basing upon their colony characteristics, gram-staining, motility and biochemical tests according to standard microbiological techniques. All the isolates were tested for their susceptibility to different antibiotics and compared to the interpretive chart zone sizes. The antibacterial effect of essential oils was conducted against all isolates by disk diffusion assay, the minimum inhibitory concentrations and the minimum bactericidal concentrations of the plant essential oils were processed by using micro-dilution technique. The results indicated that all the different strains of *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Escherichia coli* that were tested against 100% concentration of essential oils; there was no recorded zone of inhibition. However, for the different strains of Methicillin Resistant *Staphylococcus aureus*, different zones of inhibition where obtained for *Nigella sativa* oil singly and combination of *Nigella sativa* with *Zingiber officinale* at different dilutions 100%, 75%, 50%, 25%, 12.5%, 6.25% and 3.12%. *Nigella sativa* oil alone presents inhibition zones at concentration 100% ranged between (10mm) to (34mm), while *Zingiber officinale* oil extracts alone had no significant antibacterial effect. In addition, combination of both oil extracts at 100% concentration showed inhibition zone greatest than the standards antibiotics which ranged between (12mm) to (52mm). Synergistic effect was noticed in combination of *Nigella sativa, Zingiber officinale* oil extracts and Antibiotics. The combination of both oil extracts of *Nigella sativa* oil and *Zingiber officinale* oil showed an excellent antibacterial and synergistic activity against Methicillin Resistant *Staphylococcus aureus.*

Introduction

The use of medicinal plants has increased world-wide due to factors such as drug failure, adverse reactions, cost of medications as well as resistance to antimicrobials by bacteria [1]. Multi-drug resistant microorganisms are regarded to resistant against more than two groups of antibiotics [2]. It has been observed that multi drug resistant microbes are getting resistance genes to the antibiotics rapidly; the

consequence is failure in the infectious diseases treatment [3]. Multi-drug resistant microorganisms are most commonly found in the hospital environment, where they can cause serious nosocomial infections, occurring particularly in the intensive care, burn unit treatment, surgical, hematological and oncological units [4]. The most notorious resistance bacteria Methicillin Resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Proteus* sp, *Klebsiella pneumoniae*, and *Escherichia coli*, rapidly acquire antibiotic resistance factors and distribute in the hospital environment [5]. Therefore, the expansion of multiple resistance and the antibiotics side effects caused an urgent need for the development of safe and non-toxic new substances to cope with this problem [6].

Essential oils (EOs) are natural products extended from vegetal materials which are characterized with their antibacterial, anti-fungal, antiviral, and antioxidant properties [7]. Essential oils consider plants protective agents however have many beneficial antimicrobial activities [8]. Among the plants, black cumin (*Nigella sativa)* seeds and ginger (*Zingiber officinale*) rhizome have been used since ancient times as a medical plant therapy [9]. The benefits of a natural product (plant extract) and their effect have been attracting attention through centuries in plant medicine either by direct addition or used in combination with other antimicrobial agents to expand their antioxidant or antimicrobial effect[10].

Presently, the most clinically important pathogenic microorganisms are identified by multiple drug resistance not only by single drug resistance. In an attempt to challenge the distribution of antibiotic resistance, currently the most commonly application is using a combination of two or more antibiotics [11]. Ginger essential oils have been mixed with other plant oil extracts and showed potential synergistic and antibacterial action [12].

Antimicrobial and synergistic activity acquired in combination of antibiotics and plants essential oils has led to lowering the effective dose of the antibiotics, and side effects [13], [14].

Present study evaluated *in-vitro* effectiveness of *N. sativa* seeds and Ginger rhizome essential oils each singly and in combination against the most resistance bacteria: *Staphylococcus aureus, Klebseilla pneumoniae*, *Acinetobacter baumannii*, *Escherichia coli*, and *Pseudomonas aeruginosa* isolated from burn patients since burn wound provides a suitable environment for colonization and proliferation of micro-organisms and lead to complications that increase morbidity and mortality .

Materials and methods

1. Plants essential oils extraction

Black cumin seeds and Ginger rhizome were obtained from market in Iraq-Sulaimani city. Essential oils from black cumin *Nigella sativa* seeds and ginger rhizome were extracted by Cleavenger's instrument and the extraction was processed at room temperature.

The black cumin seeds were crushed and then hydro distillated for 3 hours by the instrument mentioned above. (100 g) was milled and extracted by adding (500 ml) distilled water. The volatile oil content was calculated as a relative percentage (v/w). The collected oil poured into dark bottle and kept at refrigerator until use [15].

The extraction of the essential oil in fresh ginger sample also was done in a distillation apparatus. The fresh ginger sample was grinded into mash using a manual blender. The 1 liter round bottom flask of the distillation apparatus was filled with about 500ml water, then 100grams of the grinded fresh ginger was added into the flask. The quick fit distillation apparatus was set on a thermostatic heating mantle. The temperature was set to 100 Celsius degree [16].

Serial dilutions of different concentrations were prepared by diluting the oil in ethanol absolute (Scharlau). Thus, concentrations of 100%, 75%, 50%, 25%, 12.5%, 6.25% and 3.12% of both *Nigella sativa* oil alone and mixture of *Nigella Sativa* and *Zingiber officinale* oil extracts were obtained and used for the investigation.

2. Sample collection, isolation and identification

Samples of blood, urine, tissue, and wound were obtained from burn patients admitted to the burn and plastic surgery hospital in Sulaimani city during the January to June 2018. Bacterial isolates consisted of gram

negative *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Escherichia coli*, *Pseudomonas aeruginosa* as well as the gram positive methicillin resistant *Staphylococcus aureus* . The isolated were identified basing upon their colony characteristics, gram-staining, motility and biochemical tests according to standard microbiological techniques.

3. Antibiotic susceptibility test

Kirby-Bauer's method/ disc diffusion method was performed for detection of antibiotic susceptibility of all isolated bacterial strains [17]. A suspension of 0.5 McFarland standards prepared from colonies of the isolated organism then by sterile cotton swabs inoculated on Mueller-Hinton agar (MHA) media. After the inoculum has been dried, antibiotic discs were applied to inoculated medium with sterile forceps and pressing down gently to ensure even contact. Using the following antibiotic discs: Vancomycin (30 µg), Gentamicin (10 µg), Amoxicillin-Clave-Acid (30 µg), Oxacillin (1 µg), Ciprofloxacin (5 µg), Meropenem (10 µg), Imipenem (10 µg), Clindamycin (2 µg), Rifampicin (30 µg), Ceftazidime (30 µg), Tobramycin (30 µg), Cefepim (30 µg), Amikacin (30 µg), and Aztreonam (30 µg) , following the inoculation plates were incubated at appropriate temperature [18]. Antibiotic susceptibility was determined from the size of the inhibition zone as resistant, intermediate, or susceptible to the antibiotic.

4. Detection of antibacterial activity of plant extracts by disc diffusion method

The diluting inoculum to 0.5 McFarland were distributed on Muller-Hinton (MH) agar media [19] .Then sterile filter paper discs were saturated with oil putted on the appropriate portion of culture mediums. While sterile filter paper discs containing distilled water alone was served as negative control and standard antibiotic vancomycin (30µg/ml) as positive reference to determine the sensitivity of the strain since vancomycin is an antibiotic of last resort for the treatment of serious life threatening infections by gram positive bacteria resistance to other antibiotics. After 10 minutes the dishes were placed in the incubator. Then the diameter of the zones of inhibition around each of the discs was measured by a ruler. Each experiment was performed in triplicate and the diameter of the inhibition zone was recorded.

5. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of essential oil extracts by Micro dilution Method

The minimum inhibitory concentration (MIC) was determined at varying concentrations of 100%, 75%, 50%, 25%, 12.5%, 6.25% and 3.12%. One ml of nutrient broth was added and then 10 µl of the test organism previously standardized at 0.5 McFarland turbidity was poured to the tubes. A control tube containing nutrient broth only was inoculated with the test organism. Following incubation at 37°C for 24 hours then examined for growth by observing for turbidity. The minimum bactericidal concentration (MBC) of the plant extract on the clinical bacterial isolates was carried out according to [20]. Briefly, 1ml bacterial culture was pipetted from the mixture obtained in the determination of MIC tubes which did not show any growth and were sub-cultured onto nutrient agar. Following incubation period the concentration at which there was no single colony of bacteria was taken as MBC.

6. Evaluation of the synergistic effect

Muller-Hinton agar (MHA) media were inoculated with the bacteria which are previously diluted to 0.5 McFarland. Then a few minutes left for dryness, the antibiotic filter paper disk were placed on the appropriate portion of inoculated media and labeled MH agar plates then sterile filter paper disk impregnated with known concentration of extracts essential oil were also placed. After the incubation period the cleared zones diameters were measured and compared with that of the antibiotic alone.

Results

1. Evaluation of antibiotics activity

The results of antibiotic sensitive testing represented in table 4.1 indicated that all the bacterial pathogens were highly resistant to many antibiotics including. The findings suggest that these isolate should be classified as multidrug-resistant.

Table 4.1: Antibiotic Susceptibility of selected gram positive and gram negative bacteria.

Isolates	Antibiotics and their resistance response													
	VAN (S) : ≥ 15mm	GEN (R):≤ 12 mm	AMC(R): ≤ 19 mm	OXA(R): ≤ 10 mm	CIP(R): ≤ 15 mm	MEM(R): ≤ 13 mm	IPM(R): ≤ 13 mm	CLI(R): ≤ 14 mm	RA(R): ≤ 16 mm	CAZ (R):≤ 14 mm	TM (R):≤ 12 mm	FEP (R):≤ 14 mm	AN (R):≤ 14 mm	ATM (R):≤ 15 mm
MRSA 1	S	R	R	R	R			R	R	R				
MRSA 2	S	R	R	R	R			R	R	R				
MRSA 3	S	R	R	R	R			R	R	R				
MRSA 4	S	R	R	R	R			R	R	R				
MRSA 5	S	R	R	R	R			R	R	R				
MRSA 6	S	R	R	R	R			R	R	R				
MRSA 7	S	R	R	R	R			R	R	R				
MRSA 8	S	R	R	R	R			R	R	R				
MRSA 9	S	R	R	R	R			R	R	R				
MRSA 10	S	R	R	R	R			R	R	R				
MRSA 11	S	R	R	R	R			R	R	R				
MRSA 12	S	R	R	R	R			R	R	R				
MRSA 13	S	R	R	R	R			R	R	R				
MRSA 14	S	R	R	R	R			R	R	R				
MRSA 15	S	R	R	R	R			R	R	R				
A. baumannii 1		R			R	R	R			R	R	R	R	
A. baumannii 2		R			R	R	R			R	R	R	R	
A. baumannii 3		R			R	R	R			R	R	R	R	
A. baumannii 4		R			R	R	R			R	R	R	R	
A. baumannii 5		R			R	R	R			R	R	R	R	
E. coli 1		R	R		R	R	R				R		R	R
E. coli 2		R	R		R	R	R				R		R	R
E. coli 3		R	R		R	R	R				R		R	R
E. coli 4		R	R		R	R	R				R		R	R
E. coli 5		R	R		R	R	R				R		R	R
K. pneumoniae 1		R	R		R	R	R			R			R	R
K. pneumoniae 2		R	R		R	R	R			R			R	R
K. pneumoniae 3		R	R		R	R	R			R			R	R
K. pneumoniae 4		R	R		R	R	R			R			R	R
K. pneumoniae 5		R	R		R	R	R			R			R	R
P. aeruginosa 1		R			R	R	R			R	R	R	R	
P. aeruginosa 2		R			R	R	R			R	R	R	R	
P. aeruginosa 3		R			R	R	R			R	R	R	R	
P. aeruginosa 4		R			R	R	R			R	R	R	R	
P. aeruginosa 5		R			R	R	R			R	R	R	R	

2. Effect of *Nigella sativa* and *Zingiber officinale* oil extracts on gram negative bacteria

All the different isolates of *Acinetobacter baumannii* against 100% of *N. sativa* and *Zingiber officinale* oil extract were complete resistance with no observed zones of inhibition. A similar pattern of results were obtained with the different strains of *E. coli*, *Klebsiela pneumonia* and *Pseudomonas aerogenosa*. So the black cumin *Nigella sativa* seeds and Ginger (*Zingiber officinale*) rhizome oil extracts not affected on the different gram negative isolates, no inhibition zone were recorded. (Fig-1-).

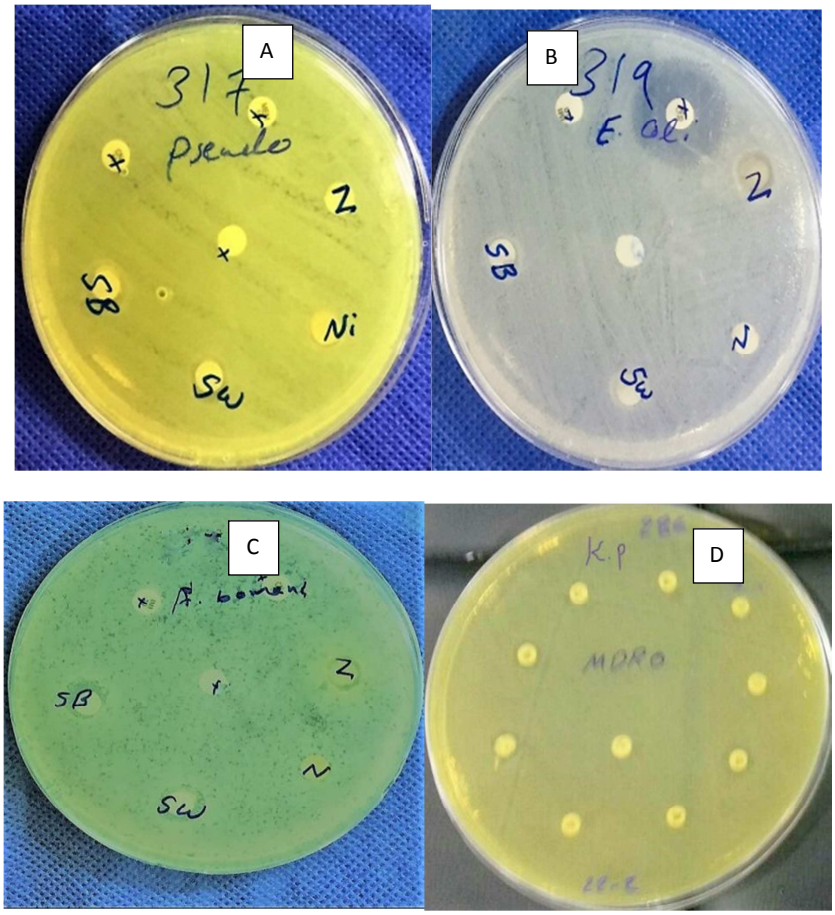

Figure 1: Antibacterial activity of oil extracts on gram negative bacteria (A: *Pseudomonas aerogenosa*, B: *Escherichia coli*, C: *Acinetobacter baumannii* and D: *Klebsiela pneumonia*) against essential oils and standard antibiotics.

3. Effect of *Nigella sativa* and *Zingiber officinale* oil extracts on Gram Positive isolates

For the Gram positive methicillin resistance *Staphylococcus aureus* bacterial isolates, zones of inhibitions to the oil concentrations were seen. Methicillin resistance *Staphylococcus aureus* was susceptible to *Nigella sativa* oil extracts recorded zone of inhibition ranged between (10 mm) to (34mm). In addition, showed the greatest inhibition zone against a combination of both oil extracts which ranged (12mm) to (52mm) (Table 4.2). However, ginger (*Zingiber officinale*) rhizome oil extracts alone had no significant antibacterial effect (Fig-2-) and (Fig-3-).

Table 4.2: Antimicrobial activity of essential oils against Methicillin resistance *Staphylococcus aureus* at 100% concentration

	inhibition zone (mm) of selected Methicillin resistance *Staphylococcus aureus*														
MRSA isolated number / Essential oils	**MRSA 1**	**MRSA 2**	**MRSA 3**	**MRSA 4**	**MRSA 5**	**MRSA 6**	**MRSA 7**	**MRSA 8**	**MRSA 9**	**MRSA 10**	**MRSA 11**	**MRSA 12**	**MRSA 13**	**MRSA 14**	**MRSA 15**
Nigella sativa	**10**	**10**	**25**	**34**	**33**	**13**	**32**	**22**	**19**	**20**	**24**	**34**	**27**	**25**	**31**
N sativa and Z *officinale* oil combination	**24**	**12**	**33**	**52**	**45**	**27**	**48**	**30**	**28**	**25**	**30**	**50**	**47**	**35**	**45**

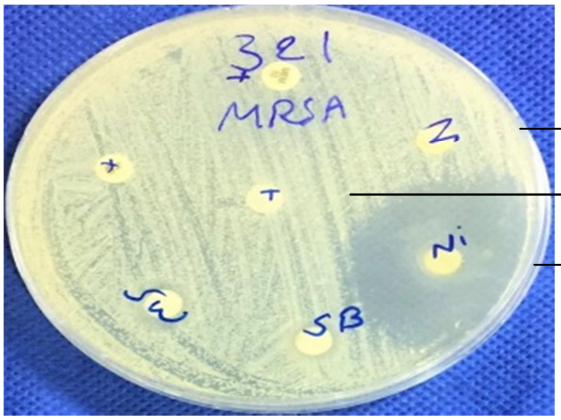

Figure 2: The antibacterial activity of *N sativa* and *Z. officinale* essential oils each alone on MRSA

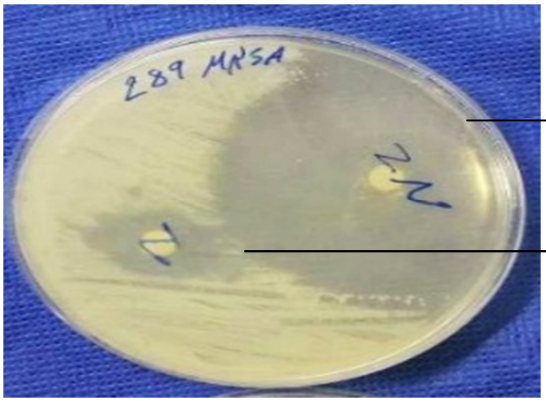

Figure 3: Essential oils combination and their synergistic effect against (MRSA).

The activity of both essential oil extracts were very minimal at low concentration, a decrease in zone of inhibition was seen with the lowest 6.25 % oil concentration. While the results showed the highest activity at concentration of 50 % oil dilutions which were the greatest inhibition zone recorded. (Fig.4 and 5).

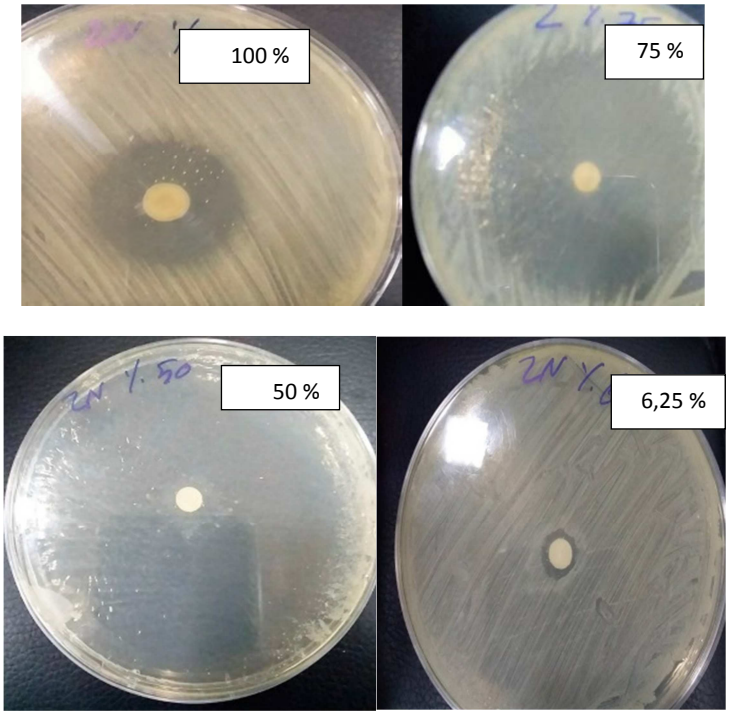

Figure 4: Sensitivity of MRSA against different concentration of black cumin seeds and Ginger rhizome combination essential oils at different concentration.

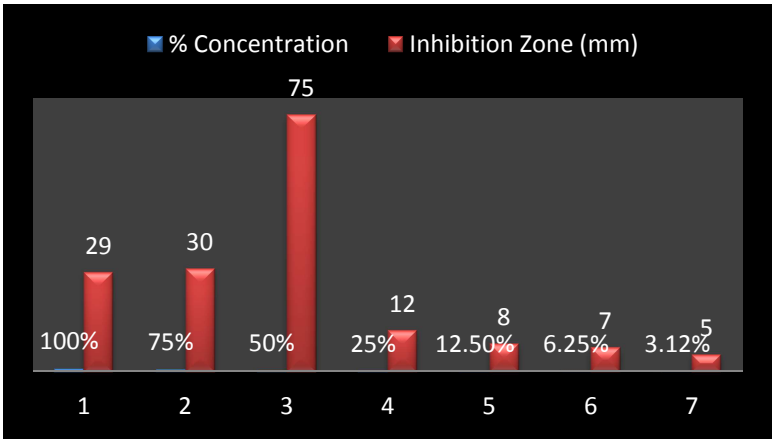

Figure 5: Showing a comparison of the zones of inhibition by different MRSA isolates to combination of *N. sativa* and ginger (*Zingiber officinale*) oil extracts at different concentrations (highest greater zone at 50% and lowest zone at 3.12% concentration.

4. Synergism effect of combination between black seed and ginger oil and standard antibiotics

Combination of both black cumin (*N. sativa*) seeds and Ginger (*Zingiber officinale*) rhizome essential oils showed an additive action (antibacterial enhancement) against Methicillin resistance *Staphylococcus aureus* isolates in a manner that combination of both oils presented inhibition zones greatest than the positive control against Methicillin resistance *Staphylococcus aureus* bacteria. (Fig-6-).

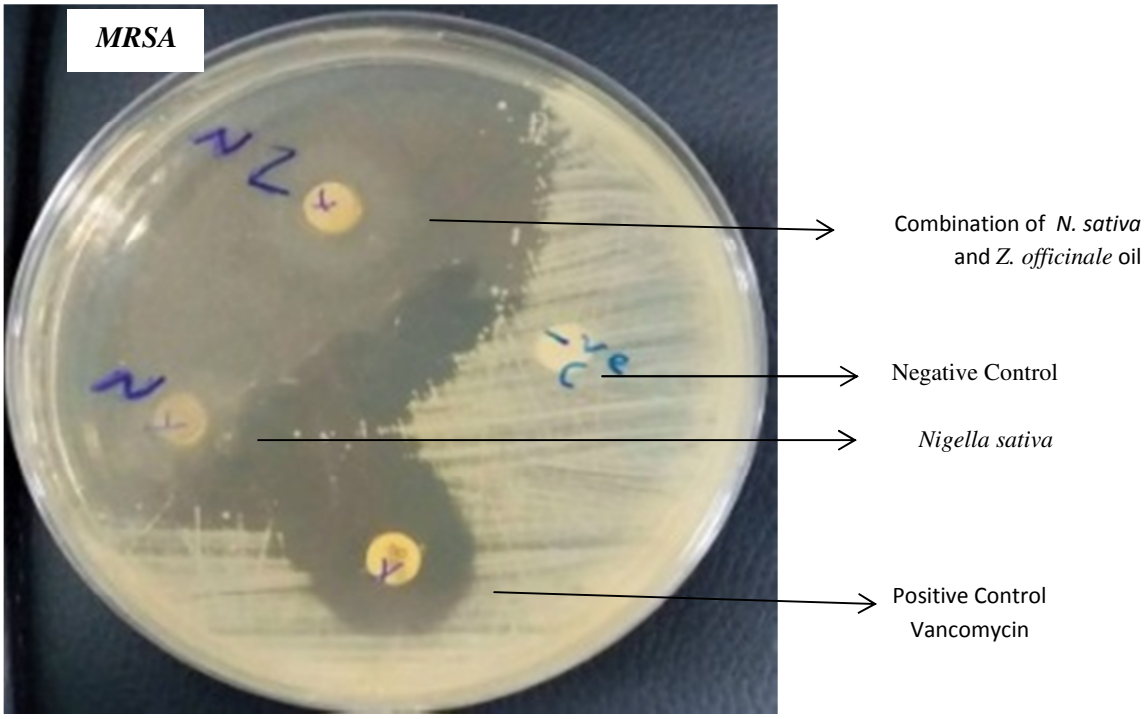

Figure 6: Essential oils and antibiotic combinations synergistic effect against the growth of MRSA.

5. The Minimum Inhibitory Concentration and the Minimum Bactericidal Concentrations against isolated bacteria

The minimum inhibition concentration of black cumin *Nigella sativa* seeds alone was 62.5 mg/ml while the minimum inhibitory concentration in combination of *Nigella sativa* and Ginger (*Zingiber officinale*) rhizome essential oils against methicillin resistance *Staphylococcus aureus* was 37.5 mg/ml while the (MBC) of black cumin *Nigella sativa* seeds alone was 100 % however in combination of both extracts was 50% of ethanol oil dilution. (Fig-7-).

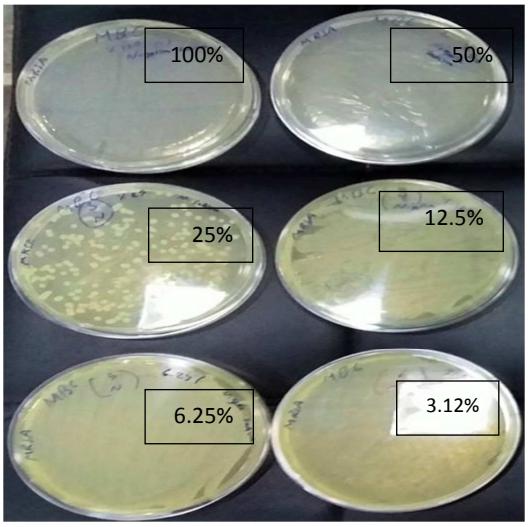

Figure 7: Minimum bactericidal concentration of serial dilutions of a combination of *Nigella sativa* and *Zingiber officinale* oils against methicillin resistance *Staphylococcus aureus (*no growth colony in 50%).

Discussions

Essential oils are the volatile liquids which formed by different parts of aromatic plants .These oils represent the most important part of the plant. Essential oils is thought to have the ability to open cell membrane channels and disruption of the cell membrane, thereby disturbing pH homeostasis and ATP production leading to cell death [21]. The findings of this study indicated that black cumin *Nigella sativa* oil extract affected all the isolated strains of MRSA, these results are compatible with those of Emeka *et al*., [22] and Salem *et al*., [23] who reported that the *Staphylococcus aureus* which is resistant to the most common antibiotics β- lactams which include methicillin, oxacillin, penicillin and amoxicillin was sensitive against different concentrations 100%, 75%, 50%, 25%, 12.5%, 6.25% and 3.12% of *N. sativa* oil extracts. It can therefore be optimistic to say that irrespective of differences in Staphylococcal strains, either based on the sites of isolation or geographical location, and the oil extract under consideration. Also, from the present findings shows the great similarity with a study done by Al-Jaafary Maryam *et al* [24] which indicated that multi drug resistant *Acinetobacter baumannii* strains and the different *Escherichia coli* strains tested against *Nigella sativa* and *Zingiber officinale* oil extract, showed a 100% resistance of the isolates to 100 % concentrations of the oil extract . While the results were contrary to the findings of Mohammed [25] who showed multi drug resistance *Acinetobacter baumannii*, and *Escherichia coli*, to be susceptible to different concentrations 100%, 80%, 50%, 40%, 30% and 20% of *N. sativa* oil dilutions indicating the possibility that the gram negative cell wall was not a hindrance to the mode of action of *N. sativa*. In the present study indicated that all multidrug resistance *Klebsiella pneumonia* and *Pseudomonas aerogenosa* isolates were resistance to both black seed (*Nigella sativa*) and ginger essential oil at 100 % concentrations which is contrary to Salman et al indicated that only *Pseudomonas aerogenosa* was sensitive to the *Nigella sativa* oil and rest *Acinetobacter baumannii* and *Klebsiella pneumonia* were insensitive [26].

The response to essential oils as antibiotics depends on the cell wall structure of different bacteria. The selective barrier functions of the outer membrane and its permeability properties determine whether antimicrobial can penetrate the gram negative bacterial cell wall. However *Pseudomonas aeruginosa* have a low level of outer membrane permeability [27]. It has been thought that the resistance of some gram negative bacteria against black cumin *Nigella sativa* essential oil extract is due to difference in their structure. In addition the thickness of peptidoglycan cell wall is another factor of difference in response against antibiotics [28].

The previous researches indicated that *Zingiber officinale* essential oil have a huge potential antibacterial and synergistic activity against many microbes [29] while it is not agreed with our results because ginger oil

showed the highest synergistic effects in combination only with black seed oil against gram positive MRSA. These variations are correlated to many factors such as geographical and seasonal conditions, climatic, the time of harvest, and the extraction techniques of the essential oil [30].

The combination of black seed *Nigella sativa* and ginger essential oil with standard antibiotic showed potential synergistic antibacterial activity. The Synergistic effects occur if the plant oil extracts act on different targets to enhance the bio availability of one and several substances of an extract. [31].

It was not ascertained whether the resistance to antibiotics by all the isolates considered in the present study was plasmid or chromosomal based.

With the results from the present findings, it can be suggested that a combination of *Nigella sativa* and *Zingiber officinale* oil extracts having shown such effective levels of anti-bacterial than *Nigella sativa* essential oil alone on methicillin resistance *Staphylococcus aureus* bacteria, so it can be useful in either the treatment as topical applications [26] or as an adjuvant [32] or for the prevention of various bacterial infections. Its antibacterial activity might be due to the active chemical component of the oil that it possesses antibacterial activity. [33]

Conclusions

It is concluded that antibacterial activity of a black cumin *Nigella sativa* seeds alone and combination of *Nigella sativa* and Ginger (*Zingiber officinale*) rhizome essential oils plant extracts would be helpful in treating antibiotic resistant gram positive MRSA bacteria isolated from burn patients.

This might be a new ways to cope with multiple drug resistant microorganisms. However, the further studies including the *in vivo* toxicity studies on their active compounds to determine pharmacodynamics and pharmacokinetics are still necessary.

Acknowledgments

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