Research Square

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Dehydrocostus lactone from Echinops kebericho Mesfin synergizes the antibacterial effects of gentamicin and ciprofloxacin

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Research Article

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Abstract

Background: Combination therapy is becoming an appealing solution to the emergence of antimicrobial resistance (AMR). This study investigated the combinatory effects of dehydrocostus lactone (DCL) and essential oil (EO) from *Echinops kebericho* when combined with selected standard antibacterial antibiotics.

Methods: The minimum inhibitory concentration (MIC) was determined using broth microdilution. Checkerboard analyses were used to determine the interactive effect. Eight serials, two-fold dilutions of conventional antibiotics and DCL/EO were prepared by diluting in broth. Dilutions of 50µl of antibiotic were dispensed in rows; while those of the EO/ DCL were dispensed in columns (1:1 v/v) of different concentrations ranging from 1/32 × MIC to 4 × MIC. In isobole analysis the EO/ DCL were combined with conventional antibiotics in nine different ratios. Isobolograms of the combination of DCL/EO with antibiotics were constructed.

Results: Out of a total of 90 different combinations evaluated, only five (5.55%) showed a synergistic effect; the majority (80%) showed no interaction, while 14.44% showed an additive effect. No antagonistic effect was observed in all combinations tested.

Beta-lactam antibiotics were non-interactive range of ΣFIC with DCL with the exception of a 3:7 DCL to penicillin combination with $\sum$FIC =1, in the additive range. All combinations of EO with selected beta-lactam antibiotics were in the non-interactive range. In this study, isobole analyses have shown the dependence of interactive effects on the ratio of combining agents. The DCL combination ratios of gentamicin or ciprofloxacin showed a synergistic effect.

Conclusion: Dehydrocostus lactone showed a synergistic effect against methicillin resistant S*taphylococcus aureus* (MRSA) when combined with ciprofloxacin or gentamicin. The observed synergistic effect could be useful for combating resistant strains. Further investigations are required on the mechanism of synergism and reversing resistance.

Background

Plants have been used as remedies for disease conditions afflicting human being. Ancient people learned from their interactions with environment that plant remedies could treat different disease via mere observation. As science keeps advancing, methods of investigation are being developed to prove these observations. Many modern medicines have been evolving from plant medicines through scientific experimentation. The efficacy and safety of medicinal plants are evaluated using standard scientific methodologies to generate knowledge for further development of leading drugs.

Plant extracts which contain multi-components could show additive, synergism, or antagonism among themselves or when used in combination with other chemicals/treatments. Interactions have been observed between phytochemicals including essential oils (EOs) and antibiotics against various organisms. Baisri *et al* evaluated 23 phytochemical-oxacillin combinations against vancomycin intermediate *S. aureus* (VISA) strains using a checkerboard broth microdilution method, of which 14 showed synergistic and additive interactions [1]. In another study, the combinations of *lavendula angustifolia* EOs with ciprofloxacin showed synergistic effects; while a combination of chloramphenicol and fusidic acid showed an additive effect against *S. aureus* [2]. Combination therapy is becoming an appealing solution to the emergence of antimicrobial resistance (AMR) and has proved effective in the treatment of tuberculosis, malaria, HIV/AIDS and cancer.

When the combined effect of two antimicrobials is equal to the sum of the individual effect, it is known as additive effect. Synergism occurs when the combined effect is greater than the sum of individual effects. Antagonism occurs when the combined effect is less than the sum of the individual effect. Combinatory effects depend not only on the kind of combined agents but also on the ratios of each combined agents [3]. Different assay techniques are used to determine such interactions. The checkerboard assay, isobole method, time-kill analysis and E-test are some of the commonly used methods for the determination of interactive effect [3].

Plant secondary metabolites display a wide array of mechanism of actions different to commonly available antibacterial agents. Essential oils synergize conventional antibacterial agents by disrupting bacterial membranes causing damage to membrane proteins and consequently cell content leakage and damage to cell wall [4]. This all will enhance antibiotics penetration in to the bacterial cells. Inhibition of bacterial efflux pumps is also responsible for the reversal effect of antibacterial resistance (Johny, Hoagland and Venkitanarayanan, 2010) as this targets are contrary to currently available. Oregano EO reduced lipase and coagulase activity in *S. aureus* (de Barros *et al*., 2009); eugenol reduced the production or activities of staphylococcal enterotoxin A and B, toxic shock syndrome toxin 1 and a-haemolysin, in *S. aureus* [3]. Cinnamaldehyde may bind to enzyme proteins to inhibit the function of bacterial amino acid decarboxylases [5]. Cell membrane disruption with carvacrol, thymol, ρ-cymene, cinnamaldehyde, cinnamic acid, eugenol, and Y-terpinene was reported [6].

The mechanisms of action of essential oils are largely different from those of conventional antimicrobial agents. They could demonstrate potential synergistic effects as antibiotics and EO attack the microbe at different targets. Membrane disruption enhances antibacterial

activities of conventional antibacterial agents by increasing the influx [6]. This has considerable importance in battling as AMR due to increased efflux mainly in Gram-negative bacteria [7].

In a previous study, We have isolated and identified dehydrocostus lactone as antibacterial lead [8]. In the present study, we investigated the interaction of dehydrocostus lactone and EO with conventional antimicrobial agents.

Methods

The extraction of the plant material, separation and isolation and identification of dehydrocostus lactone have previously been described [8].

Microorganisms

The bacteria strain used in this study was methicillin resistant *S. aureus* (NCTC 12493).

Preparation and storage of reagents

Resazurin was prepared in a concentration of 0.015% by dissolving 0.015 g in 100 ml of PBS, vortexed, sterilized by microfiltration and stored at 4^0C [9].

Preparation and serial dilution of the extract solution

Essential oils and DCL were dissolved in dimethyl sulfoxide (DMSO) for antibacterial activity assay. The final concentration of DMSO was ≤ 2%. The stock solution of the extract prepared was 5mg/ml, and it was serially diluted with broth.

Preparation and standardization of inoculum

Clinical and Laboratory Standards Institute (CLSI) guideline was used to prepare the inoculum. McFarland 0.5 turbidity standards equivalent to $1x10^8$CFU/ml was prepared using inoculum densitometers. It was then further diluted to obtain the desired cell density of 5×10^5 CFU/ml [10]. Briefly, an equivalent of 0.5 McFarland suspensions ($1x10^8$CFU/ml) inoculum was prepared and then diluted 1:20 to yield $5x10^6$CFU/ml using saline media. Then, 0.02 ml of this suspension was inoculated into 0.18 ml of broth yielding a final concentration of bacteria approximately equal to $5x10^5$ CFU/ml.

Determination of Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined using the broth micro-dilution method [11]. CLSI guideline procedures and safety precautions were strictly followed [12]. The 96-well plates were dispensed first with 100 µl MHB, then EO and DCL of 100 µl were added in the first well and serially diluted up to the tenth well. The excess 100 µl was removed from the tenth well. Finally, 100 µl of MHB mixed with inoculum was added from the first to the eleventh well. The 11th well contained the broth without the intervention, sterility control, while the 12th well contained broth without inoculum and intervention, fertility control. The final volume in each well was 200 µL.

The bacteria were incubated for 24h at 35- 37^{o}C. The MIC of DMSO was also evaluated as negative control, while ciprofloxacin was a positive control. Prior to use, the broth was checked for sterility and fertility. Microbial growth was indicated by an irreversible change in color from the blue of resazurin to pink resofurin after 20 hours of incubation. Measurement of optical density (OD) at 620nm were taken at 0, 2 4 18 and 24 hours. Graph of time versus absorbance (OD) versus concentration was plotted at time 0h, 2h, 4h, 18h, and 20h. The plot at 18h and 20 h were compared with resazurin assay value for MIC.

Checkerboard and Isobole Assay Method

The checkerboard assay was performed according to the previously described method with slight modifications [13]. Eight serial, two-fold dilutions of conventional antibiotics and EO/DCL were prepared by diluting in broth. Dilutions of 50µl of antibiotic were dispensed in rows; while that of the EO/ DCL were dispensed in columns (1:1 v/v) of different concentrations ranging from 1/32 × MIC to 4 × MIC. Finally, broth containing inoculum density of 5x105 CFU/ml of bacterial suspension was inoculated in each well. The 96-well plates were then incubated at 35-37oC for 20 h.

In isobole analysis the EO/DCL were combined with conventional antibiotics in nine different ratios i.e. 9: 1; 8: 2; 7: 3; 6: 4; 5: 5; 4: 6; 3: 7; 2: 8 and 1: 9. The ratio is proportional to each components MIC value. For example, 5:5 ratios were 4xMIC1:4MIC2 where MIC1 and MIC2, respectively represented MIC of EO/ DCL and conventional antibacterial agents. In similar way, 6:4 was equivalent to (6*4MIC1)/5: (4*4MIC2)/5. So, the starting concentration of an EO/DCL to conventional antibacterial agents X: Y was determined as:

$$X{:}\,Y{:}\,(\frac{x*4*MIC1}{5}){:}\,(\frac{y*4*MIC2}{5})$$

Where: X: Y = the given ratio of EO/DCL to conventional antibacterial agent

MIC1 = MIC of EO/DCL

MIC2: MIC of conventional antibacterial agents

Lots of combination ratio can be prepared, but these combination ratios were selected based on feasibility and a similar previous work [3]. These combination ratios were serially diluted to determine the MIC value of each combination pair.

The fractional inhibitory concentration (FIC) was defined as the MIC of a compound in combination divided to MIC of the compound alone.

$$FICa = \frac{MICa_{in_{combination_{with_b}}}}{MICa_{alone}}$$

$$FICb = \frac{MICb_{in_{combination_{with_a}}}}{MICb_{alone}}$$

$$FIC_{Index} = FICa + FICb$$

Summation of the FIC index (ΣFIC) was expressed in terms of FIC and described as synergistic, additive, non-interactive, and antagonistic [14]. The interpretations were synergistic (ΣFIC ≤ 0.5), additive (ΣFIC > 0.5–1.0), non-interactive ΣFIC (> 1.0–≤4.0), or antagonistic (ΣFIC > 4.0) (van Vuuren and Viljoen, 2011). Isobole of the combination of the DCL/EO with antibiotics was constructed. The ratio points falling below and/or on the (0.5, 0.5) line of the isobologram were interpreted as synergistic. Points between (0.5, 0.5) and/or on the (1.0, 1.0) line were interpreted as additive and points above the (1.0, 1.0) line and (4.0, 4.0) were defined as non-interactive or antagonistic if the points lie above (4.0, 4.0).

Results

Combination of Dehydrocostus lactone or Essential Oil with Antibacterial Agents

Interactive effect of DCL and the EO with selected antibacterial drugs were evaluated in different combinations ratios using isobole method. Nine different combination ratios of isolated compound and the EO with five different antibacterial agents (penicillin, ampicillin, cloxacillin, gentamicin, and ciprofloxacin) were examined. A total of 90 different combinations were evaluated of which five showed synergistic effect. Of the 90 different combinations, 5.55% were synergistic; while the majorities (80%) were non-interactive. Additive interactions were 14.44%, and no antagonistic effect was observed in all combinations tested (Table 1).

Table 1
Fractional inhibitory concentration (FIC) and FIC index for different combination ratio of the isolated compound and the essential oil with five different antibacterial drugs using isobole method

With DCL		Penicillin G			Ampicillin			Cloxacilin			Gentamicin			Ciprofloxacin		
	Combination ratio	FIC DCL	FIC PCN	£FIC	FIC DCL	FIC AMP	£FIC	FIC DCL	FIC CLO	£FIC	FIC DCL	FIC GEN	£FIC	FIC DCL	FIC CIP	£FIC
1.	9:1	1.50	0.75	2.25	1.50	1.50	3.00	0.75	1.00	1.75	1.00	0.50	1.50	0.75	0.50	1.25
2.	8:2	0.50	0.75	1.25	0.75	0.75	1.50	1.00	0.75	1.75	0.75	1.00	1.75	1.00	1.00	2.00
3.	7:3	1.00	1.50	2.50	1.00	1.00	2.00	1.50	1.00	2.50	1.00	0.13	1.13	0.75	0.13	0.88
4.	6:4	0.50	1.00	1.50	0.75	1.00	1.75	0.75	2.00	2.75	0.50	0.13	0.63	1.00	0.13	1.13
5.	5:5	1.00	1.00	2.00	1.50	0.75	2.25	2.00	2.00	4.00	0.25	0.13	0.38	0.25	0.13	0.38
6.	4:6	0.75	0.75	1.50	1.50	1.00	2.50	1.50	0.75	2.25	0.25	0.25	0.50	0.25	0.13	0.38
7.	3:7	0.50	0.50	1.00	2.00	0.50	2.50	1.00	1.00	2.00	0.75	0.13	0.88	0.50	0.13	0.63
8.	2:8	0.75	1.00	1.75	1.00	0.75	1.75	2.00	0.75	2.75	1.00	0.25	1.25	1.00	0.13	1.13
9.	1:9	1.00	2.00	3.00	0.75	0.75	1.50	1.00	1.50	2.50	0.50	0.25	0.75	0.50	0.25	0.75
With EO																
10.	9:1	1.50	1.00	2.50	1.00	1.50	2.50	1.50	1.00	2.50	1.50	0.50	2.00	1.00	0.50	1.50
11.	8:2	0.50	0.75	1.25	0.50	0.75	1.25	0.75	0.75	1.50	0.75	1.00	1.75	0.50	1.00	1.50
12.	7:3	0.75	1.50	2.25	0.50	1.50	2.00	1.00	1.00	2.00	0.50	0.50	1.00	0.25	0.25	0.50
13.	6:4	0.75	1.00	1.75	1.00	1.00	2.00	1.00	2.00	3.00	0.37	0.25	0.62	0.25	0.50	0.75
14.	5:5	1.00	1.50	2.50	0.75	1.50	2.25	0.75	2.00	2.75	0.75	0.50	1.25	0.37	0.25	0.62
15.	4:6	0.75	0.75	1.50	0.75	1.00	1.75	0.50	0.75	1.25	0.25	0.50	0.75	0.50	0.50	1.00
16.	3:7	1.00	0.75	1.75	0.50	1.00	1.50	0.50	1.00	1.50	0.75	0.25	1.00	0.25	1.00	1.25
17.	2:8	0.50	1.00	1.50	1.00	0.75	1.75	0.50	1.50	2.00	1.00	0.50	1.50	0.50	0.50	1.00
18.	1:9	0.50	2.00	2.50	1.50	0.75	2.25	1.50	1.50	3.00	1.00	0.25	1.25	1.00	0.25	1.25

Note: FIC = Fractional inhibitory concentration, £FIC = Fractional inhibitory concentration index, PCN = Penicillin G, DCL = Dehydrocostuslactone, AMP = Ampicillin, CLO = Cloxacilin, GEN = Gentamicin, CIP = Ciprofloxacin,

Combination of Dehydrocostus lactone with Antibacterial Agents

Thirty five non-interactive, 6 additive and 4 synergistic DCL-antibacterial drugs combinations were observed. The interaction was illustrated using the isobologram. Penicillin G, ampicillin or cloxacilin were non-interactive with DCL. Exception was 3:7 DCL to penicillin combination which showed $\sum$FIC = 1, in the additive range. The interaction was observed to be dependent on the ratio of combined constituents. No synergistic or antagonistic interaction was observed with all combination ratios as shown in Fig. 1.

Dehydrocostuslactone combinations with ciprofloxacin and gentamicin showed synergistic effect against MRSA strains. DCL-ciprofloxacin combination in the ratio of 1:1 and 4:6 had £fIC = 0.38; while DCL-gentamicin combinations in the ratio of 1:1 and 4:6 showed £fIC = 0.38 and 0.5, respectively. Three ratio combinations in both cases showed additive effect, while four ratio combinations did not show interactions at all. In DCL-ciprofloxacin combination, 6:4 and 2:8 combinations showed same £fIC = 1.13. There were no antagonistic ratio combinations in both cases as shown in Fig. 2.

Combination of Essential Oil with Antibacterial Agents

Thirty-seven non-interactive, 7 additive and 1 synergistic interactions were observed between EO and antibacterial drugs. All combinations of EO with beta-lactam antibiotics (penicillin, ampicillin and cloxacilin) were in the non-interactive range as shown in Fig. 3. There were no synergistic, additive or antagonistic interactions.

Four combination ratios of EO with gentamicin showed additive interaction (6:4, 7:3, 4:6 and 3:7); while other combinations fell in the non-interactive range. Synergistic effect was shown by 7:3 combination of EO with ciprofloxacin; while 1:1, 6:4 and 4:6 ratio combination of EO

with ciprofloxacin showed additive interactions. The remaining combinations fell in the non- interactive ranges as shown Fig. 4.

Discussion

The combinatory antibacterial effect of DCL and EO with selected antibiotics against MRSA was explored in this study. The most commonly used antibiotics but the one losing their efficacy due to antimicrobial resistance emergence was selected to investigate the interaction in the present study.

Five of the 90 different combinations were synergistic; while the majorities (80%) were in the non-interactive range. Additive interactions accounted for 14.44 %, and no antagonistic effect was observed. Non-interactive effects were observed particularly between β-lactams and DCL or EO; while most synergistic effects were observed between DCL and gentamicin or ciprofloxacin. No synergistic or antagonistic interaction was observed between DCL and β-lactam antibiotics. Isobole analyses in this study showed the dependence of interactive effects on the ratio of combining agents, which aligned with that of the previous study [3].

In another study, various ratios of *Lavandula angustifolia* showed synergistic interactions against S. *aureus* and *C. albicans,* with higher ratio of *Lavandula angustifolia* EO compared to conventional antibacterial agents [15]. Essential oils combinations with conventional antimicrobials showed synergism. For example, thymol and carvacol showed synergism with penicillin against *E.coli* and *S. typhimurium* [16]. Recent reviews concluded that antimicrobial agents and EO interaction resulted in? a good number of synergistic effects [6, 17]. A previous study showed a possible synergistic activity against mycobacterial strains between sesquiterpene lactones (DCL and costunolide) [18]. However, to the best of our knowledge, there has been no study conducted to evaluate the combinatory effect between DCL and various conventional antibacterial agents. This study is the first of its kind to evaluate the interaction between DCL and selected antibacterial agents.

The combination of EOs and antibacterial agents provides a multi-targeted antimicrobial activity which reverses microbial resistance. The possible mechanism for the synergistic activity could be attributed to membrane disruption, inhibition of efflux system and depletion of specific porins of the bacteria by EOs making the bacteria more susceptible to conventional antibacterial agents [17]. Dehydrocostus lactone being a component of EOs and having high lipophilic nature could be expected to exert synergistic activity through the same mechanism as EOs do. A considerable synergistic effect was observed between DCL and gentamicin or ciprofloxacin but not with β-lactams insights some mechanism connection. Explanation of the possible mechanisms is, however, beyond the scope of this study, and the role of each constituent in such interaction has not yet been explored thoroughly and requires further studies. Establishment of the mechanism for the synergism leads to the development of combination therapy which will thwart the health impact of AMR.

Our recent review on clinical studies on topical anti-infective EOs [19] and case report on their use in preliminary treatment of pulmonary tuberculosis has provided a general picture of potential use of EOs in combination therapy. Topical Eucalyptus EO-based mixture on top of clindamycin resulted in smell reduction, anti-inflammatory, re-epithelization, and improvement of quality life [20]. Inhalation of TTO improved symptoms and turned the sputum negative in two patients [21]. The *E. kebericho* M. EOs and a component isolated from it, DCL, could be anticipated to have a clinical relevance for further development.

As the interactive effect depends on the ratio of combining agents, it is important to conduct a number of such studies which will systematically identify such combinations, determine safety profile and conduct animal model study. The methodology employed should also characterize the characteristic nature of EOs. The checkerboard method and isobole analysis remains the most preferred method for developing new antibacterial agents. Isobole analysis is a preferred method as it gives chances to examine different ratio combination [3], and the current study also proved dependence of interactive effect on the variability of combining agents.

In conclusion, the observed synergistic effect of gentamicin and ciprofloxacin could be useful for combating resistant strains and reducing their adverse effects. The more we understand the mechanism underlying the synergism, the more the chance of developing safer and effective EO-antibiotic combinations to combat the antimicrobial resistance. Further investigations are, therefore, required on the mechanism of synergism and reversing resistance. Overall, no antagonistic effect was observed. A number of combinations were non-interactive, but synergistic interaction was observed between DCL and gentamicin or ciprofloxacin.

Declarations

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was reviewed and approved by Faculty Research Committee and approved by the Research Ethics Committee (REC) of Mbarara University of Science and Technology, Uganda. It was also registered with Uganda National Council for Science and Technology (UNCST) with reference number HS398ES. There is no human participation in this study.

CONSENT FOR PUBLICATION

Not applicable

COMPETING INTERESTS

The authors declare no competing interests.

AVAILABILITY OF DATA AND MATERIALS

The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

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AUTHOR CONTRIBUTIONS

SD conceived the research idea and conducted antimicrobial activity study. EM, JB, and PA developed the concept, monitored and mentored the proposal development. SD wrote the draft manuscript. All authors revised, edited and approved the final manuscript.

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Figures

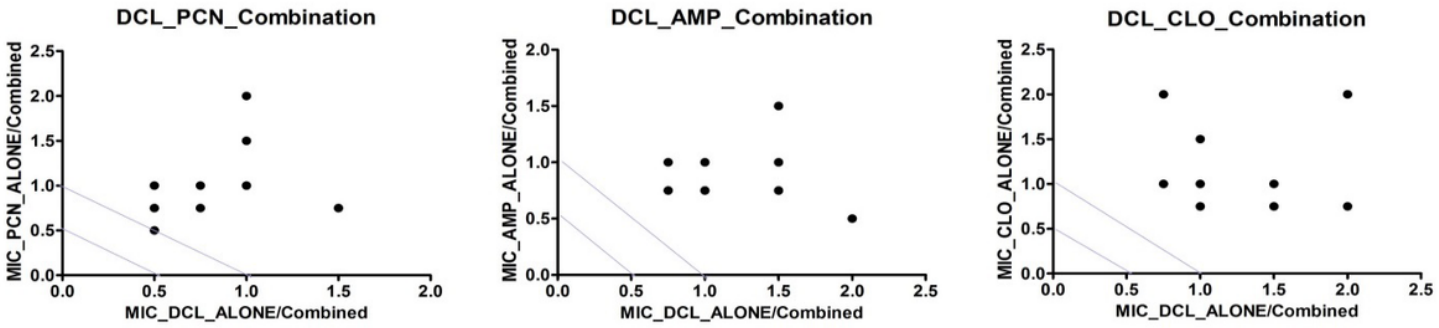


Figure 1

Isobologram representation of the combinations of DCL with penicillin, ampicillin and cloxacillin against MRSA strains. The axes represent the FIC values for each combined agents, the points indicate the £FIC and the lines cut off point

Where; DCL = dehydrocostuslactone, PCN = penicillin, AMP = ampicillin, CLO = Cloxacllin

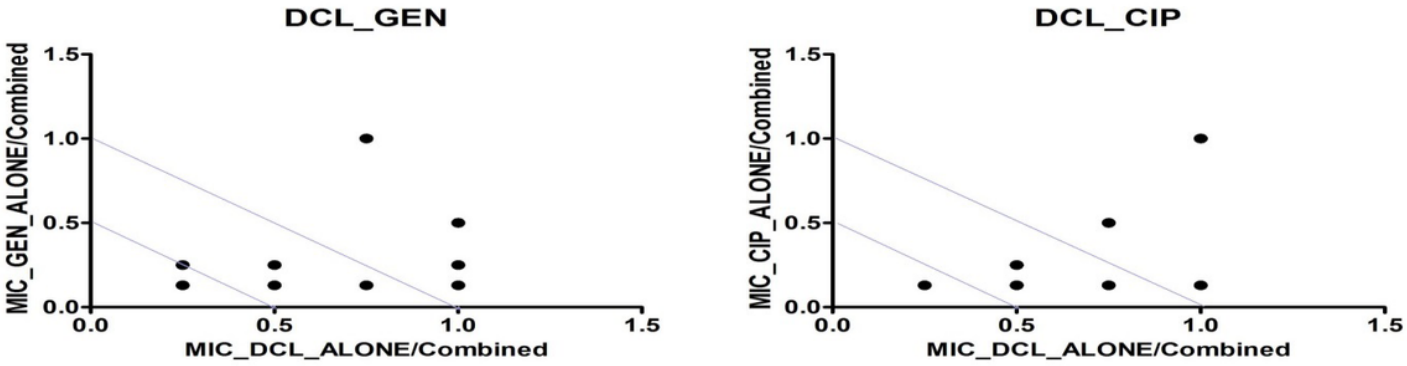


Figure 2

Isobologram representation of the combinations of DCL combination with gentamicin and ciprofloxacin against MRSA strains. The axes represent the FIC values for each combined agents, the points indicate the £FIC and the lines cut off point. Where; DCL= Dehydocostuslactone, GEN = Gentamicin, CIP = Ciprofloxacin

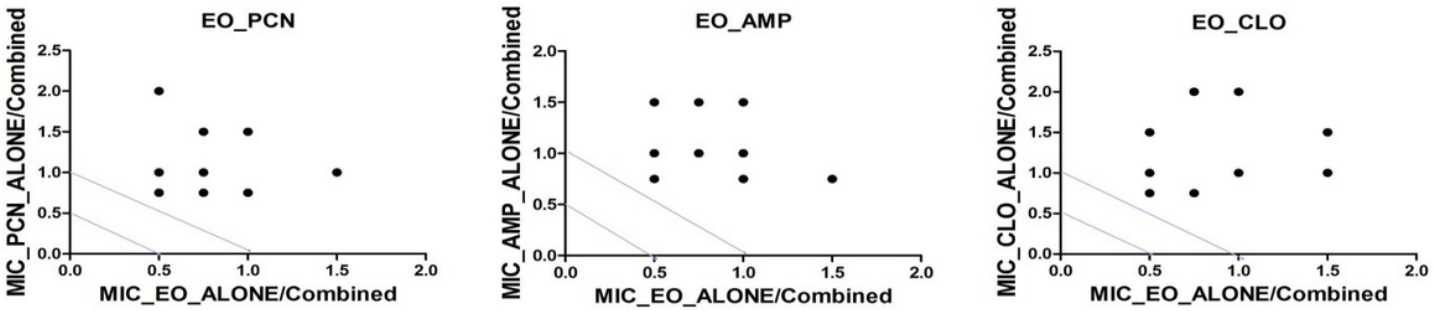


Figure 3

Isobologram representation of the combinations of EO combination with penicillin, ampicillin and cloxacilin against MRSA strains. The axes represent the FIC values for each combined agents, the points indicate the £FIC and the lines cut off point

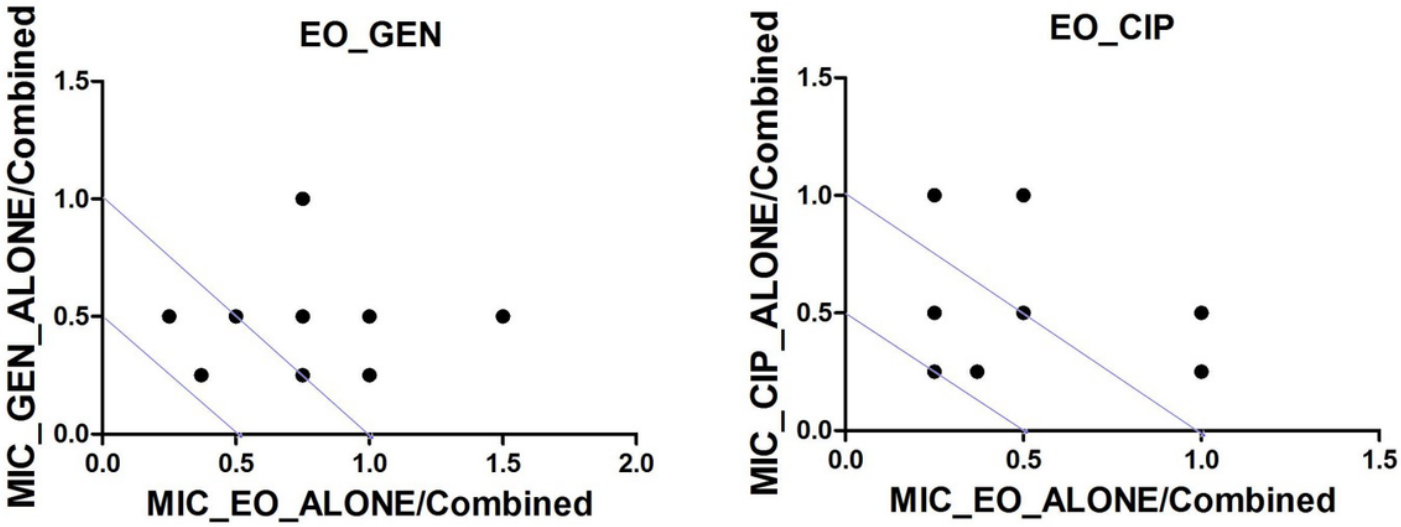


Figure 4

Isobologram representation of the combinations of EO with gentamicin and ciprofloxacin against MRSA strains. The axes represent the FIC values for each combined agents, the points indicate the £FIC and the lines cut off point. Where; EO= Essential oil, GEN = Gentamicin, CIP = Ciprofloxacin