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Evaluation of the Antibacterial Activity of *Chrysomya albiceps* Larval Extract and its Synergistic Effects with Antibiotics

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Abstract: Antimicrobial resistance is currently a significant global problem. The medical field is currently suffering from a shortage of effective treatments to prevent infections since the emergence rate of multidrug resistance has exceeded the rate of discovery and creation of new effective drugs. This study evaluates the antibacterial activity of the excretions/secretions of Egyptian calliphorid larvae, *Chrysomya albiceps* (*C. albiceps*), in vitro on the development of five pathogenic bacterial species (methicillin-sensitive *Staphylococcus aureus* ATCC 29213, methicillin-resistant *Staphylococcus aureus* ATCC BAA-1680, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli*_ATCC 25922 and *Salmonella typhi* ATCC 19430) via different assays. Resazurin-based 25turbidimetric assay (RTBA) proved that *C. albiceps* larval exosecretions (ES) was effective against all examined bacterial species, which also determined the minimum inhibitory concentration (MIC) for each bacterium. By using a colony-forming unit (CFU) assay, MICs of *C. albiceps* ES exhibited bacteriostatic activity against all tested bacteria. All the bacterial models could not recover after 24 hrs of the incubation period. However, high concentration (200 mg/ml) showed bactericidal activity towards *P. aeruginosa*. The potency of *C. albiceps* extract toward gram-negative bacteria has been proven by agar disc diffusion assay which failed to show any antibacterial efficiency of ES toward gram-positive bacteria. Synergism between larval ES and commercial antibiotics against all bacterial species was observed. The present data is promising and using these combinations can lead to a reduction in the minimum dose required for effective antimicrobial effects which is important because it may decrease both the risk of side effects and the costs of treatment of infectious diseases.
Keywords: Antibacterial activity; MRSA; MSSA; Crude extract; *Chrysomya albiceps*.

1. Introduction

The development of antibiotics significantly enhanced human health and quality of life [1]. However, excessive and unsuitable use of antibiotics had led to increased bacterial resistance to marketed antibiotics. Additionally, during the past three decades, the number of novel antibiotics that have been approved has considerably and consistently dropped [2]. Therefore, the creation of novel antimicrobial agents, such as substitute medications based on antimicrobial peptides, is urgently needed [3].

Due to the biology of insects, especially the immature stages of true flies, and their capacity to breed and survive in septic environments such as cadavers, corpses, wounds, and decaying organic materials, they are contaminated with a wide variety of microorganisms, some of which are pathogens [4]. Insect larvae have been found to produce and secrete powerful anti-microbial compounds to defend themselves from the negative effects of these microbes [5,6,7]. These compounds have been considered as sources of novel antibiotics, particularly for the treatment of diseases resulting from bacteria that are resistant to multiple drugs, like the methicillin-resistant *Staphylococcus aureus* (MRSA) [8,9].

Since the dawn of civilization, larval treatment has been used therapeutically to remove necrotic tissue [10]. The initial discovery of larval therapy was carried out by Dr William Baer, an American orthopedic surgeon [11].

According to previous studies [12, 13, 14], the larvae of the greenbottle fly, *Lucilia sericata* (Meigen), can effectively cure necrotic wounds, particularly chronic wounds where other therapies have failed. In the UK, larvae from this facultative fly species that cause myiasis are the only ones used for larval therapy (LT), which involves applying maggots to wounds.

The best way to describe the three main pathways that result in the larval debridement of necrotic tissue is reported by [15, 16]:

a) Debridement: the removal of gangrenous or necrotic tissue based on the application of proteolytic enzymes (trypsin and collagenases that disintegrate dead tissue chymotrypsin-like collagenases) [17, 18].

b) Disinfection: Larvae kill infectious bacteria by coming into contact with highly potent exosecretion (ES) that has antimicrobial characteristics, either inside the insect digestive tract [19] or inside the lesion itself. Ammonia secreted by larvae is unfavorable medium for bacterial growth [20]. As a result, the growth of biofilms is prevented and eliminated [21, 22].

c) Regeneration: Granulated tissue must be stimulated to proliferate [22, 23, 24]. Hepatocyte growth factor (HGF), a crucial component in the healing of cutaneous wounds, has been strongly correlated with larval therapies.

Calliphorid flies can occupy a wide range of niches due to the diversity of their behavior and life history. *Chrysomya albiceps* is a calliphorid group that has been proven in vitro to possess antibacterial potentials and debridement effectiveness [25]. Therefore, the objective of the current investigation was to determine the minimum inhibitory concentrations (MIC) of larval extract of *C. albiceps* against gram-positive and gram-negative bacteria and to evaluate the possible synergistic interaction between the larval extract of *C. albiceps* with commonly used antibiotics at MICs against five pathogenic bacteria.

2. Materials and methods:

2.1. Maintenance of larvae:

C. albiceps colonies were created from collections in the natural environment [26] using the proper traps containing a rabbit corpse as bait. According to [27], collected specimens were screened and identified. Selected adults were maintained at a constant temperature of (27 ± 3ºC) and a relative humidity of (30 ± 5%) with a constant supply of sugar and water inside (30x30x30 cm) wooden cages with two sides of the glass and three sides of the wire. Fresh beef meat was placed to stimulate oviposition. Hatching larvae were permitted to feed on raw ground beef in an incubator at 30ºC.

2.2. Collecting larval excretions/secretions (larval ES):

ES from laboratory strains of *C. albiceps* larvae was obtained according to the method of [28] with some modifications. The developed third instar larvae were collected from the meat and transferred to a sterile petri-plate for washing in 70% ethanol for 3 minutes before being rinsed in sterile distilled water three times and soaked on filter paper (Whatman No. 1). One hundred washed larvae were transferred to a 100 ml sterile beaker containing 10 ml de-ionized water then incubated in the dark at 37º C for 1hr. After incubation, the larvae were removed and put in a freezer for five minutes to render them inactive. In order to reveal cellular content and gut secretions, the obtained extract was added to the deactivated larvae in porcelain miller for manual homogenizing, where the gut contains antimicrobial components [29]. Subsequently, the resultant larval homogenate was transferred to sterile Eppendorf tubes and centrifuged at 4000 × g for 30 minutes at 4º C then, a clean glass beaker was used to gather the obtained supernatant. Finally, the resultant brownish supernatant was lyophilized, and stored at -40º C to preserve antibacterial activity and endure long-term storage [30]. Before ES being used, lyophilized materials were resuspended into de-ionized water at a final concentration of 200 mg/ml. Then the final extract was sterilized with a Minisart® cellulose acetate membrane syringe filter with pores 0.2 microns in size.

2.3. Representative bacterial species:

Both gram-positive bacterial strains (methicillin-sensitive *Staphylococcus aureus* (MSSA) ATCC 29213 and methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC BAA-1680) and gram-negative strains (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Salmonella typhi* ATCC 19430) were chosen according to their geography, diversity growing habitats [28] and causing several illnesses [31, 32]. All selected strains were a kind gift from Animal Health Research Institute, El – Dokky, El – Giza, Egypt.

A single colony was taken off from a stock of nutrient agar plates for each bacterium Barnes et al. [28] According to Teh et al. [33] colonies were inoculated into a sterile 5 ml of Mueller Hinton broth (MHB) and incubated at 37ºC for 24 hrs. The overnight bacterial suspension was adjusted to McFarland 0.5% (media with bacteria at 1.5 x10^8 CFU/ml). From this suspension, final dilutions of 10^6 were obtained using ten-fold serial dilutions.

2.4. Antibiotics agents:

Purified powders of vancomycin for MSSA and MRSA, gentamycin for *E. coli* and *P. aeruginosa*, and ciprofloxacin for *S. typhi*. were procured from Oxoid Ltd, Basingstoke, Hampshire, England.

2.5. Determination of antibacterial characteristics of ES:

2.5.1. Liquid culture assays:

2.5.1.a. Resazurin-based turbidimetric assay (RTBA) and determination of Minimum Inhibitory Concentration (MIC):

According to the [33] procedure, a resazurin solution was prepared, 337.5 mg of resazurin powder was dissolved in 50 ml of sterile distilled water in a cleaned beaker. The solution was homogeneously mixed in a sterile vortex mixer for one hour. Because resazurin solution is sensitive to light, its preparation steps were carried out in complete darkness and was stored in a brown bottle. In order to prepare the 96-well plates, 100 µL of sterile Mueller-Hinton broth (MHB) was poured into all wells. A 100 µL of the following components was added into the first well of each column; sterile distilled water (D.W) in the first column, filter sterilized *C. albiceps* ES (200 mg/ml) in second column, another sterile distilled water (D.W) in the third column. The previously three columns represented sterility broth control columns, the bacterial suspension as a growth control in fourth column. Both fifth and sixth columns are tested columns containing antibiotic solution and sterile ES (200mg/ml) respectively. Before broth microdilution was applied, each first well's content was mixed thoroughly. A different, sterile pipette was then used to transmit100 µL of this content into the second well (2^{-2}). This serial microdilution was performed until the eighth well (2^{-8}) while the last 100 µL was discarded. Thus, 5 µL of diluted bacterial suspension was added to all wells except sterile columns to obtain a concentration of 0.5 x 10^5 cell/ml. The plates were then incubated at 37 °C for 24 hrs After the incubation period, all wells received 5 µL of previously prepared resazurin solution and were incubated at 37°C for additional 4hrs. The inhibition of growth can be detected when the solution in the well remains blue after incubation, while color alteration from blue to pink or colorless was recorded as a positive result. MIC was described as the lowest concentration of ES prior at which color change was observed [34, 35, 36].

2.5.1.b. Colony forming unit (CFU) assay:

Bacterial suspensions previously grown overnight in MH medium at 37º C were diluted to a density of McFarland 0.5 in MHB. Serial 1: 10 dilutions of the suspensions were performed to obtain a final dilution containing about 1.5 x 10^6 CFU/ml. According to CLSI [37] scale, MICs from vancomycin, gentamycin, and ciprofloxacin against tested bacteria were prepared. Three experimental groups were set up: 1) Bacterial group (positive control) containing the diluted suspensions from

the previous pathogenic bacteria. 2) Antibiotic group (negative control) containing the prepared concentrations from the three antibiotics and diluted bacterial suspensions 1.5 x 10^6 CFU/ml. 3) Tested group containing *C. albiceps* ES at their MICs determined by resazurin assay in our study against bacterial strains used and the bacterial suspensions. Aliquots of 100 µL from each experimental group were plated on BHI agar Petri dishes at the start of the experiment, and after 2, 4, 6, 10, and 24hrs, Petri dishes were incubated at 37 ºC (according to the reading period set). Then the colonies were manually counted and recorded.

2.5.1.c. Effect of ES dose on colony-forming units:

In this work, two different volumes of *C. albiceps* ES (100 and 300 µL), which have a concentration of 200 mg/ml were tested. Both high volume (300 µL) and the low one (100 µL) were mixed with 3 ml of bacterial species suspensions that were diluted 10 times serially to 0.5 x 10^5 CFU/ml. Sample controls were prepared by replacing the volumes of *C. albiceps* ES by distilled water. 0.1 ml of the tested and control samples were plated onto Muller Hinton agar for plate counting at an initial time (t =0) and after 18 hrs. After the plates were incubated for 24 hrs at 37°C, bacterial cell counts were estimated.

2.5.2. Solid culture assay:

2.5.2.a. Zone of inhibition assay:

Microbial growth inhibition was assessed using a classical disc diffusion method [38]**.** The studied strains were grown for one day before assay evaluation. Sterile filter paper discs (6mm in diameter) were distributed on the agar plates, which were inoculated with the tested bacterial suspension 1.5 x 10^8 cell/ml. The paper discs were then impregnated with 20 µL or 50 µL of ES (concentration 200 mg/ml) and dried inside the laminar flow cabinet. The plates were subsequently incubated at 37º C for 24 hrs. The observed zones of inhibition in (mm) were traditionally measured. For preparing a positive control sample, blank paper discs were designed using 20 µL of vancomycin, gentamycin, and ciprofloxacin at (MIC) concentrations according to CLSI 2020 [37], and sterile de-ionized H_2o was applied as a negative control sample. The assay experiment was carried out in triplicates.

2.5.2.b. Synergistic effect of the larval extract with antibiotics:

To evaluate the in vitro interaction between different concentrations of *C. albiceps* ES and certain antibiotics, a standard agar disc diffusion assay was used. Tested bacterial suspensions were prepared at 0.5 McFarland turbidity 1.5 x 10^8 cell/ml. After that, 0.1 ml of these bacterial suspensions were inoculated on the BHI agar plates' surface. Six sterile paper discs (6 mm in diameter) were placed regularly on the surface of BHI agar plates, the first disc was impregnated by 25 µL of *C. albiceps* ES (200 mg/ml), and the second one was impregnated by the same prepared volume of the commercial antibiotics (AB), gentamycin for *E. coli* and *P. aeruginosa*, vancomycin for MSSA and MRSA and ciprofloxacin for *S. typhi* at a concentration equal to MIC according to CLSI 2020 [37]**.** While the following four discs were soaked in a mixture of 25 µL containing different concentrations of larval extract and selected antibiotics. The obtaining concentrations in the discs of each plate were (100% ES, 80% ES + 20% AB, 60% ES + 40% AB, 40 % ES + 60% AB, 20% ES + 80% AB and 100% AB). The prepared discs were dried under complete aseptic conditions inside the laminar flow cabinet. The plates were subsequently incubated at 37º C for 24 hrs. After incubation, the growth inhibition zone was quantified by measuring the diameter of the clear zone of inhibition in mm, this assay was done in triplicate.

2.6. Statistical analysis

The found results were described as the mean ± standard error values. SPSS software (version 22.0) was applied for data analysis and statistical calculations. The antibacterial activity of ES in the colony-forming unit was evaluated using the Chi-square test, while the rest of the obtained data was analyzed using One-way analysis of variance (ANOVA).

3. Results

3.1. Determination of MIC of (ES) using Resazurin-based turbidimetric assay (RTBA): The MICs of *C. albiceps* were higher as 100 mg/ml for both MSSA and MRSA shown in Table 1, which can be detected in the first well. However, the lower MICs of the positive control (vancomycin) were found at the sixth and fourth wells, which was corresponding to 1.56 mg/ml and 6.25 mg/ml for MSSA (Plate 1a) and MRSA (Plate 1b), respectively.

Regarding gram-negative bacteria (Plate 2), ES displayed high efficiency against *P. aeruginosa* with MIC (25mg/ml), where MIC of gentamycin against *P. aeruginosa* was detected at the fifth well (3.12 mg/ml). While the MIC of gentamycin against *E. coli* was equivalent to (1.56 mg/ml), MICs of larval ES against *E. coli* and *S. typhi* were the same (50 mg/ml), but MIC of ciprofloxacin against *S. typhi* was (3.12 mg/ml).

Table 1: MICs of standard antibiotics and *C. albiceps* larval extract against tested bacteria:

Microorganism	Antibiotics (100 mg.ml^{-1})	*C. albiceps* Larval extract (200 mg ml^{-1})
Gram-positive bacteria		
MSSA	Vancomycin (1.56)	100 mg ml^{-1}
MRSA	Vancomycin (6.25)	100 mg ml^{-1}
Gram-negative bacteria		
P. aeruginosa	Gentamycin (3.12)	25 mg ml^{-1}
E. coli	Gentamycin (1.56)	50 mg ml^{-1}
S. typhi	Ciprofloxacin (3.12)	50 mg ml^{-1}

MSSA: methicillin sensitive *S. aureus*, MRSA: methicillin resistant *S. aureus*, *P. aeruginosa: pseudomonas aeruginosa*, *E. coli: Escherichia coli*

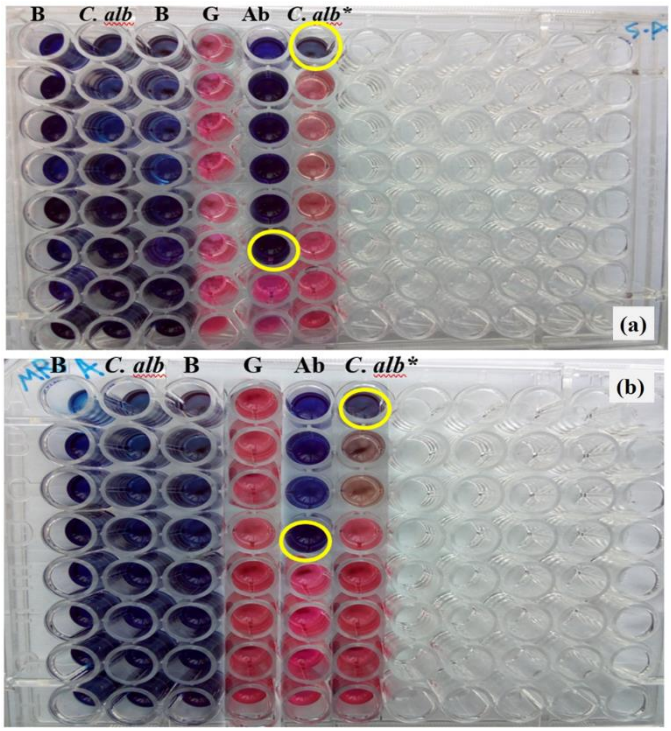
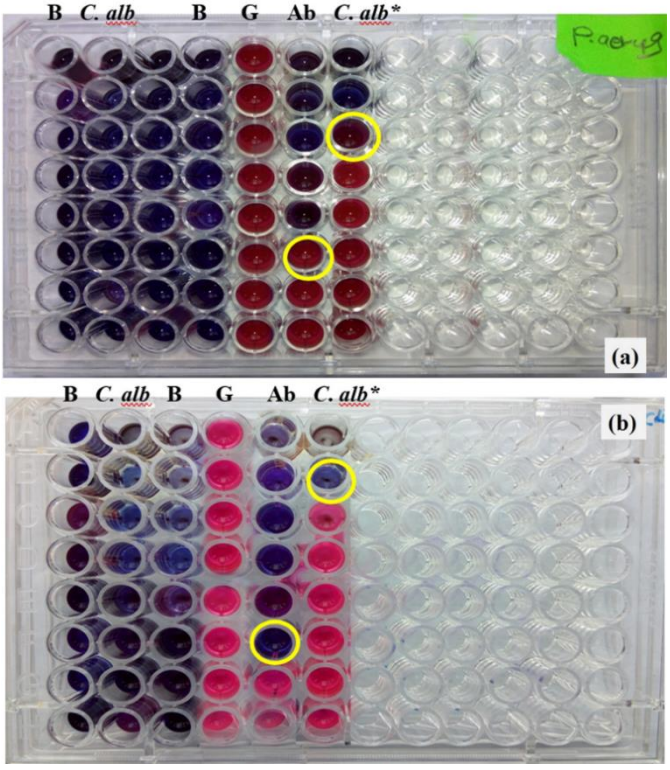


Plate 1. Microtiter plate after overnight incubation and addition of resazurin dye (**a):** MSSA, (**b):** MRSA.
B = sterile broth; *C. alb* = *C. albiceps* larval extract sterility control; G = growth control; Ab =vancomycin; *C. alb** = *C. albiceps* extract tested; the yellow circle indicates the MIC.



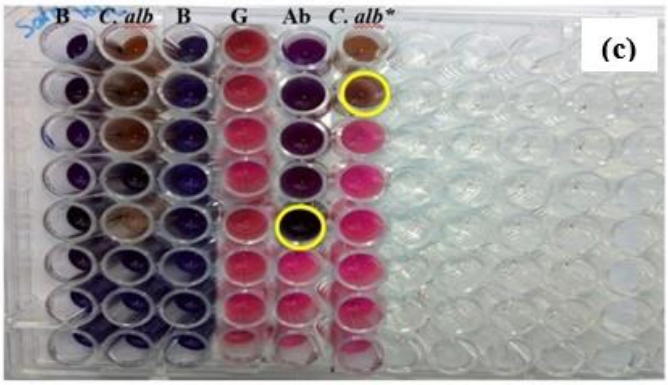


Plate 2. Microtiter plate after overnight incubation and addition of resazurin dye (**a):** *P. aeruginosa*, (**b):** *E. coli*, and (**c):** *S. typhi.* B = sterile broth; *C. alb* = *C. albiceps* larval extract sterility control; G = growth control; Ab = gentamycin for *P. aeruginosa* and *E. coli*, vancomycin for *S. typhi*; *C. alb** = *C. albiceps* extract tested; the yellow circle indicates the MIC.

3.2. Determination of the antibacterial activity of MIC of *C. albiceps* ES using (CFU) assay:

The dynamic growth of the five bacterial species under investigation in the control experiment lacked the typical lag period. Following a typical bacterial growth curve, the growth began early and immediately entered the log phase **(**Figures **1 and 2)**. During the experiment, *C. albiceps* ES showed bacteriostatic activity with different observed levels against the five bacterial species. All of the studied bacterial species could not recover through 24 hrs incubation period.

3.2.1. Gram-positive bacteria:

3.2.1.a. MSSA:

The dynamic of growth of MSSA control and following incubation for 2 hrs with ES of *C. albiceps* are not statistically different by the end of two hours. However, the antibacterial activity of ES became significantly efficient compared with the control group, and the ES continued to inhibit the growth of MSSA until the end of the experiment (82.17 % bacterial count reduction, $p < 0.001$) **(**Figure **1a)**.

3.2.1.b. MRSA:

C. albiceps ES showed significant inhibitory activity and slight reduction in the bacterial count of MRSA starting from the first 2 hrs ($p = 0.001$). Then plateau in the bacterial count was observed in the period between 10 and 24 hrs. The highest reduction in MRSA count (69.65 %) was observed at 4 hrs compared to the control **(**Figure **1b)**.

3.2.2. Gram-negative bacteria:

3.2.2.a. *P. aeruginosa*:

Slight growth of *P. aeruginosa* was observed for the first two hours. Then slight decrease in the bacterial count was detected, and a continual decrease over the 24 hrs experimental period was observed. Significant inhibition of bacterial growth ($p < 0.001$) in comparison with the control was recorded, and the reduction of bacterial growth reached 72% at the end of the experiment **(**Figure **2a)**.

3.2.2.b. *E. coli*:

Following incubation with the extract, the growth curve of *E. coli* showed variable bacterial count relative to the starting population that fluctuated between a few decreases and increases. The reduction of bacterial cells reached to 75.97% compared to the control over 4 hrs **(**Figure 2b**)**. ES did not allow any bacterial growth to occur in a period between 10 and 24 hrs.

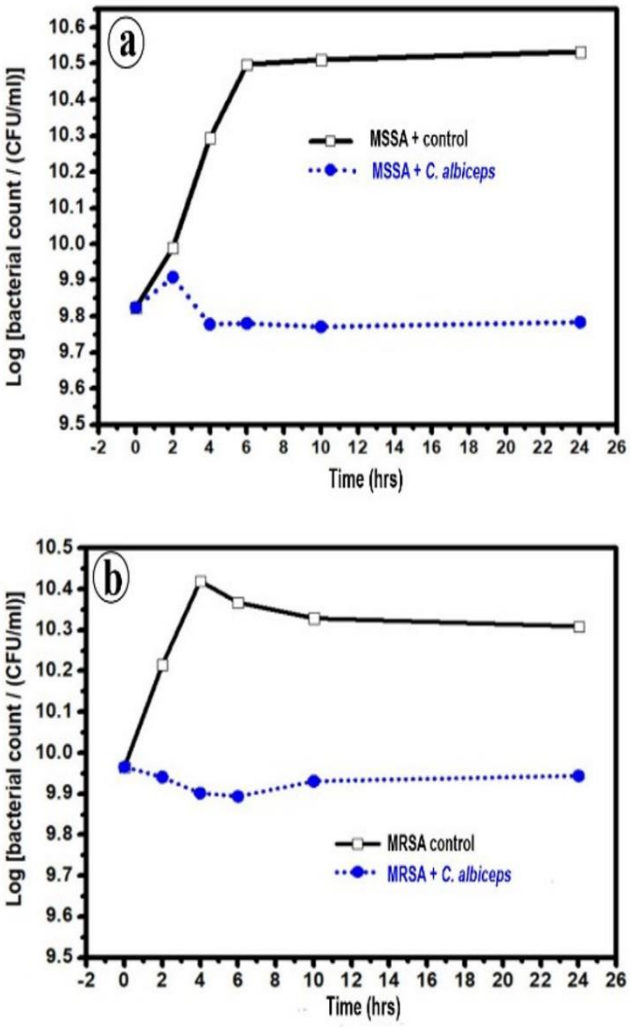


Figure 1. A typical 24-hrs growth curve of **(a):** MSSA and **(b):** MRSA in control and with the addition of MIC-determined concentration of *C. albiceps* ES.

3.2.2.c. *S. typhi*

Growth curve of *S. typhi* following incubation with *C. albiceps* extract exhibited stability to some extent in the number of initial bacterial populations. The extract displayed about 67% reduction in bacterial growth within 2 hrs in the comparable growth seen in the control. *S. typhi* completely stopped growing between 4 and 24 hrs period. There was a highly significant difference in the percentage of survival bacterial cells between control and treated ($p < 0.001$) throughout the experimental period. **(**Figure 2c**)**.

It is important to note that selected used antibiotics showed bactericidal activity against tested bacterial spp.

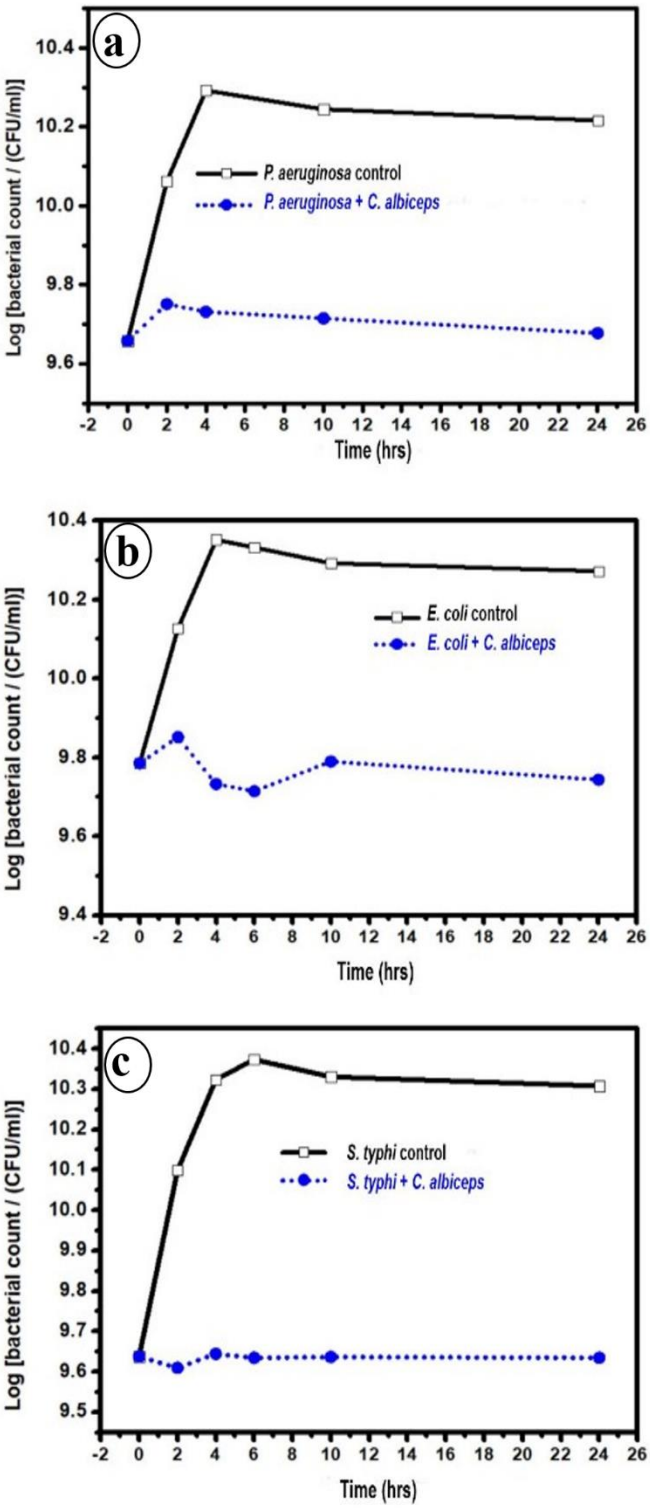


Figure 2. A typical 24-hrs growth curve of **(a):** *P. aeruginosa*, **(b):** *E. coli* and **(c):** *S. typhi* in control and with the addition of MIC-determined concentration of *C. albiceps* ES.

3.3. Determination of the antibacterial activity of different doses of ES using (CFU) assay:

Table 2 showed the antibacterial activity of two doses (100, 300 µL) of *C. albiceps* ES at a 200 mg/ml concentration**.** For gram-positive bacteria, both doses reduced the bacterial count

of MSSA and MRSA after 18 hrs. However, one-way ANOVA test revealed that both doses of ES were significantly potent only against MSSA compared to the control ($p < 0.001$). For gram-negative bacteria, both doses of ES significantly killed bacterial cells and thus retarded the colonial formation of *E. coli* and *S. typhi* ($p < 0.001$) after 18 hrs compared to the corresponding controls. Regarding *P. aeruginosa*, both doses displayed bactericidal action, where no bacterial cells were observed over 18 hrs. Nevertheless, there was no discernible difference between the antibacterial activity of ES at 100 and 300 µL against any of the studied bacterial species other than MSSA ($p < 0.001$).

Table 2: Antibacterial activity of two doses of *C. albiceps* larval extract using (CFU) assay:

Excretions /Secretions (ES)	Gram-positive bacteria			
	MSSA (CFU mm^{-1})		MRSA (CFU mm^{-1})	
	At 0 h	At 18 h	At 0 h	At 18 h
Positive control	$7.70x10^7$	$3.83 x10^8$	$4.55 x10^7$	$1.03 x10^8$
100 µL	$7.70x10^7$	$2.88 x10^8$	$4.55 x10^7$	$0.90 x 10^8$
300 µL	$7.70x10^7$	$1.41 x10^8$	$4.55 x10^7$	$0.88 x 10^8$
Excretions /Secretions (ES)	**Gram-negative bacteria**			
	P. aeruginosa (CFU mm^{-1})		*E. coli* (CFU mm^{-1})	
	At 0 h	At 18 h	At 0 h	At 18 h
Positive control	$4.05x10^7$	$1.52 x10^8$	$9.98 x10^7$	$3.14 x10^8$
100 µL	$4.05x10^7$	0.00	$9.98 x10^7$	$9.90 x10^7$
300 µL	$4.05x10^7$	0.00	$9.98 x10^7$	$7.68 x10^7$
Excretions /Secretions (ES)	*S. typhi* (CFU mm^{-1})			
	At 0 h	At 18 h		
Positive control	$4.05x10^7$	$1.95 x10^8$		
100 µL	$4.05x10^7$	$7.67 x10^7$		
300 µL	$4.05x10^7$	$8.1 x10^7$		

MSSA: methicillin-sensitive *S. aureus*, MRSA: methicillin-resistant *S. aureus*, *P. aeruginosa: Pseudomonas aeruginosa*, *E. coli: Escherichia coli*, *S. typhi*: *Salmonella typhi*, CFU: colony forming unit, hr: hour, mm: millimeter.

3.4. Determination of the antibacterial activity of ES using disc diffusion assay:

Agar disc diffusion assay was applied throughout the investigation of the antibacterial activity of *C. albiceps* ES against the selective pathogenic bacterial models. The results demonstrated that as little as 20 µL (200 mg/ml) maggot extract was able to inhibit the growth of both *P. aeruginosa* and *E. coli*, while only 50 µL showed the apparent potency of maggot ES against *S. typhi* with the resulting of 13.07 ± 0.18 mm inhibition zone (Figure 3 and Plate 3**).** The inhibition zones of larval ES against gram-negative bacteria were significantly smaller in diameter than the corresponding controls. However, the disc diffusion assay failed to show any antibacterial activity of maggot ES against gram-positive bacteria either with 20 µL or 50 µL of ES at 200 mg/ml concentration, where no zones were observed around the disc.

3.5. Evaluation of synergistic effect between the larval extract and antibiotics:

We evaluated in vitro synergism between *C. albiceps* extract and antimicrobial drugs (vancomycin, gentamycin, and ciprofloxacin) against selective bacterial strains using the disc diffusion method. As shown in Figure 4 and (Plates 4, 5) there was a remarkable synergism between the larval extract and antibiotics used against all studied bacterial species.

The best synergistic effect of vancomycin against gram-positive bacteria (MSSA, MRSA) appeared till a percentage of (60% VA + 40% ES) where no significant reduction was detected in the diameter of inhibition zones when compared to the corresponding controls ($p < 0.01$). However, the other remaining mixtures of vancomycin and ES exhibit a gradually decreasing in the diameter of MRSA and MSSA inhibitory zone compared with antibiotic alone.

Concerning gram-negative bacteria, larval ES inhibits bacterial growth synergistically with both gentamycin and ciprofloxacin. The results of the combination revealed that ES and gentamycin were more effective toward *E. coli* than *P. aeruginosa*, where there was a significant difference in the diameters of the inhibition zone starting from (20% CN +80% ES) for *E. coli* and from (40% CN + 60% ES) for *P. aeruginosa*. The combination of ciprofloxacin and ES at a percentage of (20% CIP + 80% ES) only showed a significant decrease in the diameter of the inhibition zone compared to the antibiotic alone ($p < 0.05$). However, all other mixtures between ciprofloxacin and ES did not exhibit any reduction in the inhibition zone diameter.

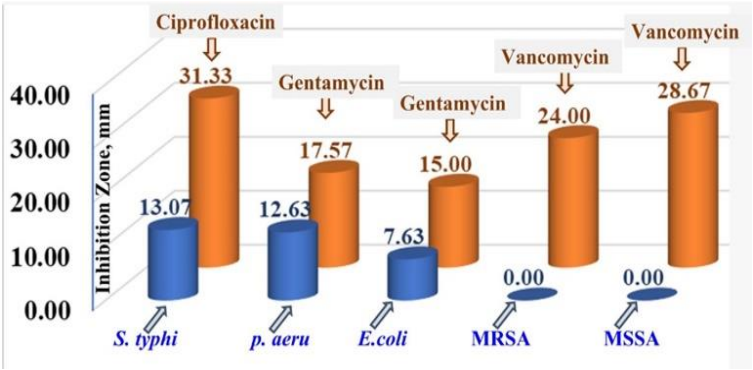


Figure 3. Effect of whole-body extract of *C. albiceps* and different antibiotics used against tested bacterial species represented by inhibition zone (mm).
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4. Discussion:

The worrying rise in the prevalence of bacteria that are resistant to antibiotics. Thus, the challenges associated with treating infections have initiated research into new antibacterial substances and the development of novel alternative strategies for treating bacterial illness.

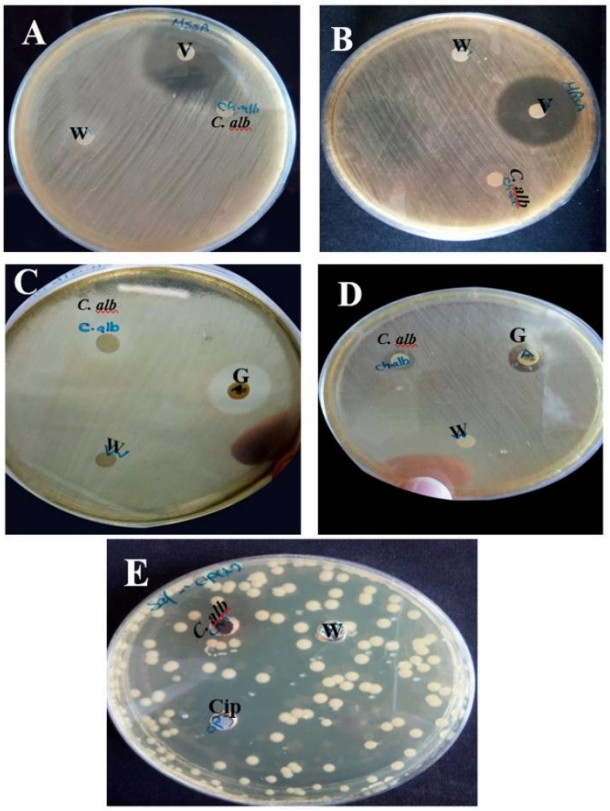

Plate 3. Inhibition zones of the five pathogenic bacterial strains in response to *C. albiceps* ES. **A**: MSSA, **B**: MRSA, **C**: *P. aeruginosa*, **D**: *E. coli* and **E**: *S. typhi C. alb: C. albiceps* larval extract, V: vancomycin, G: gentamycin, Cip: ciprofloxacin and W: distilled water.

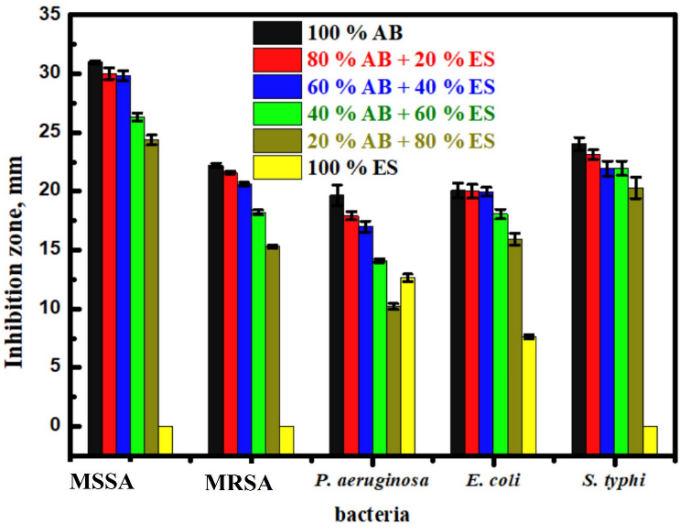

Figure 4. Synergistic activity between *C. albiceps* ES and different antibiotics used against bacterial species tested.

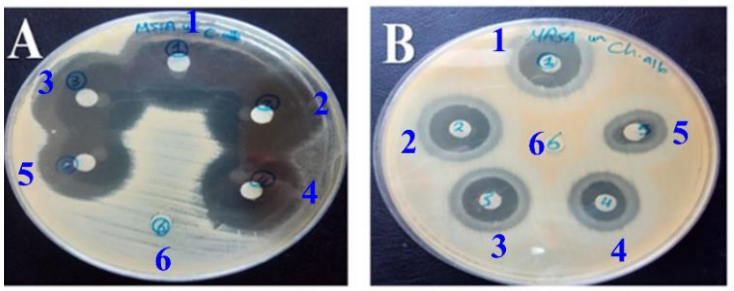

Plate 4. Effect of vancomycin alone and in combination with *C. albiceps* larval extract on growth of **(A)** = MSSA, (**B)** = MRSA. 1=100% AB, 2 = 80% AB+20% ES, 3 = 60% AB+40% ES, 4 = 40% AB+60% ES, 5 = 20% AB+80% ES; 6 = 100% ES.

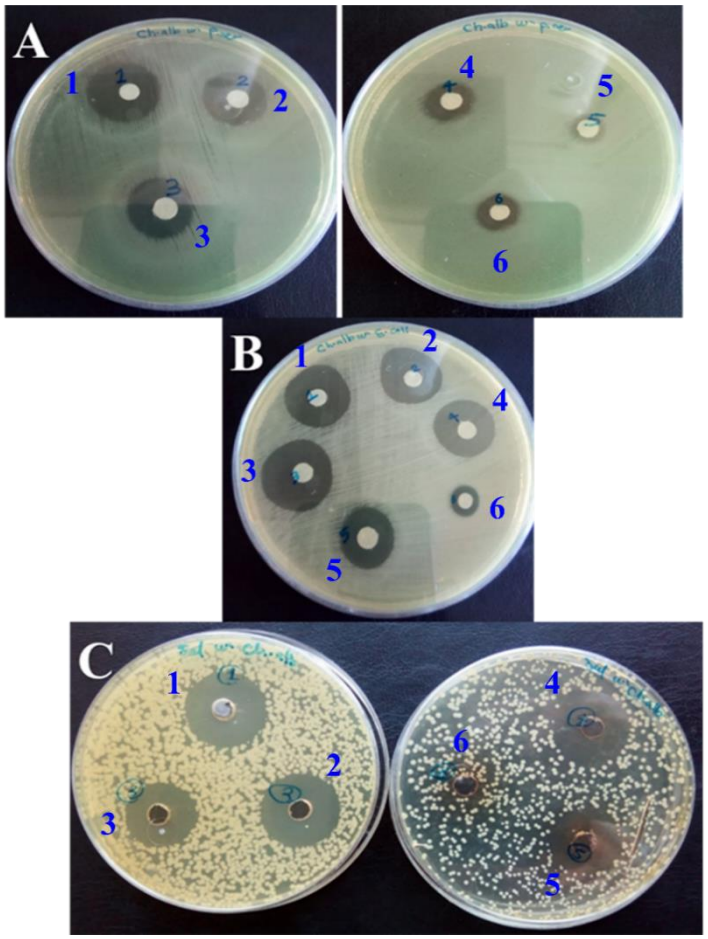

Plate 5. Effect of gentamycin alone and in combination with *C. albiceps* larval extract on growth of (**A**): *P. aeruginosa*, (**B**): *E. coli*. Effect of ciprofloxacin alone and in combination with *C. albiceps* larval extract on growth of (**C):** *S. typhi*, 1=100% AB, 2 = 80% AB+20% ES, 3 = 60% AB+40% ES, 4 = 40%AB+60%ES, 5 = 20% AB+80% ES; 6 = 100% ES.

Insect peptides have been known to successfully eradicate various bacterial infections, such as helicobacter infections, impetigo, and diabetic foot ulcers [39]. It is well known that the larvae of the calliphoridae family live in carrion and come into contact with abundant pathogens, suggesting that larvae may be able to synthesize antimicrobial peptides and other substances

possessing activity against bacteria and have the potential for the development of natural medicine [40, 41]. The current study examines the impact of *C. albiceps* larval ES on suppressing some gram-negative and gram-positive bacterial growth. Although *C. albiceps* ES was obtained from non-infected larvae that were reared on raw beef, the results of the present study showed that *C. albiceps* larvae ES has an inhibitory impact against all of the examined microorganisms in vitro. This confirms that many of insect antimicrobial peptides have been constitutively produced from secretory cells [42] and do not require bacterial induction. No detectable differences in larval ES antibacterial activity between infected and non-infected larvae were reported [30], indicative of constitutive activity.

Based on our findings in the resazurin assay, MIC of *C. albiceps* ES was higher (100 mg/ml) against gram-positive bacteria (MSSA, MRSA) than those found against gram-negative bacteria *P. aeruginosa* (25 mg/ml) and (50 mg/ml) for both *E. coli* and *S. typhi*. This suggests that the larval ES was more effective against gram-negative than gram-positive bacteria. The results also indicate that a finite concentration of ES is required to exert antibacterial activity, at least in vitro, which varied according to the bacterial species.

These findings are in partial agreement with those obtained in a previous study by [33] who used the same assay and reported that the extract of *Lucilia cuprina* was an inhibitor to the growth of both gram-positive (MSSA and MRSA) 100 mg/ml while our results very low than [33] for gram-negative (*P. aeruginosa* and *E. coli*) with MIC whilst, the same authors noted that the larval extracts of *Sarcophaga peregrina* and *Musca domestica* were only effective against MRSA at MIC of 100 mg/ml. However, Teh et al. [43] demonstrated that the methanolic extract of *L. cuprina* Larvae could inhibit more than 50 % of *P. aeruginosa* and *E. coli* with MIC of 0.78 and 1.56 mg/ml, respectively, using conventional turbidimetric assay. To the best of our knowledge, resazurin assay is a more reliable antibacterial assay than turbidimetric assay because it is simple, more sensitive, and does not need a spectrophotometer to detect bacterial growth [33]. The wide spectrum inhibitory effect of larval extract of other flies was reported previously using the turbidimetric assay. Park et al. [44] detected MIC of black soldiers *Hermetra illucens* ES against MRSA (25 mg/ml) and (12.5 mg/ml) against both *E. coli* and *P. aeruginosa*. As reported in [45] study, *Sarcophaga argyrostoma* ES was more effective against gram-negative *P. aeruginosa* and *E. coli* (MIC = 0.125 mg/ml), while higher concentrations are required to inhibit the growth of gram-positive *S. aureus* (0.25 mg/ml) and *Bacillus subtilis* (0.5 mg/ml).

The results of CFU assay revealed that at the concentrations tested (MIC detected by resazurin assay), ES of *C. albiceps* could not only inhibit the growth of all tested bacterial strains but also slightly reduce the initial number of bacteria for at least part of a 24 hrs incubation. The viable count data for *C. albiceps* ES indicated that the highest reduction (82 %) was observed for MSSA, while the lowest (56.8 %) for MRSA was compared to the corresponding controls at the end of the experiment. The larval ES always maintains an antibacterial activity over 24 hrs experimental period. The results suggest that the degree of potency of *C. albiceps* ES varied according to the bacterial species. It can be concluded that the control of the growth of all tested bacterial strains for up 24 hrs can be achieved by using MICs of *C. albiceps* ES. Some of our results coincide with previous studies, Barnes et al. [28] reported that both *L. sericata* and *Calliphora vicina* ES could reduce *E. coli* count by 84% and 63%, respectively over 24 hrs. They also recorded 55% of *P. aeruginosa* count reduction by *L. sericata* ES. Arora et al. [29] observed 30% of MSSA growth inhibition by *L. cuprina* larval ES over 24 hrs. Previously published studies recorded the short-lived action of bacterial growth inhibition by larval extracts. Barnes et al. [28] noticed that *C. vicina* ES reduced *P. aeruginosa* growth up to 8 hrs and allowed regrowth to occur between eight and 24 hrs. Masiero et al. [46] examined the ES of *Cochliomyia macellaria* and observed a reduction of *S. aureus* and *P. aeruginosa* only during the first 2 and 4 hrs, respectively. Recently, Dallavecchia et al. [47] reported that *S. aureus* was reduced by ES of *C. vicina* for up to 4 hrs only. It's important to note that total bacterial killing could not be observed throughout the present experiment by using the detected MIC of *C. albiceps* ES against bacterial strains. In contrast to our results, Jaklič et al. [48] demonstrated that *L. sericata* ES reduced the population of MSSA by 100 %. The bactericidal effectiveness of both *L. senicata* and *Calliphora vicina* against *Bacillus cereus* was reported by [28]. Additionally, Ratcliff et al. [40] stated that ES from the three calliphords (*C. megacephala*, *C. albiceps*, and *C. putoria*) were able to inhibit completely *E. coli* growth and also there was no significant difference between the three species ES and penicillin control at any time during the experiment. It can be concluded that the antibacterial efficiency of the larval extract depends on many factors, including characteristics of target microorganism (the genus, species, and strain), characteristics of larval material (bioactive compounds as well as the method of extraction) and chemical composition such as concentration [48]. The inhibitory activity of *C. albiceps* larval ES against different bacterial strains may be due to the presence of the defensins group. The defensins are less than 5 KDa in molecular weight and large amount of isolated insect peptides belonged to this group [30, 38, 49].

These peptides provide insects with the ability to combat many pathogenic bacteria [29]. In the present study, we note that ES of *C. albiceps* has good bactericidal activity against *P. aeruginosa* within 18 hrs by employing doses of ES both 100 and 300 µL (200 mg/ml). The bactericidal effect of *C. albiceps* ES against *P. aeruginosa* in our assay has been supported the results of [33], who reported that extract of *L. cuprina* larvae showed a significant bactericidal effect on all tested bacteria (*P. aeruginosa*, *E. coli*, *S. aureus*, and MRSA) at a concentration of 100 mg/ml. On the other hand, larval ES of *C. albiceps* exerted bacteriostatic effect against MSSA, MRSA, *E. coli*, and *S. typhi* with both doses (100 and 300 µL) while 300 µL was more effective in bacterial count reduction than 100 µL after treatment for 18 hrs. In contrast to our results, Chaiwong et al. [50] demonstrated that the same doses from *C. megacephala* extract only had an inhibitory effect on *E. coli*. However, both *S. aureus* and *P. aeruginosa* showed significantly increased viable cell count when treated with these doses. It can be concluded that the bactericidal activity of *C. albiceps* ES against *P. aeruginosa* can be achieved only at a high concentration of the whole crude extract. This finding was confirmed by previous data of [51],

who reported that ES of *L. sericata* showed its bactericidal activity only at high concentrations of whole, non-purified exosecretion.

Our results from the experiment with bacteria in disc diffusion agar assay revealed that ES of *C. albiceps* inhibit *P. aeruginosa* and *E. coli* in a dose-dependent pattern with as little as 20 µL (200 mg/ml) being effective. However, only 50 µL showed apparent activity towards *S. typhi*. No clearent zones were observed by applying the same volumes of ES against gram-positive bacteria, despite the antibacterial activity of MICs of *C. albiceps* ES against all tested bacteria, either gram-positive or gram-negative, when liquid culture assay was performed. This suggests that the antibacterial activity of ES towards the same strain of bacteria varied under a range of media conditions (liquid or solid). Kerridge et al. and Jaklic et al. [**30, 48**] demonstrated that larval extract of *L. sericata* showed activity against some bacterial liquid culture previously undetected by solid media agar diffusion assay, which means lower sensitivity of agar diffusion compared with liquid culture assay. Therefore, the agar diffusion method required very high concentrations of *L. sericata* ES to exhibit its activity [**30, 52**]. In this regard, Auza et al. [**53**] reported that the antibacterial activity of black soldier flies *(H. illuceus)* extract increased with the increased level of extract concentration against *E. coli*, *S. typhimurium*, and *P. aeruginosa* using disc diffusion method. Thus, it can be concluded that conditions of media used during performing assays would have influenced the effectiveness of antibacterial activity exhibited by larval ES. Therefore, it is very important to select the correct bioassay in order to detect the antibacterial activity of Larval ES against a range of pathogenic bacteria [**54**].

In the present study, the combination between antibiotics and larval ES was tested by the disc diffusion method, and synergistic effects were observed on the basis of the diameter of the inhibition zone at sub-MIC of antibiotics. The results revealed that no growth inhibition towards gram-positive (MSSA, MRSA) was observed with 25 µL ES (at 200 mg/ml). However, the growth suppression of these bacterial strains was recorded by combining the larval ES with all different fractions of vancomycin. While the addition of ES to vancomycin at 40% MIC showed a significant decrease in the diameter of the inhibition zone compared to vancomycin with a concentration of 100% against MSSA and MRSA. It can be concluded that the addition of larval ES helped enhance or potentiate vancomycin activity at sub-MIC levels. Previous studies indicated that using larval extracts of *L. sericata* combined with antibiotics promotes a significant reduction of the minimum inhibitory concentrations of antibiotics for bacterial strains [**55**]. Furthermore, an association of ES with gentamycin at 60% MIC exhibited a stronger synergistic effect against *P. aeruginosa* and *E. coli* where there was no significant difference in the diameter of the inhibition zone compared with the gentamycin alone. On the other hand, a positive interaction is created when ES and gentamycin at 40% and 20% are combined, which results in an inhibitory effect greater than the individual effects of ES against *P. aeruginosa* and *E. coli*, respectively.

The combination of *C. albiceps* with ciprofloxacin enhanced antibiotic activity till 40 % MIC and showed high efficacy with no significant difference in the diameter of the inhibition zone compared to antibiotics with a concentration of 100 %. A similar synergistic effect between ciprofloxacin at 60, 80 % MICs and *L. sericata* ES against *S. aureus* was reported by [**46**].

It is interesting to report that synergism between different bacterial species was observed between the extract of *C. albiceps* and different antibiotics used in the experiment, suggesting that there is no specificity of different used antibiotics, which indicates that the crude extract of *C. albiceps* larvae might include a mixture of compounds that potentiate the effectiveness of different antibiotics. It can be concluded that larval extract in combination with antibiotics, increases their activity and decrease the doses of antibiotics and, subsequently, their side effect [**55**]. The ability of maggot extracts to act synergistically with antibiotics is considered a new method that helps in solving the problem of bacterial resistance.

It's important to note that the collected ES of *C. albiceps* have a dark brown color. This suggests that melanization process may occur and lead to the biosynthesis of melanin (phenolic biopolymer), which is involved in immunity and cuticular hardening in insects [**56, 57, 58**]. The latter authors reported that the reactive oxygen molecules O_2^-, H_2O_2, and OH^-**.** can be created during the melanization cascade and have antibacterial activity.

5. Conclusion:

All the features of our findings provide important evidence that larval extract of *C. albiceps* has a range of antibacterial properties against both gram-positive and gram-negative bacterial strains tested. Also, our results indicated the bactericidal activity of *C. albiceps* larval extract against *P. aeruginosa*, while bacteriostatic activity was observed against the remaining bacterial models. *C. albiceps* larval ES has the potential to be a source of chemical compounds that can modify antibiotic resistance, as shown by the synergy detected between the extract and classical antibiotics. This synergy allows a lower dose of traditional antibiotics to be used while maintaining antibacterial activities, which may be helpful for delaying the emergence of resistance and that could be extremely beneficial in the battle against *P. aeruginosa* and the management of other nosocomial infections. Moreover, the combination of antibiotics with larval extract could be an important basis for developing a new approach in resistance modifying agents. This approach will allow us to drive the real potential of these combinations for being translated into clinical treatment.

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