CHAPTER IV

RESULTS AND DISCUSSION

4.1 PRODUCTION OF STERILE LARVAL EXTRACT FOR ANTIBACTERIAL ASSAYS

In the present study, homogenization of *L. cuprina* late second-instar larvae was performed manually using 15-ml glass Dounce homogenizer. This classical manual method of homogenization uses mechanical force applied by hand to disrupt tissue and cells gently and effectively. In a dounce system, the mortar (cylinder made of borosilicate glass) and pestle come bundled and are specially crafted for use with each other to ensure a tighter fit and improves homogenization efficiency. During homogenization, an adequate amount of methanol was added from time to time and the lamina flow of methanol through the annular space between the pestle and the mortar wall resulted in different fluid (methanol) speeds existing over the diameter of the cell and the resulting shear forces disrupted the cell and extracted the cellular content (Figure 4.1a) (Dennison 2003). Prior to homogenization, the Dounce homogenizer was autoclaved to avoid contamination.
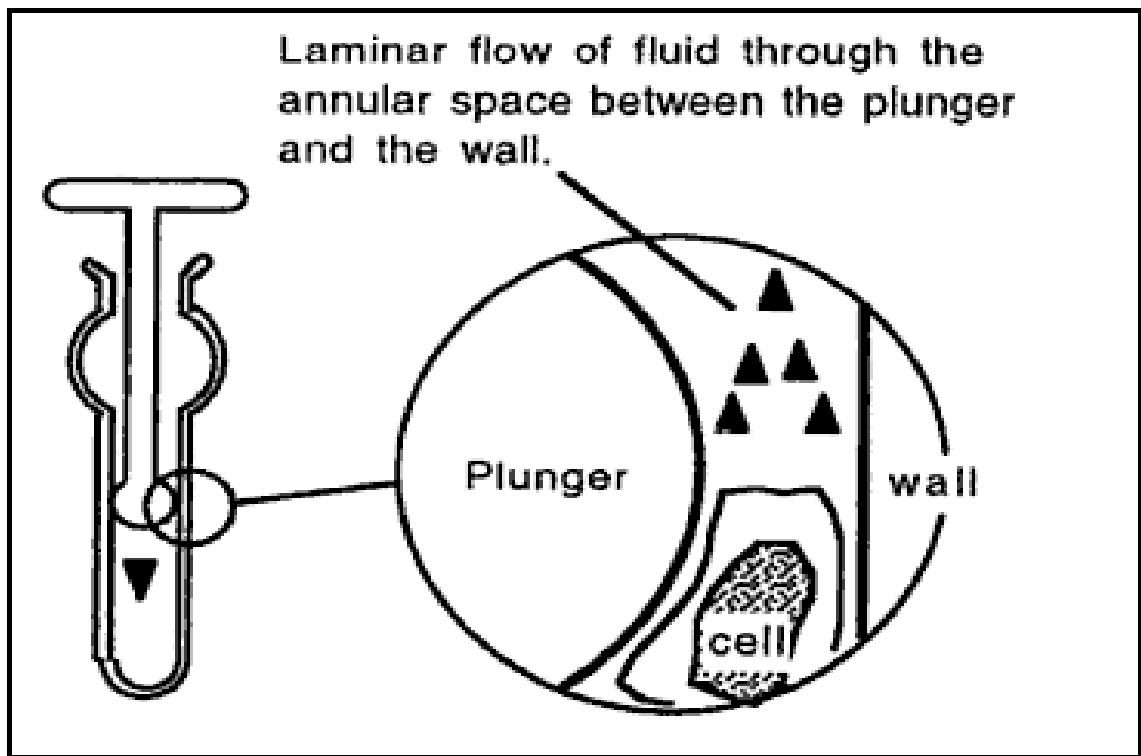
Figure 4.1a: Enlarged view of a Dounce homogenizer

Figure 4.1b illustrates the final product of methanol extraction of *L. cuprina* late second-instar larvae after methanol-homogenization, centrifugation and subsequent vacuum-concentration of the resulted larval supernatant. The final product, *L. curpina* larval extract and its suspension (Figure 4.1c) appears in bright yellow colour. However, Bexfield *et al.* (2004) reported that the collected *L. sericata* larval excretions/secretions (ES) was dark-brown in colour. This suggested that the active ingredient(s) retained in *L. cuprina* larval extract is/are different to that/those of *L. sericata* larval ES. In other words, *L. cuprina* larval extract might possess different antibacterial constituents against bacteria as compared to *L. sericata* larval ES.

Collection of excretions/secretions (ES) from live, aseptically reared, late second-instar larvae is time-consuming and therefore costly to perform as series of aseptic measures would need to be taken to maintain the sterility of larvae and larval ES. Besides, it is difficult to assure and keep the larvae constantly and actively excreting in order to attain a high yield of ES. Thomas *et al.* (1999) have reported that the volume of secretions from sterile larvae of *L.* sericata was limited. Hence, the present study adopted methanol extraction method to produce whole-body extract from late second-instar larvae of *L. cuprina* for antibacterial assays instead.

Another rationale of producing whole-body extract instead of ES is that the whole-body extract may contain the entire bio-products of larvae, which included feces, cutaneous and oral secretions. There was evidence to substantiate that the active antibacterial agent(s) was/were contained in the feces as Robinson and Norwood (1933) reported that the content of the hind intestine of *L. sericata* larvae which had fed within
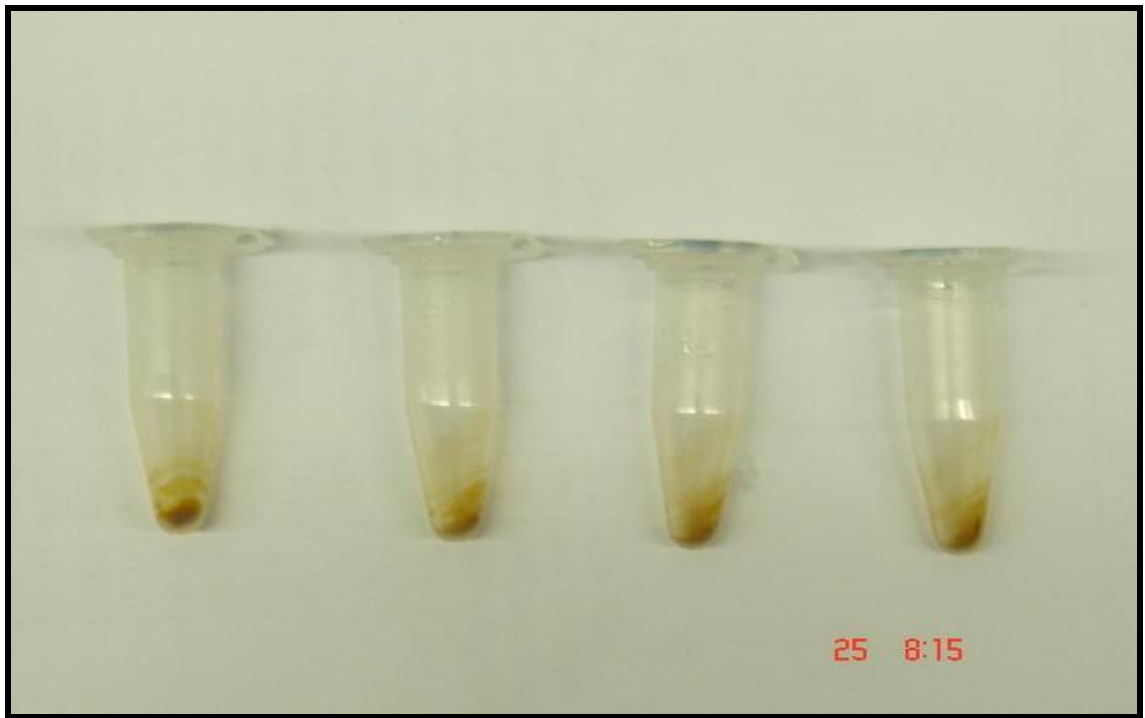
Figure 4.1b: Vacuum-concentrated larval extract of *L. cuprina*

Figure 4.1c: Suspension of *L. cuprina* larval extract (200 mg/ml) for antibacterial assays

osteomyelitis wounds was sterile, while those of the crop and stomach were heavily contaminated.

In the present study, non sterile, late second-instar *L. cuprina* larvae that had been reared on raw, decaying cow liver were employed in the production of larval extract, in order to simulate as closely as possible conditions encountered in maggot therapy (Simmons 1935). Nevertheless, the larval extract was cultured for sterility prior to use. Only larval extract that had been proven sterile and free from bacterial contaminants was used for antibacterial assays. Findings from other studies (Simmons 1935; Huberman *et al.* 2007) have demonstrated that the larval ES or extract of *L. sericata* would be collected under septic conditions and then sterilized without apparent loss of potency against tested bacteria. Moreover, excretion from sterile larvae was also considered less typical of that produced by larvae worked in bacteria-infected wounds (Simmons, 1935).

4.2   ANTIBACTERIAL ASSAYS

4.2.1   Turbidometric Assay

Turbidometric assay (TB) or spectrophotometric assay was adopted to demonstrate the inhibition effects of *L. cuprina* larval extract on the growth of the seven selected wound pathogenic bacteria. Bacterial growth can be defined as the orderly increase in the quantity of all cellular components and in the number of cells. Due to the limited increase in cell size and rapid cell division, bacterial growth is often measured by increase in cell number.

In turbidometry, an increment in turbidity or optical density (OD) generally indicates an increase in the number of bacteria present, although under certain circumstances a small increase in OD may be due to an increase in bacterial size caused by swelling prior to lysis (Thomas *et al.* 1999). Nonetheless, this assay can be automated and provides a rapid, convenient method for monitoring changes in cell number in small volume of bacterial suspension. This simple and rapid assay allows extensive kinetic studies even in the presence of low larval extract concentrations and volumes and is capable of detecting inhibitory level below those recorded for well or disc diffusion assay (Patton *et al.* 2006).

In this study, both bacterial growth in the controls (without larval extract) and test samples (with larval extract) were expressed in the ratio of OD before incubation to OD after incubation. A bigger OD ratio implies higher growth rate. Results from the TB assay (Figure 4.2.1a) showed that there was a conspicuous difference between the OD ratio of test samples and controls.

Further statistical analysis by independent Student's *t*-test (Table 4.2.1a) had demonstrated that the *in vitro* growth of *S. aureus*, MRSA, *S. epidermidis*, *S. pyogenes*, *K. pneumoniae*, *P. aeruginosa* and *E. coli* in the test samples (overnight incubation with larval extract) were significantly lower as compared to the normal bacterial growth in the controls. These results indicated that *L. cuprina* larval extract possessed significant (p<0.001, n=30) antibacterial activity against all bacteria tested, which are the potential Gram-positive and Gram-negative wound pathogen.

The results obtained are partially in agreement with the previously published studies, in which the investigators concluded that *L. sericata* larval ES exhibited
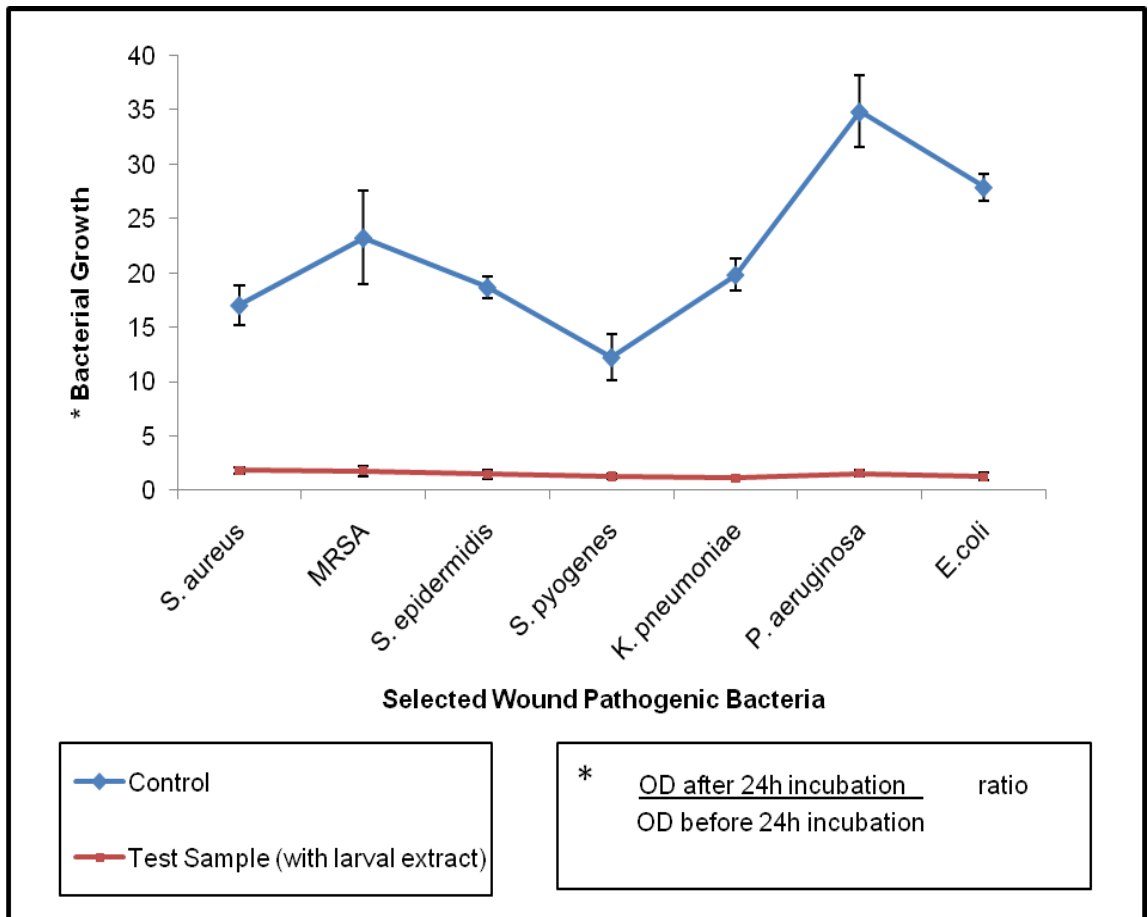
Figure 4.2.1a: Effect of *Lucilia cuprina* larval extract on bacterial growth

Table 4.2.1a: Comparison of mean OD ratio of controls and test samples at 630 nm for seven bacteria tested

Bacterial Species	TB Assay	Mean ±SD	t-statistics [a] (df)	p value
S. aureus	Control	17.02 ±1.88	25.26 (18)	*<0.001
	Test Sample	1.82 ± 0.26		
MRSA	Control	23.23 ±4.32	15.58 (18)	*<0.001
	Test Sample	1.80 ±0.47		
S. epidermidis	Control	18.67 ±0.99	50.95 (18)	*<0.001
	Test Sample	1.48 ± 0.40		
S. pyogenes	Control	12.23 ±2.09	16.46 (18)	*<0.001
	Test Sample	1.28 ± 0.21		
K. pneumoniae	Control	19.82 ±1.51	39.19 (18)	*<0.001
	Test Sample	1.11 ± 0.09		
P. aeruginosa	Control	34.84 ±3.29	31.87 (18)	*<0.001
	Test Sample	1.54 ± 0.30		
E. coli	Control	27.89 ±1.22	66.57 (18)	*<0.001
	Test Sample	1.25 ± 0.33		

[a] Two-tailed unpaired Student's t-test
*Significant value at p=0.05

significant inhibitory effects against a range of Gram-positive and Gram-negative bacteria, which were *S. aureus,* a clinical isolate of MRSA, *E. coli* and *P. aeruginosa* (Bexfield *et al.* 2004). However, the growth of *S. epidermidis* was not inhibited by the larval ES from *L. sericata* even with an increased dose of larval ES or a reduced bacterial inoculum (Bexfield *et al.* 2008) using the TB assay. This suggested that *L. cuprina* larval extract has a broader spectrum of antibacterial activity compared to *L. sericata* larval ES.

However, a study published recently by Arora *et al.* (2010) reported opposing results though they employed the same blowfly species *L. cuprina* larvae for antibacterial bioassays. In their study, one of the methods that they used to extract the metabolites from late second-instar larvae was by incubating 500 larvae in a 200 ml-conical flask with 2 ml phosphate-buffered saline (PBS) for 24 hours. The resultant liquid in the flask was pipetted out, centrifuged at 10, 000 x g for five minutes and sterilized using 0.2 μm syringe filter. Nonetheless, they failed to demonstrate the antibacterial activity of *L. cuprina* larval ES against methicillin-susceptible *S. aureus* (MSSA) and *E. coli* but have instead observed bacterial growth promotion after ES addition compared to the controls.

Nevertheless, with some modifications in the above method, Arora *et al.* (2010) incubated 100 larvae in 200 μl of PBS in dark for one hour, then collected and processed the final larval ES as mentioned above. In contrast to the results obtained previously, they were able to detect 30% of bacterial growth inhibition from the initial bacterial inoculum for MSSA. However, the ES had no significant inhibition against *E. coli.*

The contrasting results between the present study and Arora *et al*. (2010) though employing the same blowfly species could be due to heavier bacterial inocula (2.0 x 10^7 cells/ml for *S. aureus* and 1.5 x 10^6 for *E. coli*) and different method of extraction for larval metabolites employed by Arora *et al*. In addition, they also explained that the antibacterial factor(s) in the collected ES may be present in much diluted form to exert significant antibacterial activity since they did not concentrate the collected ES via lyophilization or other dehydration methods.

Additionally, statistical analyses by independent Student's *t*-test revealed that the mean OD ratios for controls and test samples were significantly different between the selected Gram-positive and Gram-negative bacteria. In the present study, the mean OD ratios for the Gram-positive bacteria in the controls were 35.36% lower than those of the Gram-negative bacteria (Table 4.2.1b). These indicated that the Gram-negative bacteria grew better than the Gram-positive bacteria in the same growth medium (BHI broth). Nonetheless, the inhibitory effect of the larval extract was significantly more pronounced on the Gram-negative bacteria as the OD or growth ratios of the Gram-negative bacteria in the test samples were 23.08% lower than those of the Gram-positive bacteria (Table 4.2.1c).

On the other hand, Figure 4.2.1b demonstrated the potency of larval extract in inhibiting bacterial growth. The potency or effectiveness of larval extract was indicated by the percentage of decrement in the OD ratio of test samples in comparisons with the OD ratio of the corresponding controls. The results showed that the larval extract exhibited high potency against all tested bacteria, with an average inhibitory potency of 92.51 $\pm$ 2.75% (Table 4.2.1d). However, statistical analysis by one-way ANOVA (Table 4.2.1d) substantiated that the larval extract was significantly potent towards at

Table 4.2.1b: Mean OD ratios for controls between the Gram-positive and Gram-negative bacteria

Bacterial Species	Mean ±SD	t-statistics [a] (df)	p value
Gram-positive	17.79 ±4.72	-7.20 (68)	*<0.001
- *S. aureus*			
- MRSA			
- *S. epidermidis*			
- *S. pyogenes*			
Gram-negative	27.52 ±6.59		
K. pneumoniae			
P. aeruginosa			
E. coli			

[a] Two-tailed unpaired Student's *t*-test
*Significant value at p=0.05

Table 4.2.1c: Mean OD ratios for test samples between the Gram-positive and Gram-negative bacteria

Bacterial Species	Mean $\pm$ SD	t-statistics [a] (df)	p value
Gram-positive	1.60 $\pm$ 0.41	3.31 (68)	*<0.001
- *S. aureus*			
- MRSA			
- *S. epidermidis*			
- *S. pyogenes*			
Gram-negative	1.30 $\pm$ 0.31		
K. pneumoniae			
P. aeruginosa			
E. coli			

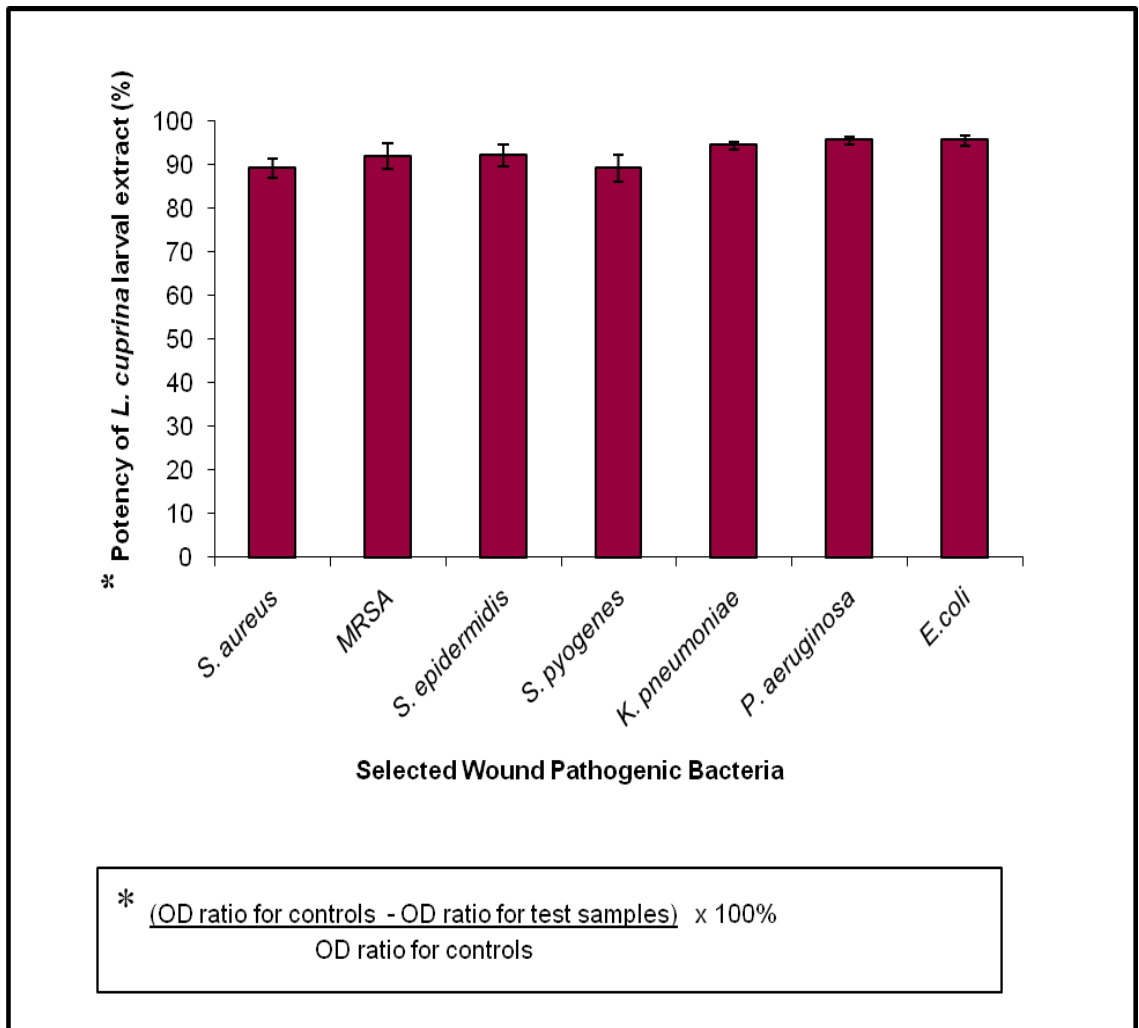[a] Two-tailed unpaired Student's t-test
*Significant value at p=0.05

Figure 4.2.1b: Potency of *Lucilia cuprina* larval extract against the seven bacteria tested

Table 4.2.1d: Mean potency of *L. cuprina* larval extract against bacteria

Bacterial Species	Potency of Larval Extract (%) (Mean ±SD)	F-statistics [a] (df)	*p* value
S. aureus	89.13 ±2.29	16.80 (6, 63)	*<0.001
MRSA	91.92 ±2.87		
S. epidermidis	91.99 ±2.49		
S. pyogenes	89.13 ±2.99		
K. pneumoniae	94.36 ±0.87		
P. aeruginosa	95.55 ±0.87		
E. coli	95.53 ±1.15		
Average Potency	92.51 ± 2.75		

[a] One-way ANOVA test
*Significant value at p=0.05

least one of the bacteria tested (ANOVA, p<0.001). Subsequent post-hoc multiple comparison test revealed that the Gram-negative bacteria *P. aeruginosa*, *E. coli* and *K. pneumoniae* were significantly sensitive to the antibacterial effects of larval extract as compared to the Gram-positive bacteria (Tukey's HSD Post-hoc Test, p<0.05). The *in vitro* growth of *P. aeruginosa*, *E. coli* and *K. pneumonia* significantly decreased by 95.55± 0.87%, 95.53 ± 1.15% and 94.36 ± 0.87%, respectively in comparisons to the corresponding controls.

Nevertheless, in the study conducted by Thomas *et al.* (1999), ES produced by sterile *L. sericata* larvae through repeated washing with sterile water or buffer exhibited significant inhibitory effect on the growth of *S. aureus*, but partial growth inhibition of MRSA and *P. aeruginosa*, and even enhanced the growth of *E. coli*. The possible explanations for the discrepancies between their results and the present study could be the different experimental conditions, including the use of different species of larvae (*L. sericata*) and larval product (*L. sericata* larval ES), and a shorter incubation period (five-hour).

On the other hand, in contrast to the results obtained by Thomas *et al.* (1999), the significant inhibitory effect on *P. aeruginosa* in the present study was supported by Huberman *et al.* (2007). They isolated and identified three molecules with antibacterial activity from the haemolymph extracts of non-sterile *L. sericata* larvae. The three isolated molecules, *p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid and proline diketopiperazine revealed antibacterial activity against *P. aeruginosa*, and the inhibitory effect was even more significant when these molecules were tested in combination. These findings suggested that the significant bacterial growth inhibition of *L. cuprina* larval extract against pathogen might not be only contributed by one antibacterial agent

alone, but could be pronounced by the synergistic effect of the corresponding antibacterial agents in the larval extract.

4.2.2   Colony-Forming Unit Assay

Colony-forming unit (CFU) assay is used to enumerate the number of viable bacteria cells in a sample containing bacteria. In conjunction with the TB assay, it is always used to elucidate the antibacterial properties (bactericidal or bacteriostatic) of an antimicrobial agent. The underlying theory for CFU assay is that a single bacterium will divide or multiply via binary fission to produce a single, macroscopic colony visible to the naked eye on agar plate. Therefore by counting the number of colonies that developed, colony-forming units (CFUs), and by taking into account the dilution factors, the concentration of bacteria in the original sample can be determined. Plates producing 30 to 300 colonies are considered within the countable range. Plates with CFUs fewer than 30 are not acceptable statistically, whilst more than 300 colonies on a plate are likely to produce colonies too close to each other to be distinguished as individual CFU (Black 2004).

In CFU assay, the potency of *L. cuprina* larval extract was determined by comparing the CFU/ml produced on the test sample plates (bacterial inocula incubated overnight with 100 mg/ml larval extract) with the control plates (overnight incubation without larval extract). Statistical analyses by independent Student's *t*-test (Table 4.2.2a) affirmed that *L. cuprina* larval extract significantly killed and hence impeded the formation of *S. epidermidis*, *K. pneumoniae*, *P. aeruginosa* and *E.coli* ($p < 0.001$, n=10) colonies on the BHIA plates as compared to the colonies formed on the BHIA control plates.

Table 4.2.2a: Comparison of mean CFU/ml of control and test sample plates for seven bacteria tested

Bacterial Species	CFU Assay	Mean ±SD (x10^9 CFU/ml)	t-statistics [a] (df)	p value
S. aureus	Control	1.07 ±0.05	0.72 (18)	0.481
	Test Sample	1.05 ±0.07		
MRSA	Control	1.10 ±0.05	-0.84 (18)	0.412
	Test Sample	1.12 ±0.02		
S. epidermidis	Control	0.58 ±0.05	8.21 (18)	*<0.001
	Test Sample	0.35 ±0.07		
S. pyogenes	Control	2.14 ±0.03	1.76 (18)	0.096
	Test Sample	1.98 ±0.29		
K. pneumoniae	Control	0.63 ±0.04	4.55 (18)	*<0.001
	Test Sample	0.53 ±0.05		
P. aeruginosa	Control	0.9 ±0.06	37.44 (18)	*<0.001
	Test Sample	0.07 ±0.02		
E. coli	Control	1.49 ±0.13	13.93 (18)	*<0.001
	Test Sample	0.64 ±0.14		

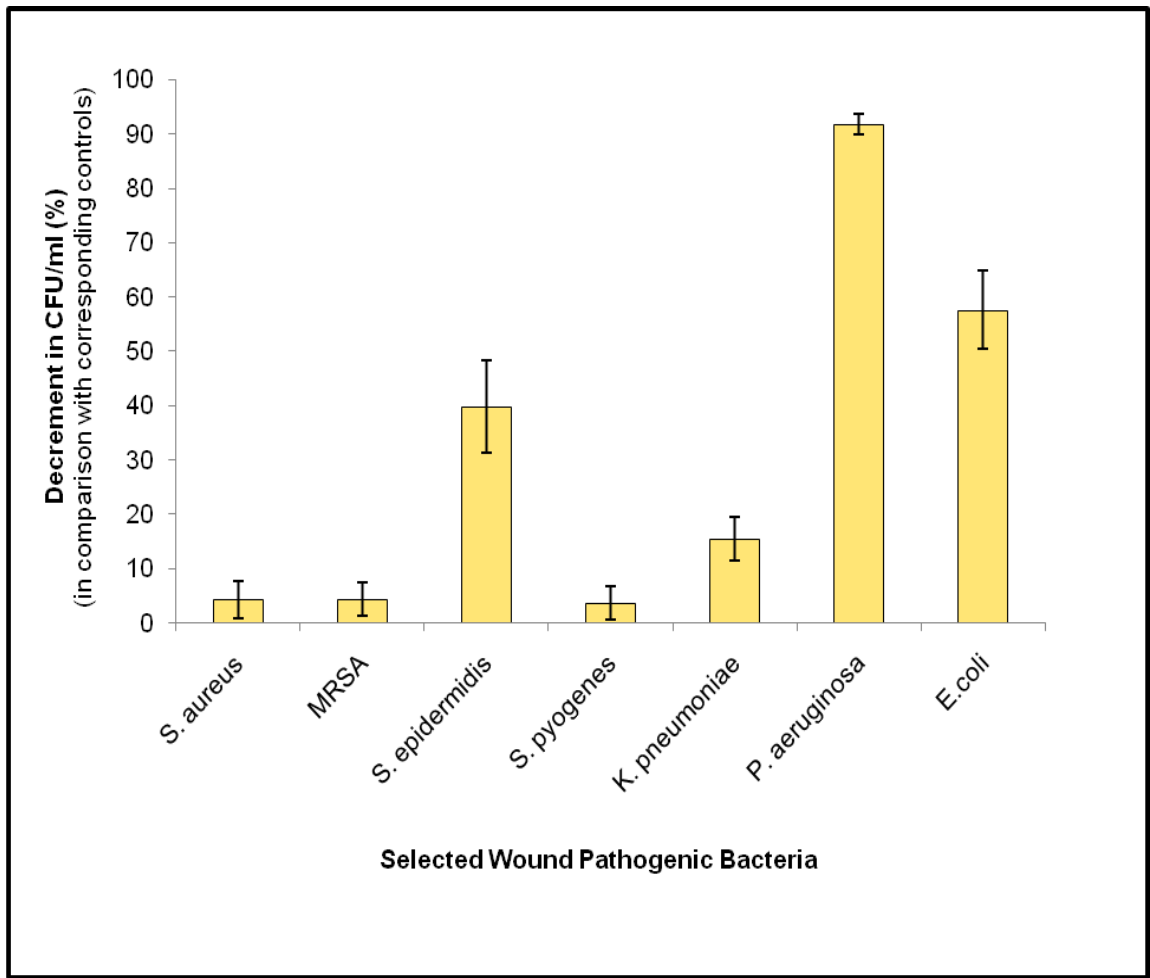[a] Two-tailed unpaired Student's *t*-test
*Significant value at p=0.05

Figure 4.2.2a: Effect of *Lucilia cuprina* larval extract on bacterial viability using CFU assay

On the other hand, Figure 4.2.2a illustrated the viability of tested bacteria in the presence of larval extract. The decrement of CFU/ml in the test sample plates was expressed as a percentage of control CFU/ml. The higher the decrement in the CFU/ml of the test sample plates as compared to the controls, the greater is the number of cells being killed, and hence the stronger is the bactericidal or killing effect of larval extract. The results revealed that there were only $4.25 \pm 3.44\%$, $4.31 + 3.12\%$ and $3.65 \pm 3.09\%$ decrement of CFU/ml in the test sample plates of *S. aureus*, MRSA and *S. pyogenes* respectively as compared to the corresponding control plates. These implied that as high as $95.75 \pm 3.44\%$ of *S. aureus*, $95.69 \pm 3.12\%$ of MRSA and $96.35 \pm 3.09\%$ of *S. pyogenes* cells were still viable after overnight incubation in 100 mg/ml *L. cuprina* larval extract as compared to the control plates (Table 4.2.2b).

The recovery of these bacteria in the solid BHI media (CFU assay) after inhibited by the larval extract in broth media (TB assay) indicated that the larval extract did not kill the bacteria but instead, restrained bacterial reproduction and this suggested a bacteriostatic or growth-inhibiting effect on the corresponding bacteria. When the aliquots were transferred from the broth medium (with larval extract) to the agar plates, the growth-stunted but viable bacterial cells resumed their growth and formed macroscopic colonies on the plates as the quantity of larval extract carried over would not be in adequate concentrations and volumes to exert the bacteriostatic effect.

On the other hand, CFU assay (Figure 4.2.2a) also revealed that $39.76 \pm 8.55\%$ of *S. epidermidis*, $15.40 \pm 3.98\%$ of *K. pneumoniae*, $91.72 \pm 1.82\%$ of *P. aeruginosa* (Figure 4.2.2b) and 57.55 ± 7.16 of *E. coli* (Figure 4.2.2c) cells were killed as a result of the bactericidal or killing effect of *L. cuprina* larval extract. Amongst these statistically

Table 4.2.2b: Percentage of viable bacterial cells in the test sample plates after overnight incubation with *L. cuprina* larval extract

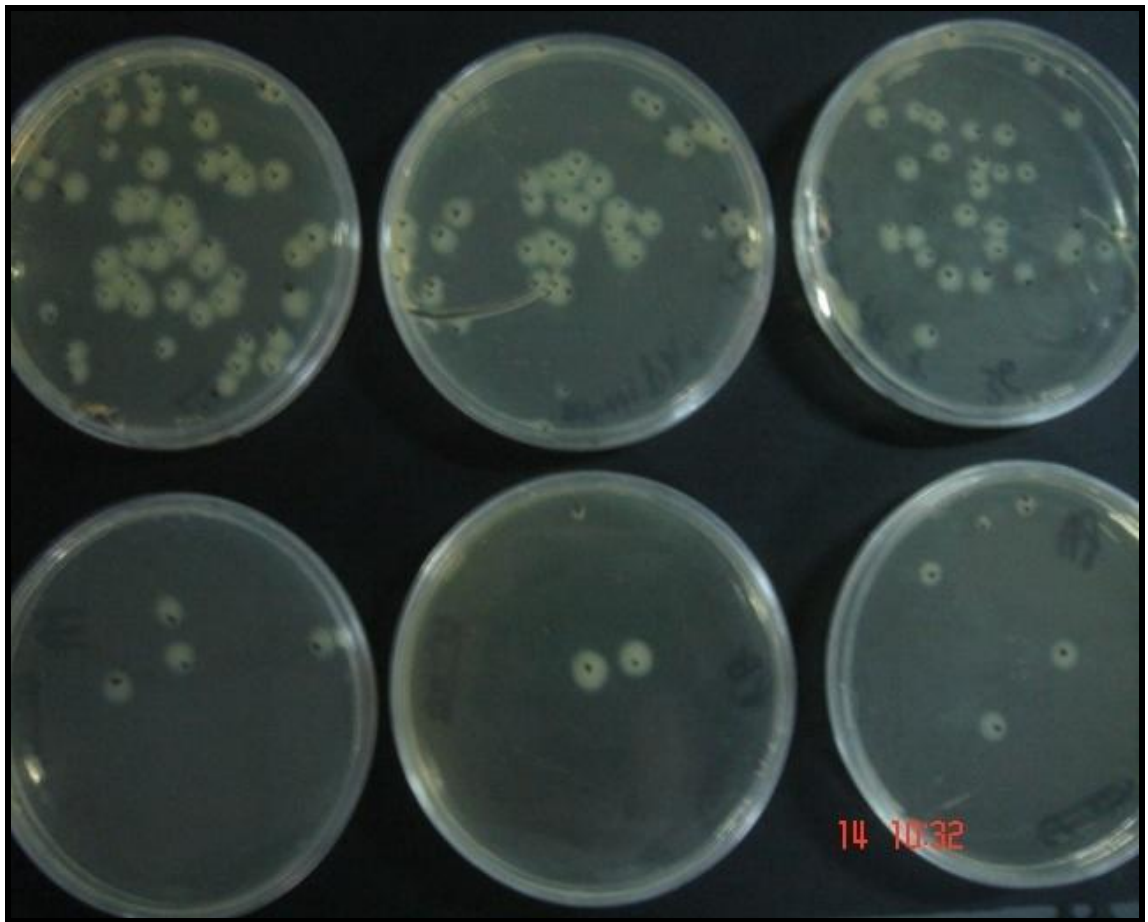| Bacterial Species	N	Percentage of Viable Cells (Mean ±SD)
S. aureus	10	95.75 ±3.44
MRSA	10	95.69 ±3.12
S. epidermidis	10	60.24 ±8.55
S. pyogenes	10	96.35 ±3.09
K. pneumoniae	10	84.60 ±3.98
P. aeruginosa	10	8.28 ±1.82
E. coli	10	42.45 ±7.16

Figure 4.2.2b: Potent bactericidal effect of *L. cuprina* larval extract on *P. aeruginosa* cultures (upper plates are the controls whilst lower are test samples)
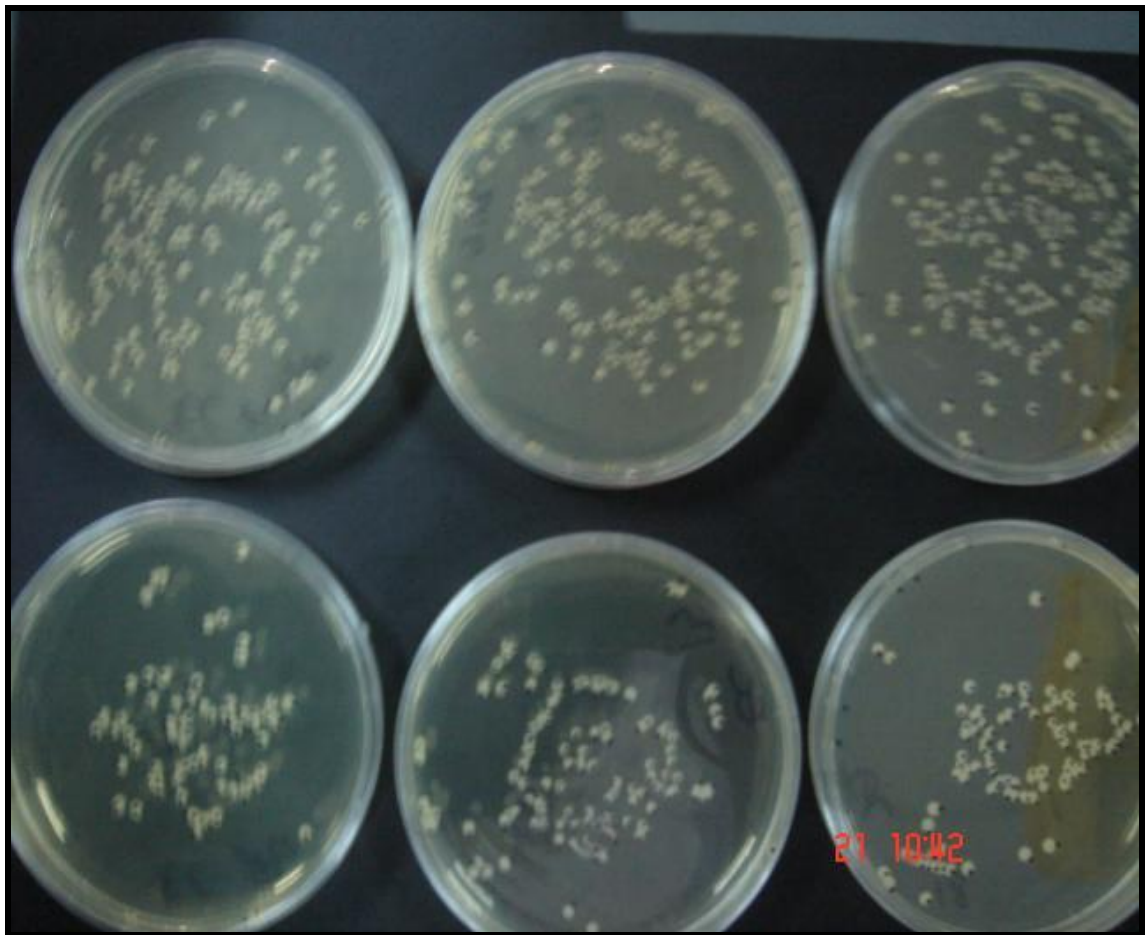
Figure 4.2.2c: Potent bactericidal effect of *L. cuprina* larval extract on *E. coli* cultures (upper plates are the controls whilst lower are test samples)

significant bactericidal effects, it was noticeable that the Gram-negative *P. aeruginosa* was most sensitive to the larval extract as the cell viability of *P. aeruginosa* in the test sample plates was only less than 10% (8.28 $\pm$ 1.82%) on BHI agar (Figure 4.2.2b).

This result once again agreed with the findings from Huberman *et al.* (2007) that the haemolymph extract of non-sterile *L. sericata* larvae exhibited significant bactericidal effect on *P. aeruginosa*. One of the identified bactericides from the extract was *p*-hydroxybenzoic acid, a phenol derivative with known antibacterial activity and is used as preservatives in food and cosmetic industries (Friedman *et al*. 2003). Besides, a recent research undertaken by Barnes *et al.* (2010) demonstrated that *L. sericata* larval ES produced by 1000 mg of larvae per ml of distilled water was more potent on the Gram-negative bacteria *E. coli* and *P. aeruginosa*.

However, there are contradictory reports that the results obtained did not agree with those reported by investigators worked on *L. sericata* larval ES. In the study conducted by van der Plas *et al.* (2007), sterile larval ES did not affect *P. aeruginosa* viability. Besides, in 1998, Jaklic *et al.* had also failed to demonstrate the growth inhibitory and bactericidal effects of filter-sterilized *L. sericata* larval extract on *P. aeruginosa* and *E.coli*. These results seemed to support the earlier hypothesized statement that *L. cuprina* larval extract exhibits different inhibition capacity from *L. sericata* larval ES.

As a whole, it is not unusual for antibacterial agents to exert both bacteriostatic and bactericidal activity depending on the bacteria tested and the concentrations of the antibacterial agent achieved at various infection sites, whilst clinically the importance of bacteriostatic versus bactericidal effect on microorganisms is under dispute.

Bacteriostatic drugs inhibit the growth and replication of microorganisms and the final eradication of pathogens at the infection sites is always accomplished by the immune system of the host. The bactericidal drugs though kill and thus eradicate pathogen, yet in heavily colonized infection site, the host's immune system is equally crucial for the final eradication of the pathogens. Moreover, the supposed superiority of bactericidal effect over bacteriostatic is of little clinical relevance when treating particularly patients with uncomplicated infections and non-compromised immune systems (Pankey and Sabath 2004).

In view of these, though *L. cuprina* larval extract exerted bacteriostatic effect on the growth of most of the tested bacteria, however, the larval capability in eliminating pathogens from the infected chronic wounds shall not be underestimated as it should be realized that the medicinal larvae that work in the wounds are continuously present to exert the antibacterial effect and accomplish more than disinfection of the wounds (Simmons 1935).

4.2.3   Agar Well Diffusion Assay

Another antibacterial assay, the agar well diffusion or zone inhibition assay was performed to determine the susceptibility or resistance of the seven selected wound pathogenic bacteria to *L. cuprina* larval extract. Diffusion or movement of antibacterial molecules through the agar matrix, and the tendency of the antimicrobial molecules to move from a region of high concentration (larval extract-containing well) to the surrounding region with lower concentration are the basis of agar diffusion assay. Following an overnight incubation, the inhibition of bacteria is evident as a clear region, called "zone of inhibition" formed around the larval extract-containing well. This

antibacterial assay is an economical and easy way for the detection of bacterial susceptibility or resistance.

Results from the agar well diffusion assay (Figure4.2.3a) revealed the apparent potency of *L. cuprina* larval extract on *P. aeruginosa*. However, this assay failed to demonstrate the antibacterial activity of larval extract against the other six selected bacteria as no zones of bacterial growth inhibition was developed around wells containing *L. cuprina* larval extract (Figure 4.2.3b, Figure 4.2.3c, Figure 4.2.3d, Figure 4.2.3e, Figure 4.2.3f, Figure 4.2.3g). Nonetheless, zones of bacterial growth inhibition were recorded for the formaldehyde positive controls for all bacteria tested (Table 4.2.3).

Figure 4.2.3h illustrated the resulted diameter of inhibition zones surrounded the well containing larval extract in the *P. aeruginosa* agar plates was 19.60 ± 1.06 mm (n=10). Beyond these zones, the larval extract was apparently too diluted for bactericidal action. This result once again concurred with the findings reported by Huberman *et al.* (2007) whereby the three antibacterial molecules (*p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid and proline diketopiperazine) isolated from the haemolymph extracts of *L.* sericata larvae demonstrated active antibacterial activity against *P. aeruginosa* in the zone inhibition assay and, furthermore, a combination of these molecules had even enhanced the inhibitory effect. The consistency of the present results with Huberman *et al.* (2007) proposes that larval extract (*L. cuprina* and *L.* sericata) is selectively inhibitory to the Gram-negative *P. aeruginosa*.

Besides, the inactivity of *L. cuprina* larval extract towards *S. aureus* and *E. coli* in the present study was in agreement with Bexfield *et al.* (2004) and van der Plas *et al.*
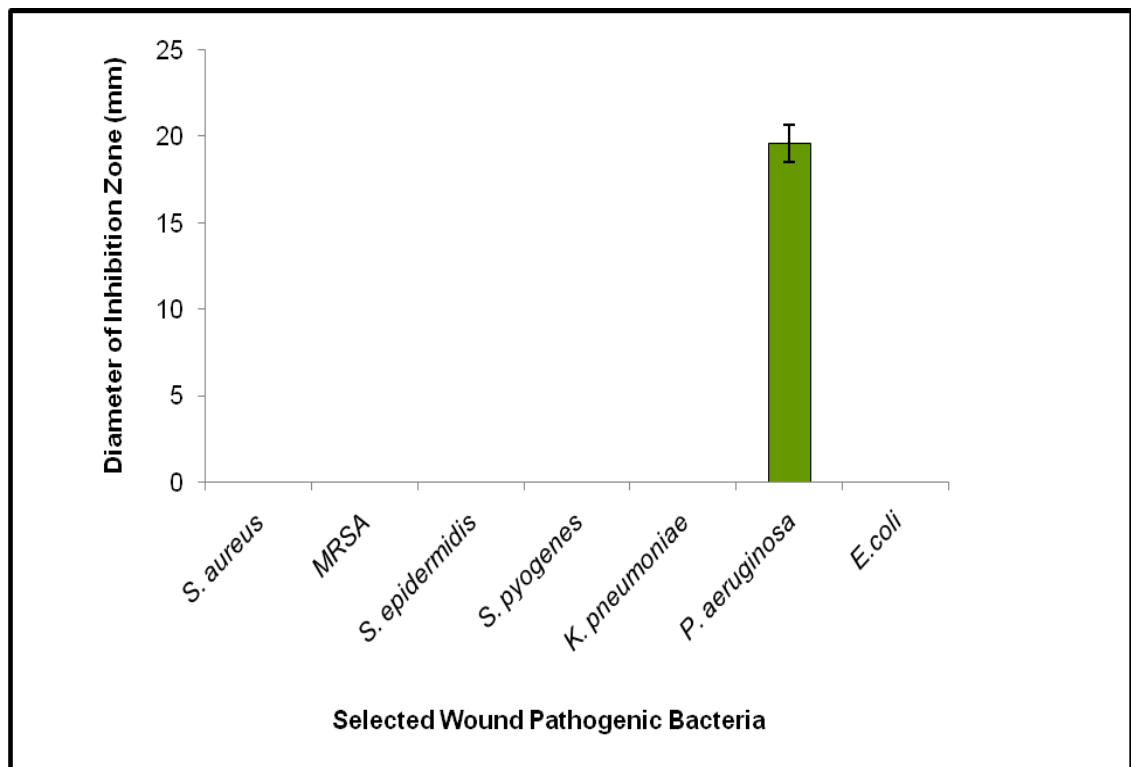
Figure 4.2.3a: Antibacterial activity of *L. cuprina* larval extract against bacteria using agar well diffusion assay
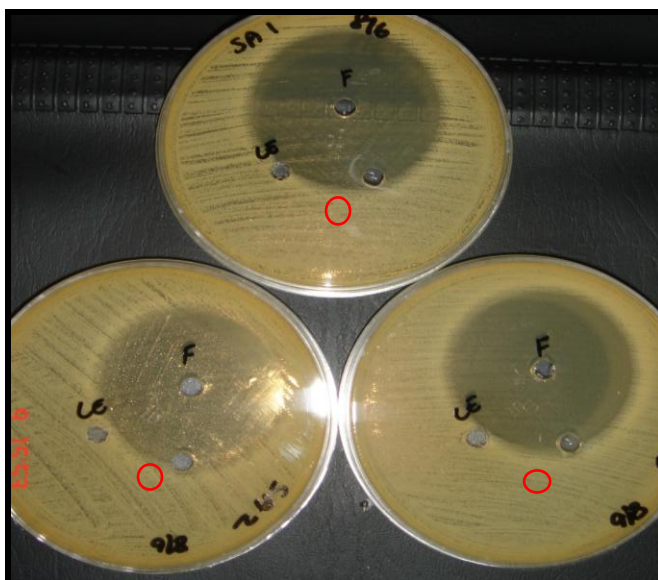
Figure 4.2.3b: Inactivity of *L. cuprina* larval extract against *S. aureus* in agar well diffusion assay
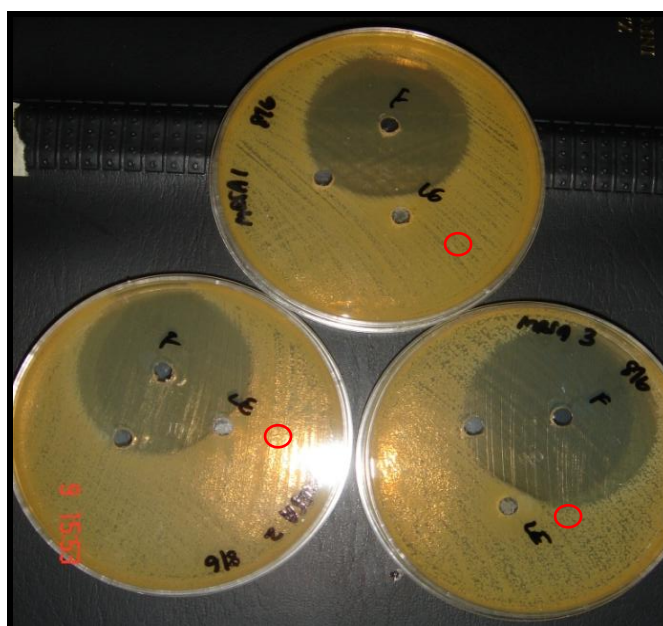

Figure 4.2.3c: Inactivity of *L. cuprina* larval extract against MRSA in agar well diffusion assay
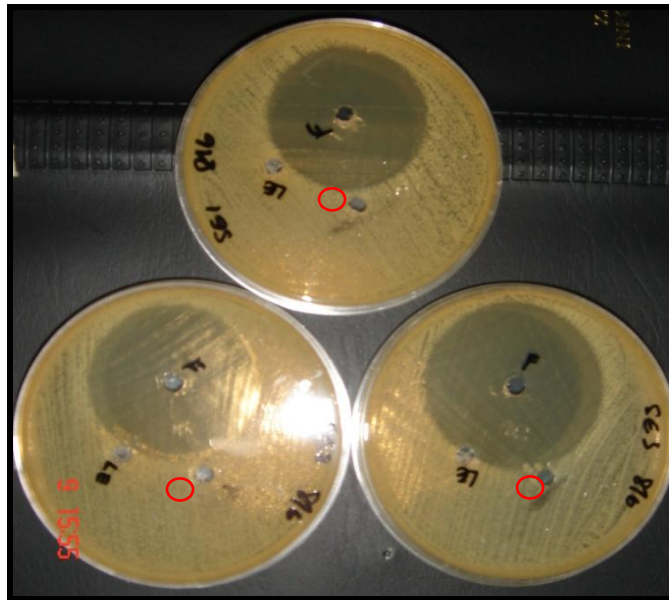
Figure 4.2.3d: Inactivity of *L. cuprina* larval extract against *S. epidermidis* in agar well diffusion assay
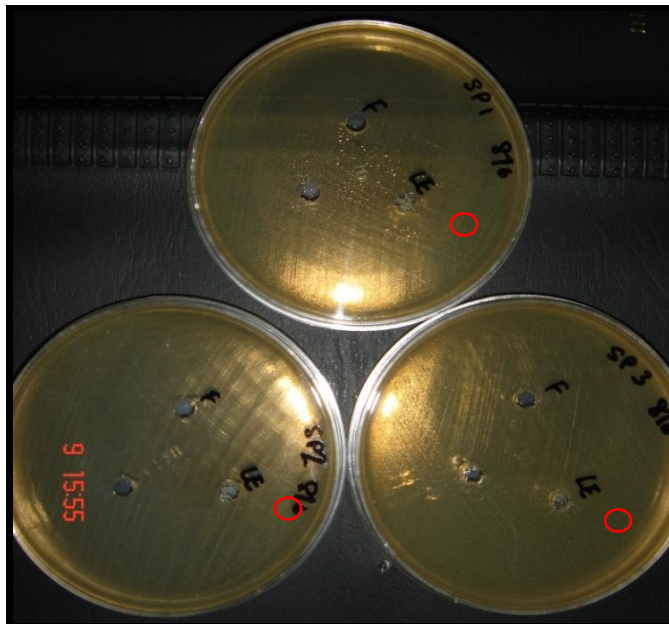

Figure 4.2.3e: Inactivity of *L. cuprina* larval extract against *S. pyogenes* in agar well diffusion assay
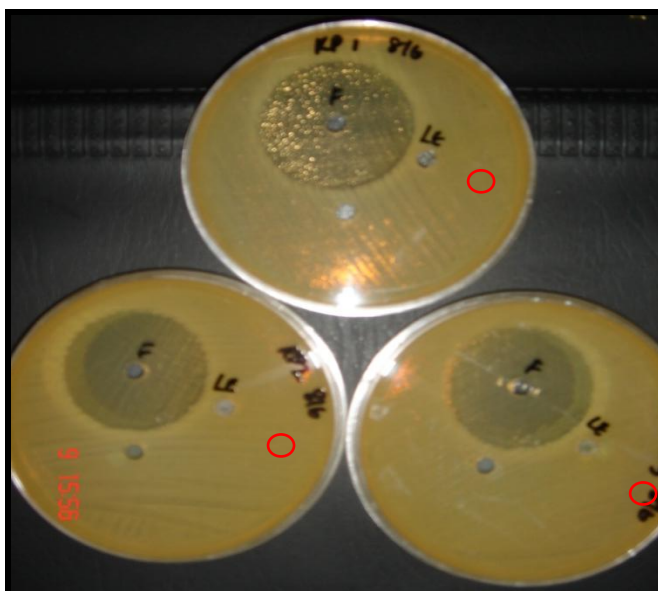
Figure 4.2.3f: Inactivity of *L. cuprina* larval extract against *K. pneumoniae* in agar well diffusion assay
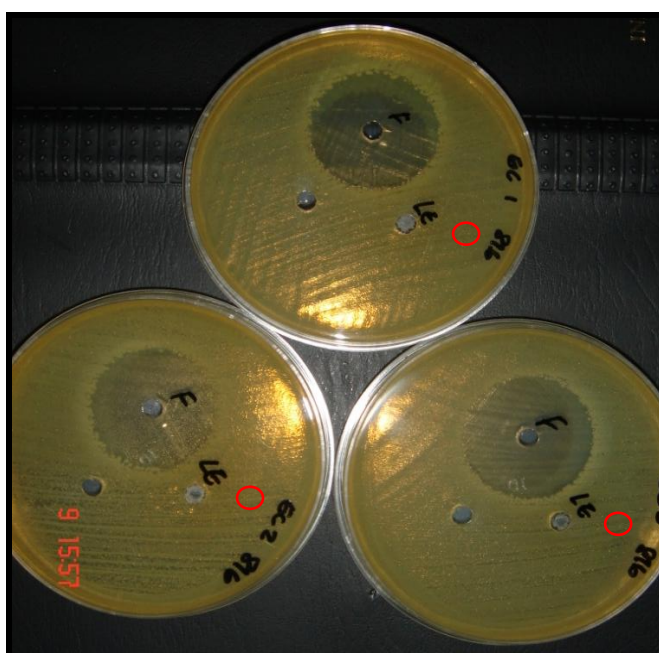
Figure 4.2.3g: Inactivity of *L. cuprina* larval extract against *E. coli* in agar well diffusion assay

Table 4.2.3: Diameter of inhibition zones produced in BHIA plates after overnight incubation

Bacterial Species	Diameter (mm) (Mean $\pm$ SD)		
	Test Sample Well (with 100 mg/ml larval extract)	Positive Control Well (with 5% formaldehyde)	Negative Control Well (with sterile distilled water)
S. aureus	0	40.6 $\pm$ 1.34	0
MRSA	0	45.8 $\pm$ 1.92	0
S. epidermidis	0	42.6 $\pm$ 1.95	0
S. pyogenes	0	50.8 $\pm$ 1.30	0
K. pneumoniae	0	37.0 $\pm$ 1.22	0
P. aeruginosa	19.60 $\pm$ 1.06	25.8 $\pm$ 0.84	0
E. coli	0	31.4 $\pm$ 1.52	0

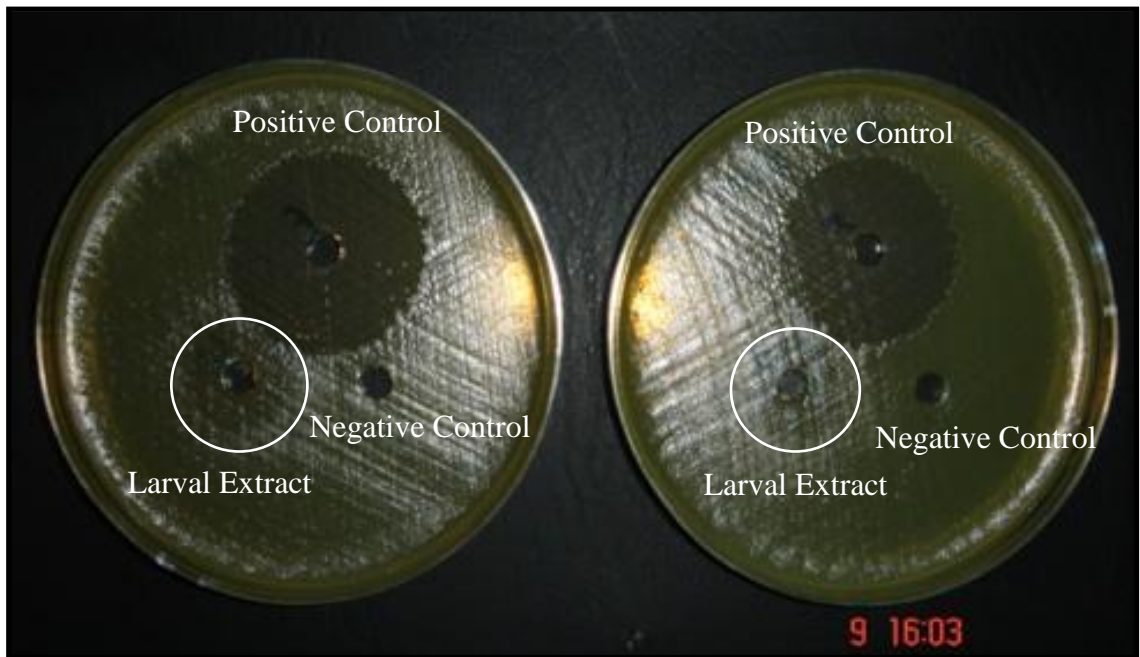**Figure 4.2.3h**: Inhibition zones of *P. aeruginosa* (white-cycle) against *L. cuprina* larval extract in agar well diffusion assay

(2007) whereby they reported that the zone inhibition assay was ineffectual in determining the inhibition activity of *L. sericata* larval ES against *S. aureus* and *E. coli*.

On the other hand, in contrast with the results obtained by Huberman *et al.* (2007), Bexfield *et al.* (2004) and van der Plas *et al.* (2007), Kerridge *et al.* (2005) observed complete inhibitory effect of *L. sericata* larval ES against the Gram-positive bacteria *S. aureus,* MRSA and *S. pyogenes* but partial inhibitory effect against the Gram-negative bacteria *P. aeruginosa* and no inhibitory activity against *E. coli* as evidenced by partial halos zone of inhibition against *P. aeruginosa* and no zones of inhibition against *E. coli.* These opposing observations could be attributable to the different methods of larval ES processing. Kerridge *et al.* (2005) used re-suspended freeze-dried *L. sericata* larval ES which was more concentrated and purified as compared to the centrifuged supernatant of ES [Bexfield *et al.* (2004), van der Plas *et al.* (2007)] for the examination of antibacterial activity.

As a whole, amongst the three antibacterial assays (TB, CFU and agar well diffusion assay) adopted in this study, the TB assay was the most sensitive assay in detecting the antibacterial activity of *L. cuprina* larval extract as substantiated by the significant growth inhibition of the seven selected bacteria (Figure 4.2.1 a). This was due to the known variation in sensitivity of different types of bioassays that had been reported previously by Millar and Ratcliffe (1987), Kerridge *et al.* (2005) and Patton *et al*. (2006). Kerridge *et al.* (2005) found that TB assay was able to detect the antibacterial activity against some bacteria, particularly the vancomycin-resistant Enterococcus (VRE) which was previously undetected by the standard agar disc or well diffusion assay. The variation in the contact times of larval extract with the test bacteria, dilutions and method of collection of ES (Millar and Ratcliffe, 1987) as well as the

diffusion of larval extract through the agar matrix in the agar well diffusion assay are the contributing factors in influencing the efficacy of the corresponding bioassays.

Owing to the sensitivity of the turbidometric assay, it was used in the determination of minimum inhibitory concentration (MIC) and the physicochemical properties of larval extract against each selected bacterial species.

4.2.4   Minimum Inhibitory Concentrations Assay

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent at which no bacterial growth is detected after overnight incubation in growth medium. Besides the utilization by diagnostic laboratories principally to confirm resistance, MIC has also most often undertaken as a research tool to determine the *in vitro* activity of new antibacterial agents (Jennifer, 2001). MIC assay can be determined by agar dilution, broth macrodilution or broth microdilution method.

To the best of the author's knowledge, the MICs of *L. cuprina* larval extract and even the extensively studied *L. sericata* larval ES for pathogenic bacteria have yet to be determined. Therefore, the present study adopted the broth microdilution antibacterial assay with some modifications to determine the MICs of *L. cuprina* larval extract for the seven selected bacteria spectrophotometrically as it is convenient and widely used for susceptibility testing of bacteria (Otvos and Cudic, 2007). For this study, the MIC endpoints of larval extract for each selected bacterial species are defined as the lowest concentration of larval extract (mg/ml) resulting in at least 50% bacterial growth inhibition relative to that of the corresponding controls.

Table 4.2.4 summarizes the MICs of *L. cuprina* larval extract against the seven selected bacteria after overnight incubation in BHI media. In this study, twofold serial microdilutions of 100 mg/ml of larval extract were performed, ensued a final dilution range of 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and 0.39 mg/ml. Again, the results obtained confirmed the apparent potency of *L. cuprina* larval extract against the Gram-negative bacteria, *P. aeruginosa* and *E. coli*. As little as 0.78 and 1.56 mg/ml larval extract were able to inhibit 59.17 ± 2.95% of *P. aeruginosa* and 56.71 ± 3.38% (n = 5) of *E. coli* comparative to the respective growth controls. Conversely, the MICs of larval extract to inhibit at least 50% of bacterial growth for all selected Gram-positive bacteria and *K. pneumoniae* are 3.13 mg/ml.

However, the manifested antibacterial activity of *L. cuprina* larval extract against *P. aeruginosa* was not supported by the findings reported by some other researchers who are working on *L. sericata* larvae. In the study undertaken by van der Plas *et al.* (2007), they reported that *L. sericata* larval ES at a concentration of 4.0 mg/ml did not affect the viability of *P. aeruginosa* in the *in vitro* killing assay (CFU assay). Furthermore, Kerridge *et al.* (2005) have demonstrated that as concentrated as 40 mg/ml of *L. sericata* larval ES was apparently inactive towards *P. aeruginosa* in the well diffusion assay. In addition, Barnes *et al.* (2010) employing the CFU assay had also revealed that as much as 1000 mg/ml *L. sericata* larval ES was needed to inhibit *P. aeruginosa* growth.

On the other hand, in contrary to the MIC of larval extract on *S. aureus* (3.13 mg/ml) and *E. coli* (1.56 mg/ml) as determined by the present study, van der Plas *et al.* (2007) reported that 2.0 mg/ml *L. sericata* larval ES was able to exhibit antibacterial activity against *S. aureus* by reducing the number of viable *S. aureus* by 73 ± 10% (n =

Table 4.2.4:   Broth microdilution MICs of *L. cuprina* larval extract against bacteria

Bacteria	MICs (mg/ ml)	Bacterial Growth Inhibition (%) (as compared to growth controls, n= 5)
S. aureus	3.13	60.90 ± 1.23
MRSA	3.13	59.66 ± 3.53
S. epidermidis	3.13	62.14 ± 3.12
S. pyogenes	3.13	60.96 ± 5.78
K. pneumoniae	3.13	62.30 ± 3.35
P. aeruginosa	0.78	59.17 ± 2.95
E. coli	1.56	56.71 ± 3.38

7) as compared with the controls. Nevertheless, in 2005, Kerridge *et al.* failed to detect the antibacterial activity of 40 mg/ml *L. sericata* larval ES against *E. coli*. However, recently, Barnes *et al.* (2010) found that 1000 mg/ml *L. sericata* larval ES was inactive towards *S. aureus* but yet was able to inhibit *E. coli*.

A review of the above findings has again demonstrated the generation of contradictory results on the potency of larval antibacterial activity against bacteria due to the variations in the sensitivity of different bioassays as discussed earlier and consequently, revealed the importance of selecting the most sensitive and appropriate bioassay to detect antibacterial activity.

Besides, it is also crucial to use growth media with a sufficiently high nutrient content for normal bacterial growth in the controls (Barnes *et al.* 2010) as this would have influenced the assessment of larval antibacterial activity as evidenced by the inconsistent results on *P. aeruginosa* and *S. aureus* obtained from the present study and the study conducted by van der Plas *et al.* (2007) who used growth medium consisted of 10 mM sodium phosphate buffer and supplemented with only 1% TSB. This medium would not be adequate for normal bacterial growth in both the test samples and controls, hence they may possibly have underestimated the antibacterial activity of larval ES since the bacterial growth inhibition capacity of ES in the test samples was determined in comparison to the growth achieved in the controls in which optimum bacterial growth was impeded.

In addition, another factor that would have compounded the inconsistency of the antibacterial activity of larval products (crude extract or ES) is the type of diluents used to collect or reconstitute the larval products. In 1935, Simmons found that maggot

excretions diluted with normal saline (0.85% sodium chloride) was much more potent against bacteria than those diluted with sterile distilled water and he explained that this might be due to the effect of some physical change. Then, Bexfield *et al*. (2004) who collected larval native ES (nES) in sterile PBS reported that PBS might exert adverse effect on bacterial growth. Moreover, van der Plas *et al.* (2007) reported that the well diffusion assay was very sensitive in detecting the antibacterial activity of freeze-dried larval ES solubilized in 0.01% acetic acid. However, they might have overlooked the possibility that the use of acetic acid (a weak acid) had enhanced the sensitivity of well diffusion assay and the antibacterial activity of the ES against the tested bacteria.

Hence, it is imperative to standardize the preparation and quantification of larval products, type of bioassay employed, growth medium, method and diluents used during the collection and dilution of larval products in order to obtain consistent and comparable results of the potency of larval products. Barnes *et al.* (2010) had demonstrated that the number of larvae, bacterial species and nutritional values of growth medium did influence the antibacterial potency of larval ES.

4.3   PHYSICOCHEMICAL PROPERTIES OF LARVAL EXTRACT

The physicochemical properties of larval extract are very important in terms of development of a disinfectant product for medical purposes. However, it should be realized that this product is not recommended to replace the use of the live larvae but rather could be utilized in infection sites where the medicinal larvae cannot be employed.

4.3.1 Robustness

As evidenced by the results shown in Figure 4.3.1a, *L. cuprina* larval extract which had been stored at -70 ℃ for 13 months (red bars) exhibited broad spectrum antibacterial activity against all bacteria tested, while retaining a high degree of robustness as compared to the freshly prepared larval extract (blue bars). These results were affirmed by independent Student's *t*-test that there was no significant loss of potency (p>0.05, n=5) in the 13-month-old *L. cuprina* larval extract against all bacteria tested as compared to the freshly prepared larval extract (Table 4.3.1a). In other words, the 13-month-old larval extract was as potent as the freshly prepared ones. The average potency of the 13-month-old larval extract was $92.76 \pm 2.35\%$ (Table 4.3.1b).

The results obtained were also supported by Duncan (1926) who investigated the bactericidal activity within the gut-content of several insects and arachnids. He observed that the dried materials collected from the gut-contents of certain insects retained the bactericidal activity after storing for at least six months. Additionally, Kerridge *et al.* (2005) had also reported that the lyophilized ES of *L. sericata* was able to withstand a long-term storage.

Nonetheless, there was a noticeable change in the colour of the 13-month-old larval extract. The colour of the long-term stored larval extract diluted in sterile distilled water was darker as compared to the control (Figure 4.3.1b). Nevertheless, these results had verified that vacuum-concentration of larval extract at 30˚C (to remove methanol by enhanced vacuum-evaporation) for seven hours did not result in detectable lost of antibacterial activity of *L. cuprina* larval extract against all bacteria tested.
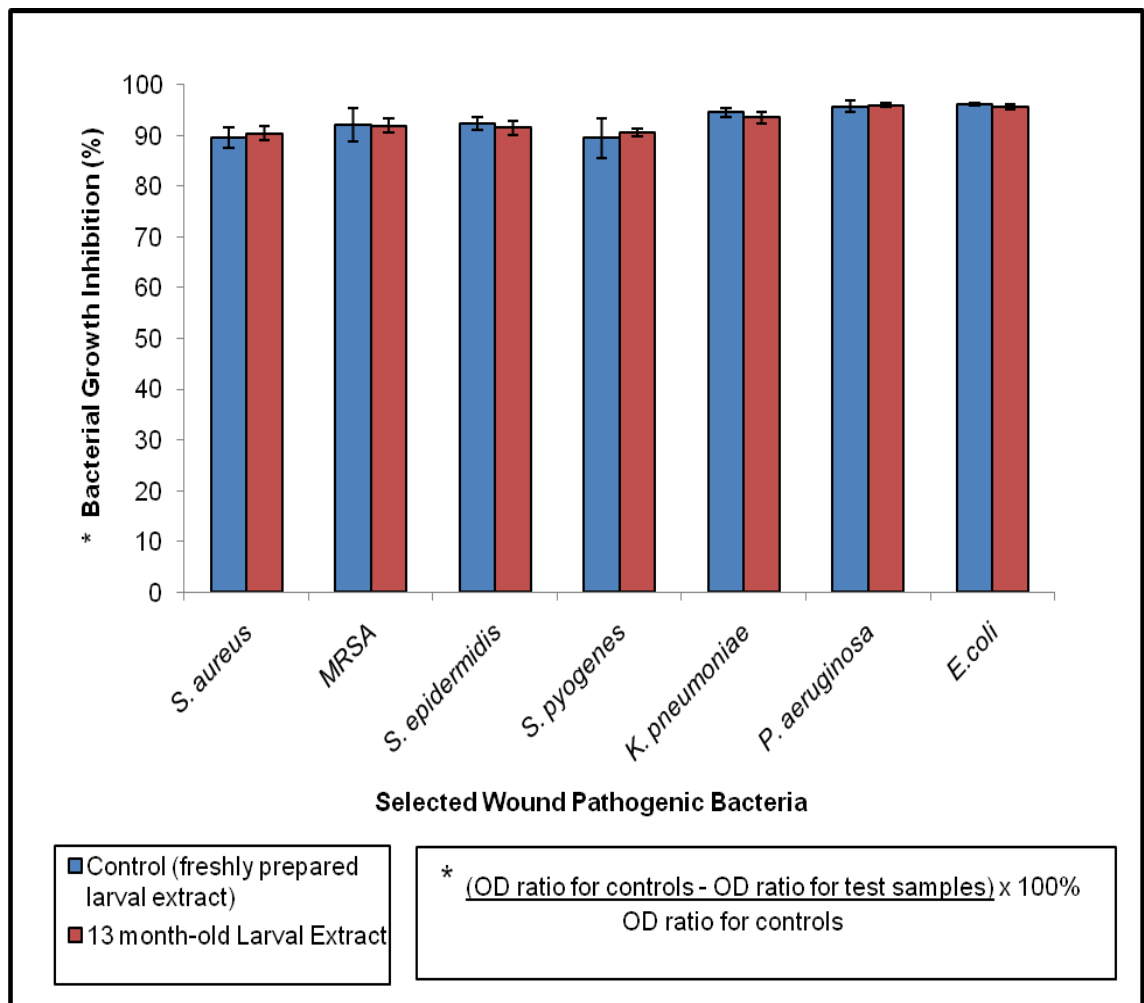
Figure 4.3.1a: Potency of 13-month-old *L. cuprina* larval extract against bacteria in comparision to the controls (freshly prepared larval extract)

Table 4.3.1a: Comparison of mean potency of freshly prepared *L. cuprina* larval extract and 13-month-old *L. cuprina* larval extract against bacteria

Bacterial Species	TB Assay	Mean ±SD	t-statistics [a] (df)	p value
S. aureus	Freshly Prepared	89.50 ±1.97	0.84 (8)	0.427
	13-month-old	90.41 ±1.41		
MRSA	Freshly Prepared	92.10 ±3.26	-0.12 (8)	0.909
	13-month-old	91.91 ±1.34		
S. epidermidis	Freshly Prepared	92.40 ±1.27	-1.08 (8)	0.310
	13-month-old	91.48 ±1.41		
S. pyogenes	Freshly Prepared	89.48 ±3.93	0.58 (8)	0.581
	13-month-old	90.50 ±0.73		
K. pneumoniae	Freshly Prepared	94.51 ±0.95	-1.43 (8)	0.192
	13-month-old	93.56 ±1.15		
P. aeruginosa	Freshly Prepared	95.71 ±1.13	0.44 (8)	0.669
	13-month-old	95.95 ±0.42		
E. coli	Freshly Prepared	96.10 ±0.32	-2.26 (8)	0.054
	13-month-old	95.49±0.51		

[a] Two-tailed unpaired Student's *t*-test

Table 4.3.1b: Mean potency of 13-month-old *L. cuprina* larval extract against bacteria

Bacterial Species	N	Potency of Larval Extract (%) (Mean ±SD)
S. aureus	5	90.41 ±1.39
MRSA	5	91.91 ±1.34
S. epidermidis	5	91.48 ±1.41
S. pyogenes	5	90.50 ±0.73
K. pneumoniae	5	93.56 ±1.15
P. aeruginosa	5	95.95 ±0.42
E. coli	5	95.49 ±0.51
Average Potency	35	92.76 ± 2.35

Figure 4.3.1b: Change of colour in the 13-month-old *L. cuprina* larval extract (right) as compared to the freshly prepared larval extract (left)

4.3.2 Heat Stability

On the other hand, to investigate the stability of the antibacterial activity of *L. cuprina* larval extract to heat, larval extract was boiled at 100 ℃ for 5 minutes or autoclaved at 121 ℃ for 20 minutes.

Results from Figure 4.3.2 demonstrated the heat-resistant antibacterial activity of *L. cuprina* larval extract against all bacteria tested. Both boiled (green bars) and autoclaved (pink bars) larval extracts retained the bacterial growth inhibitory effect as no significant loss of potency was observed ($p > 0.05$, n=5) when compared with the corresponding controls (freshly prepared larval extract) (Table 4.3.2a, Table 4.3.2b). Additionally, the average potency of the boiled and autoclaved larval extracts were $94.18 \pm 1.65\%$ and $93.36 \pm 1.53\%$ respectively (Table 4.3.2c).

The same observations had been reported by Simmons (1935) and Bexfield *et al.* (2004) who worked on *L. sericata* larval ES. As early as in 1935, Simmons proved that *L. sericata* larval ES autoclaved at 110˚C for 20 minutes was of heat-resistant properties and did not loss its antibacterial activity. He then concluded that the active principle(s) within the larval ES is of a non-viable nature. Furthermore, Bexfield *et al.* (2004) have also found that the heat-treated native ES (boiled at 100˚C for 8 minutes) from the larvae of *L. sericata* significantly reduced the CFUs to 23% of the control of *S. aureus*, whilst the native ES enhanced bacterial growth to 123% of the control. They elucidated that these observations may be somewhat due to the deactivation of specific inhibitor(s) or activation of the antibacterial factor(s).
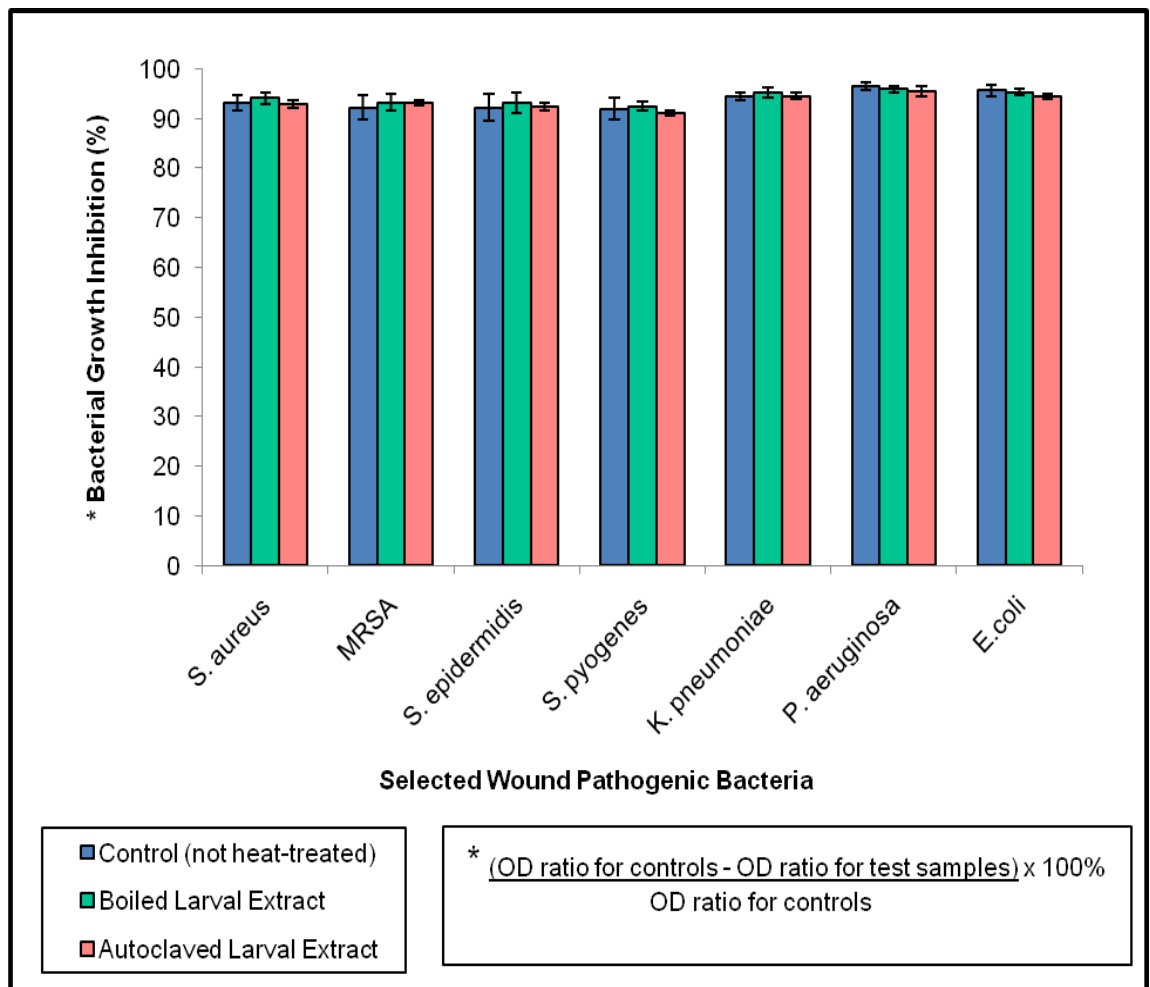
Figure 4.3.2: Potency of heat-treated *L. cuprina* larval extract against bacteria

Table 4.3.2a: Comparison of mean potency of freshly prepared *L. cuprina* larval extract and boiled *L. cuprina* larval extract against bacteria

Bacterial Species	TB Assay	Mean ±SD	t-statistics [a] (df)	p value
S. aureus	Freshly Prepared	93.20 ±1.56	1.025 (8)	0.335
	Boiled	94.07 ±1.07		
MRSA	Freshly Prepared	92.18 ±2.48	0.784 (8)	0.456
	Boiled	93.22 ±1.60		
S. epidermidis	Freshly Prepared	92.19 ±2.64	0.648 (8)	0.535
	Boiled	93.14 ±1.96		
S. pyogenes	Freshly Prepared	91.96 ±2.13	0.489 (8)	0.638
	Boiled	92.48 ±0.97		
K. pneumoniae	Freshly Prepared	94.44 ±0.78	1.327 (8)	0.221
	Boiled	95.22 ±1.04		
P. aeruginosa	Freshly Prepared	96.56 ±0.73	-1.558 (8)	0.158
	Boiled	95.87 ±0.67		
E. coli	Freshly Prepared	95.62 ±1.23	-0.569 (8)	0.585
	Boiled	95.27 ±0.66		

[a] Two-tailed unpaired Student's *t*-test

Table 4.3.2b: Comparison of mean potency of freshly prepared *L. cuprina* larval extract and autoclaved *L. cuprina* larval extract against bacteria

Bacterial Species	TB Assay	Mean ±SD	t-statistics [a] (df)	p value
S. aureus	Freshly Prepared	93.20 ±1.56	-0.491 (8)	0.636
	Autoclaved	92.81 ±0.81		
MRSA	Freshly Prepared	92.18 ±2.48	0.745 (8)	0.478
	Autoclaved	93.03 ±0.51		
S. epidermidis	Freshly Prepared	92.19 ±2.64	0.150 (8)	0.884
	Autoclaved	92.37 ±0.72		
S. pyogenes	Freshly Prepared	91.96 ±2.13	-0.898 (8)	0.395
	Autoclaved	91.08 ±0.59		
K. pneumoniae	Freshly Prepared	94.44 ±0.78	0.065 (8)	0.950
	Autoclaved	94.47 ±0.68		
P. aeruginosa	Freshly Prepared	96.56 ±0.73	-2.122 (8)	0.067
	Autoclaved	95.37 ±1.03		
E. coli	Freshly Prepared	95.62 ±1.23	-2.007 (8)	0.080
	Autoclaved	94.40±0.59		

[a] Two-tailed unpaired Student's *t*-test

Table 4.3.2c: Mean potency of boiled and autoclaved *L. cuprina* larval extract against bacteria

Bacterial Species	N	Potency of Boiled Larval Extract (%) (Mean ±SD)	Potency of Autoclaved Larval Extract (%) (Mean ±SD)
S. aureus	5	94.07 ±1.07	92.81 ±0.81
MRSA	5	93.22 ±1.60	93.03 ±0.51
S. epidermidis	5	93.14 ±1.96	92.37 ±0.72
S. pyogenes	5	92.48 ±0.97	91.08 ±0.59
K. pneumoniae	5	95.22 ±1.04	94.47 ±0.68
P. aeruginosa	5	95.87 ±0.67	95.37 ±1.03
E. coli	5	95.27 ±0.66	94.40±0.59
Average Potency	35	94.18 ± 1.65	93.36 ± 1.53

Nevertheless, Kerridge *et al.* (2005) who also investigated the heat stability of *L. sericata* larval ES have reported that boiling of larval ES resulted in a complete loss in antibacterial activity against MRSA and they stated the possible explanation for the failure to detect the activity of the boiled ES was the lower sensitivity of the agar well diffusion assay as compared to liquid culture assay.

4.3.3 Freeze-Thaw Stability

Besides robustness and thermal stability, the freeze-thaw stability of larval extract is one of the important physicochemical properties to be considered in developing a novel disinfectant as well. Results from Figure 4.3.3 demonstrated that the larval extract which had been freeze-thawed for ten cycles was as potent as the control against all bacteria in the TB assay and this result was affirmed by independent Student's *t*-test as there was no significant loss of potency ($p > 0.05$, n=5) (Table 4.3.3a) in the repeatedly freeze-thawed larval extract as compared to the freshly prepared ones and yet had an average potency of $92.24 \pm 3.27\%$ (Table 4.3.3b).

These results were again agreed with the findings reported by Bexfield *et al.* (2004) and Kerridge *et al.* (2005) whereby they affirmed that the antibacterial activity of *L. sericata* larval ES which has undergone several freeze-thaw cycles remained unaffected.
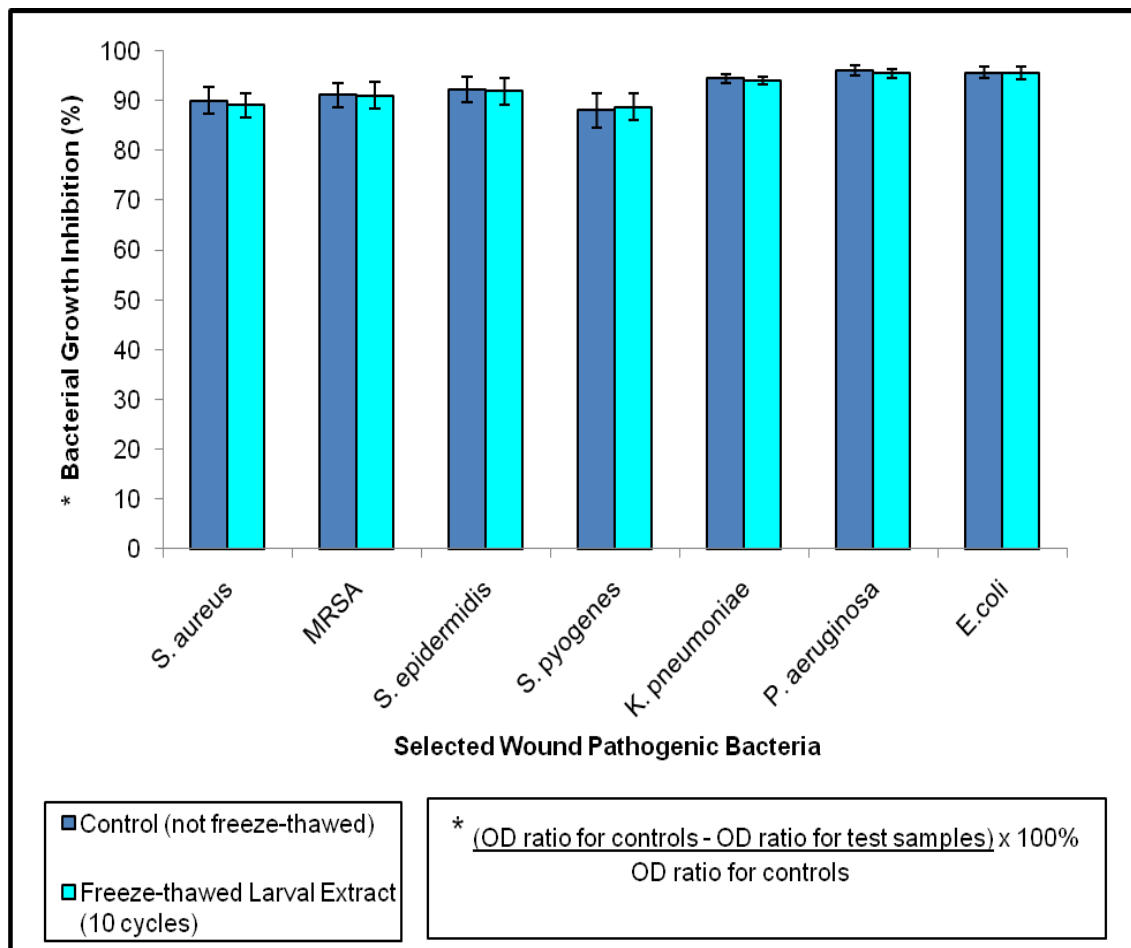
Figure 4.3.3: Potency of freeze-thawed *L. cuprina* larval extract against bacteria

Table 4.3.3a: Comparison of mean potency of freshly prepared *L. cuprina* larval extract and freeze-thawed *L. cuprina* larval extract against bacteria

Bacterial Species	TB Assay	Mean $\pm$ SD	*t*-statistics [a] (df)	*p* value
S. aureus	Freshly Prepared	89.08 $\pm$ 2.47	-0.517 (8)	0.619
	Freeze-thawed	89.92 $\pm$ 2.70		
MRSA	Freshly Prepared	91.05 $\pm$ 2.70	-0.049 (8)	0.962
	Freeze-thawed	91.13 $\pm$ 2.43		
S. epidermidis	Freshly Prepared	91.85 $\pm$ 2.66	-0.202 (8)	0.845
	Freeze-thawed	92.19 $\pm$ 2.64		
S. pyogenes	Freshly Prepared	88.69 $\pm$ 2.65	0.340 (8)	0.742
	Freeze-thawed	88.03 $\pm$ 3.40		
K. pneumoniae	Freshly Prepared	94.05 $\pm$ 0.70	-0.668 (8)	0.523
	Freeze-thawed	94.39 $\pm$ 0.90		
P. aeruginosa	Freshly Prepared	95.43 $\pm$ 0.85	-1.045 (8)	0.327
	Freeze-thawed	96.05 $\pm$ 1.01		
E. coli	Freshly Prepared	95.57 $\pm$ 1.25	-0.072 (8)	0.945
	Freeze-thawed	95.62 $\pm$ 1.22		

[a] Two-tailed unpaired Student's *t*-test

Table 4.3.3b: Mean potency freeze-thawed *L. cuprina* larval extract against bacteria

Bacterial Species	N	Potency of Larval Extract (%) (Mean ±SD)
S. aureus	5	89.92 ±2.70
MRSA	5	91.13 ±2.43
S. epidermidis	5	92.19 ±2.64
S. pyogenes	5	88.03 ±3.40
K. pneumoniae	5	94.39 ±0.90
P. aeruginosa	5	96.05 ±1.01
E. coli	5	95.62 ±1.22
Average Potency	35	92.24 ± 3.27