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RESEARCH ARTICLE

Activity of two serine proteases and anti-bacterial studies on both excretory/secretory products and whole body homogenates of third instar larvae of *Chrysomya megacephala*

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Abstract

The present study revealed the activity of both trypsin and chymotrypsin in both excretory/secretory products and homogenates of whole body larvae of third instars larvae of *Chrysomya megacephala*. The optimum pH activity for trypsin and chymotrypsin enzymes was at pH 9 in the excretory/secretory products and for both of them and at pH 7 in homogenates of whole body larvae for the two enzymes. The present study also evaluate the potential anti-bacterial activity of both excretory/secretory products and homogenates of whole body larvae of third instars' larvae of *Chrysomya megacephala* against gram -ve bacteria (*Escherichia coli,Pseudomonas aeruginosa*) and gram +ve bacteria (*Staphylococus aureus,Bacillis subtilis*) The excretory/secretory products showed antibacterial activity against *Escherichia coli, Staphylococus aureus,Bacillis subtilis.*
The minimum inhibitory concentrations (MIC) were 125ug/ml, 31.25ug/ml and 31.25ug/ml respectively. The homogenates of whole body larvae showed antibacterial activity against *Escherichia coli, Staphylococus aureus,Bacillis subtilis.*The minimum inhibitory concentrations (MIC) was 125ug/ml,31.25ug/ml,15.63ug/ml, respectively. Both the excretory/secretory products and homogenates of third instars larvae of *Chrysomya megacephala* have no effect on *Pseudomonas aeruginosa.*

INTRODUCTION

Many dipteran species are capable of infesting living vertebrate hosts (a condition termed myiasis). Maggot therapy is essentially artificially induced myiasis, performed in a controlled environment by experienced medical practitioners. Maggot therapy has the following three beneficial effects on a wound: debridement, disinfection and enhanced healing. Research into the debridement mechanisms underlying maggot therapy has revealed that maggots secrete a rich soup of digestive enzymes while feeding, including carboxypeptidases A and B , leucine aminopeptidase [**Vistnes et al. 1981**], collagenase [**Ziffren et al.,1953**] and serine proteases (trypsin-like and chymotrypsin-like enzymes) [**Casu et al.,1994**]. The majority of wounds are polymicrobial, hosting a range of both anaerobic and aerobic bacteria [**Bowler and Davies 1999**]. Antimicrobial treatment of clinically infected and non-healing wounds, should, therefore, encompass broad-spectrum antimicrobials in order to cleanse the wound effectively. The application of maggots to an infected wound results in the rapid elimination of such infecting microorganisms **[Courtenay 1999].** The most frequently isolated pathogen from acute and chronic wounds is *Staphylococcus aureus.*

Chrysomya megacephala (F.), the Oriental latrine fly, is a common blow fly species of medical importance in many parts of the world, including Egypt. Adults may feed on food sources including nectar, animal carcasses, garbage, and other filth materials, or even human food. Therefore, it is possible that mechanical transfer of potential disease causing pathogens, such as bacteria, viruses, protozoa, and helminthes eggs, to human food may occur [**Sukontason 2000**]. Larvae of this species are known to cause myiasis in several mammal species, including humans [**Kumarasinghe 2000**]. Another facet of medical importance of this blow fly is its association with human corpses and its relevance to forensic entomology. Many researchers have reported that specimens of *C. megacephala* were found connected with cases of human death [**Sukontason 2005**].
The aim of the present study is to investigate the activity of both trypsin and chymotrypsin enzymes in both excretory/secretory (ES) products and homogenates of whole larval bodies of third instars larvae of *C. megacephala* and their corresponding optimum pH using biochemical reactions as only molecular studies were estimated before. Also the antibacterial effect of both ES products and homogenates of whole larval bodies was investigated against some bacterial strains.

2-MATERIALS AND METHODS:

2.1 Rearing of insect:

The laboratory colony of *C. megacephala* used in this study was established in the Department of Entomology, Faculty of Science, Helwan University. *C. megacephala* was reared following the reported protocol [**Gabre et al., 2005**]. They were identified according to the mentioned method [**Zumpt 1965**]. Adults from the stock colony of *C. megacephala* were kept in cages (38×38×56 cm) at 25±3°C, 14h photoperiod and 60–70% R.H. The cages were made with a wooden floor, a glass roof, and wire gauze on three of the sides. The fourth side was wooden with a circular hole fitted with a cloth sleeve to facilitate daily feeding, cleaning of the cage, and removal of eggs. Adults were supplied daily with granular sucrose, water, and pieces of liver.
Water was supplied by dipping a piece of cotton as a wick in a bottle filled with water, and the liver was provided in a Petri- dish. Egg batches were removed daily and transferred to a fresh piece of chicken placed in a rearing enamel bowl (35 cm in diameter) covered with muslin secured with a rubber band. At the prepupal stage, dry autoclaved sawdust was added to the bowl as a medium for pupation. Pupae were sieved from the sawdust and transferred to adult cages described above for adult emergence.

2.2 Preparation of crude extracts from insect larvae:

Takes place by two methods:

1-Collection of larval secretions: The ES products were collected according to modifications of reported method [**Bexfield et al., 2004**]. Native excretions/secretions (nES) were collected by incubating third instars larvae of *C. megacephala* in a small quantity (50,000 larvae/ 500 ml) of sterile distilled water for 1h at 30°C in darkness. The sterile liquid was siphoned from the containers and centrifuged at 10,000×g for 5 min to remove particulate material, after which the supernatant was collected and separated .Part of it was stored at 4 °C for enzyme assay and the other part was lypophilized for antibacterial tests.
2- Preparation of larval homogenate: Whole third instar of insect larvae were (20,000 larvae in 500 ml) homogenized in distilled water and centrifuged at 10,000×g for 5 min, the supernatant was collected and separated. Part of it was stored at 4c for enzyme assay and the other part was lypophilized for antibacterial tests.

2.3 Determination of total protein :

The total protein was estimated using Kits for colorimetric determination of serum total protein (BIOSCOPE).

2.4 Enzyme assay:

The activity of trypsin was measured according to a modified method of [**Erlanger et al.,1961**].The reaction medium contained 0.5ml of appropriate buffer at pH values (7,8 and 9),30 ul of enzyme solution and 30 ul of N-benzoyl-DL-arginine-P-nitroanilide Hcl (BApNA).The reaction medium was incubated for 10 minutes at room temperature and then the reaction was stopped by 0.5ml of 30% glacial acetic acid. Control contained all components and under same conditions but without enzyme solution.The change in activity was measured at 410nm.
The activity of chymotrypsin was measured according to a modified method of [**Hummel 1959**].The reaction medium contained 0.5ml of appropriate buffer at pH values (7, 8 and 9),200 ul of enzyme solution and 200 ul of N-benzoyl-L-Tyrosine ethylester (BTEE).The reaction medium were incubated for 10 minutes at room temperature and then the reaction was stopped by 0.5ml of 30% glacial acetic acid. Control contained all components and under same conditions but without enzyme solution. The change in activity was measured at 410nm.The biochemical reactions were analyzed using Prism program and ANOVA test was carried.

2.5 Antimicrobial assay:

Agar well diffusion method

The antibacterial activity of synthesized compounds was determined using agar well diffusion method [**Scott 1989**]. All the compounds were tested *in vitro* for their antibacterial activity against Gram positive bacteria (*Staphylococcus aureus, Bacillus substilis),* and Gram negative bacteria (*Escherichia coli , Pseudomonas aeruginosa)* using nutrient agar medium. Ampicillin and gentamycine were used as standard drugs for Gram positive, Gram negative activity respectively. DMSO was used as solvent control. The compounds were tested at a concentration of 1 mg/ml against bacterial strains.

The sterilized media was poured onto the sterilized Petri dishes (20-25 ml, each petri dish) and allowed to solidify. Wells of 6 mm diameter was made in the solidified media with the help of sterile borer. A sterile swab was used to evenly distribute microbial suspension over the surface of solidified media and solutions of the test compounds were added to each well with the help of micropipette. The plates were incubated at 37°C for 24 hrs for antibacterial activity. This experiment was carried out in triplicate and zones of inhibition were measured in mm. scale.

Determination of minimum inhibitory concentration (MIC)

The MIC was determined by the broth microdilution method using 96-well micro-plates [**Saini et al.,2005**, **Bhuiyan et al., 2011**]. The inoculate of the microbial strains was prepared from 24 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. Each sample (1.0 mg) was dissolved in DMSO (1 mL) to obtain 1000 µg/mL stock solution. A number of wells were reserved in each plate for positive and negative controls. Sterile broth (100 µL) was added to the well from row B to H. The stock solutions of samples (100 µL) were added to the wells in rows A and B. Then, the mixture of samples and sterile broth (100 µL) in row B was transferred to each well in order to obtain a twofold serial dilution of the stock samples (concentration of 500, 250, 125, 62.5, 31.3, 15.6 and 7.81,3.9,1.95, 0.98 and 0.49 µg/mL). The inoculums (100 µL) were added to each well and a final volume 200 µL was obtained in each well. Plates were incubated t 37°C for 24 hrs for antibacterial activity. Bacterial growth was indicated by the presence of turbidity and a pellet at the bottom of the well.

3- RESULTS

The total protein was 4 mg/ml for ES products and 0.5 mg/ml for homogenates of whole larvae of third instars larvae of *C. megacephala*.The present study revealed the activity of both trypsin and chymotrypsin in both excretory/secretory products and homogenates of whole body larvae of third instars larvae of *C. megacephala*. The optimum pH activity for trypsin and chymotrypsin enzymes was at pH 9 in the excretory/secretory products for both enzymes and at pH 7 in homogenates of whole body larvae. Also the present study also evaluate the potential anti-bacterial activity of both excretory/secretory products and homogenates of whole body larvae of third instars larvae of *C. megacephala* against gram -ve bacteria (*Escherichia coli,Pseudomonas aeruginosa*),gram +ve bacteria (*Staphylococus aureus,Bacillis subtilis*) The excretory/secretory products showed antibacterial activity against *Escherichia coli, Staphylococus aureus,Bacillis subtilis.*The minimum inhibitory concentrations (MIC) were 125ug/ml,31.25ug/ml,31.25ug/ml, respectively. The homogenates of whole body larvae showed antibacterial activity against *Escherichia coli, Staphylococus aureus,Bacillis subtilis,*.The minimum inhibitory concentrations (MIC) was 125ug/ml,31.25ug/ml,15.63ug/ml, respectively. Both the excretory/secretory products and homogenates of third instar larvae of *C.megacephala* have no effect on *Pseudomonas aeruginosa.*

Table (1): The optimum pH of both trypsin and chymotrypsin enzymes in both excretory/secretory (ES) products and whole body homogenates (WH) of third instar larvae of *Chrysomya megacephala*

Enzyme	PH=7 ES mean±S.D.	PH=8 ES mean±S.D.	PH=9 ES mean±S.D.	PH=7 WH mean±S.D.	PH=8 WH mean±S.D.	PH=9 WH mean±S.D.
Trypsin	0.523±0.29	0.841±0.09	1.06±0.10	0.317±0.069	0.065±0.014	0.013±0.01
Chymotrypsin	1.99±0.48	2.57±0.05	2.56±0.15	0.16±0.02	0.05±0.013	0.145±0.02

Fig (1): Trypsin- like activity at different pH values using BApNA in ES products of third instars larvae of *Chrysomya megacephala*

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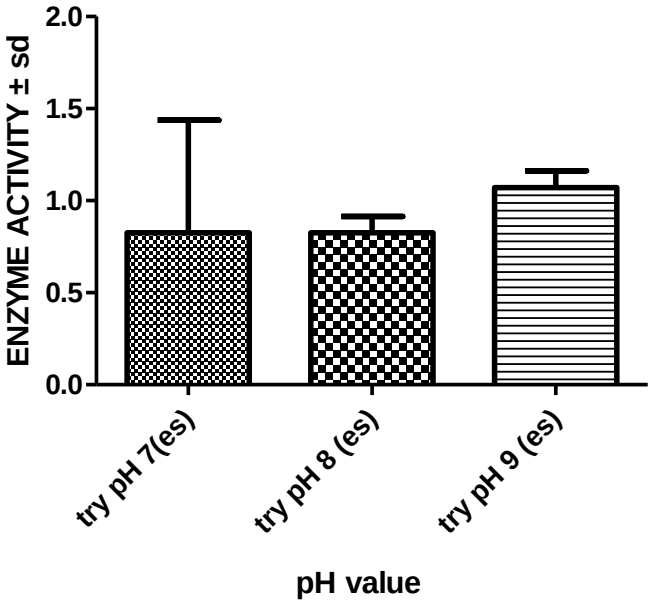

Fig (2): Trypsin – like activity at different pH values using BApNA in homogenates of whole body of third instars larvae of *Chrysomya megacephala*

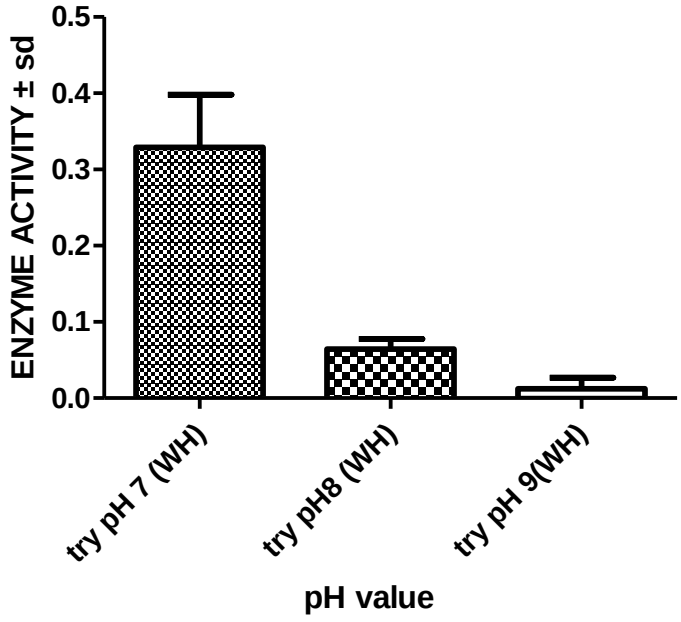

Fig (3) : Chymotrypsin - like activity at different pH values using BTEE in ES products of third instars larvae of *Chrysomya megacephala*

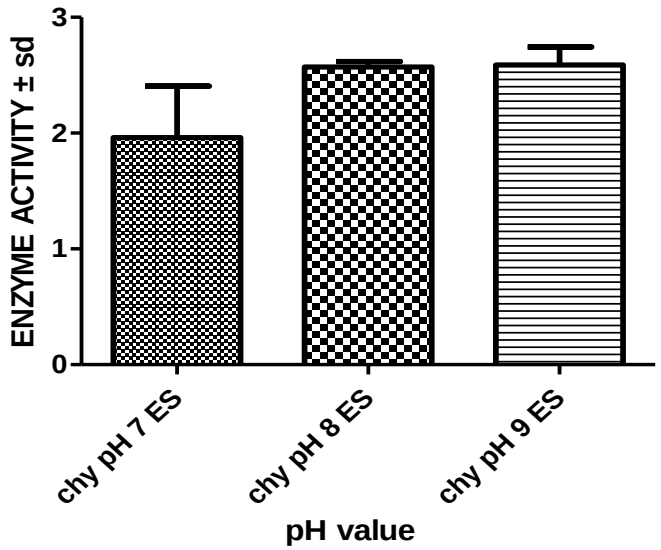

Fig (4) : Chymotrypsin - like activity at different pH values using BTEE in whole body homogenates of third instars larvae of *Chrysomya megacephala*

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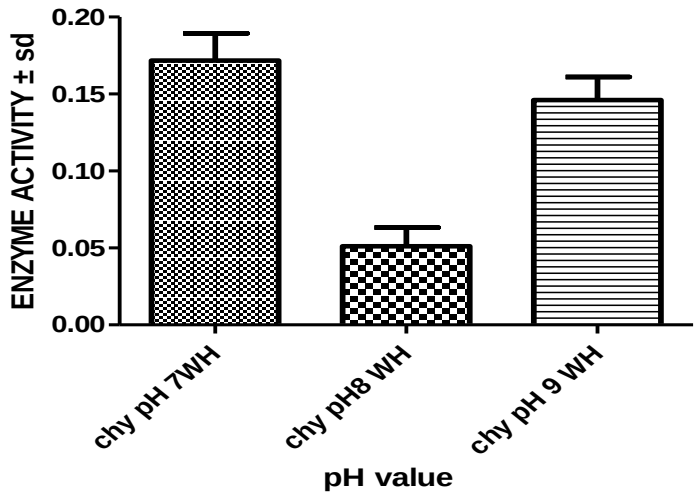
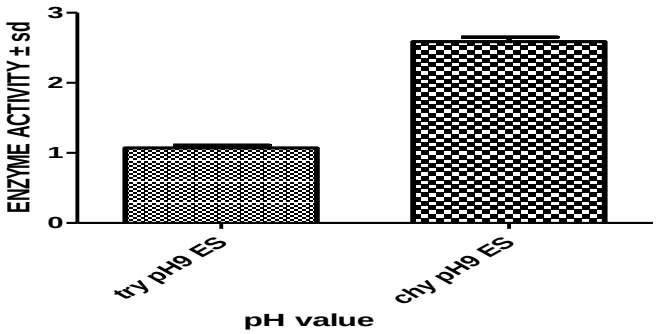


Fig (5): Comparison between Trypsin-like activity and Chymotrypsin-like activity at their optimum pH values in ES products of third instar larvae of Chrysomya *megacephala*



Fig(6)**:** Comparison between Trypsin-like activity and Chymotrypsin-like activity at their optimum pH values in whole homogenates body of third instar larvae of *Chrysomya megacephala*

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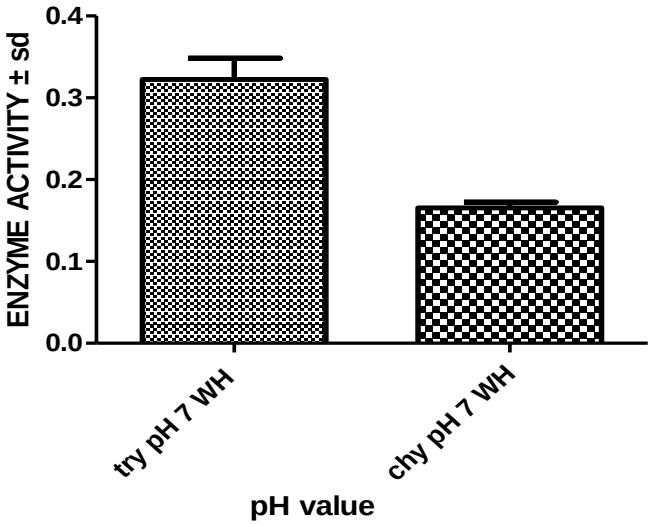

Table(2): Mean zone of inhibition in mm ± Standard deviation beyond well diameter (6 mm) produced on gram positive bacteria using (1mg/ml) concentration of tested samples. Minimum inhibitory concentration (MIC) was determined.

Tested micro-organisms(Gram positive bacteria)	e/s ofthird instar larvae	MIC µg	Homogenates of whole larvae	MIC µg	Standard *Ampicillin*
Staphylococcus aureus (RCMB 010028)	17.2±0.55	31.25	18.1±0.35	31.25	27.4+±0.18
Bacillis subtilis (RCMB 010067)	18.7±0.67	31.25	19.4±0.64	15.63	32.4±0.10

Table(3): Mean zone of inhibition in mm ± Standard deviation beyond well diameter (6 mm) produced on gram negative bacteria using (1mg/ml) concentration of tested samples. Minimum inhibitory concentration (MIC) was determined.NA expressing no activity.

Tested micro-organisms(Gram negative bacteria)	e/s ofthird instar larvae	MIC µg	Homogenates of whole larvae	MIC µg	Standard *Gentamicin*
Pseudomonas aeruginosa (RCMB 010043)	NA	NA	NA	NA	17.3±0.15
Escherichia coli (RCMB 010052)	13.6±0.39	125	14.0±0.64	125	22.3±0.18

Disscussion

The occurrence of different digestive enzymes in the alimentary canal of insects is frequently said to depend mainly on the chemical composition of the diet ingested by the animals [**Wigglesworth 1965**].Enzymes responsible for the complete hydrolysis of proteins down to amino acids are the proteases. Proteases are enzymes acting on peptide bonds and include exopeptidases and endopeptidases. Endopeptidases are divided into sub-classes on the basis of catalytic mechanism. Serine proteases are endopeptidases and have a serine and a histidine in the active site [**Terra and Ferreira 1994**]. The serine proteases (SP) are the dominant class of proteolytic enzymes in many insect species [**Terra et al., 1996**]. SP carry out a diverse array of physiological functions, the best known being digestion, blood clotting, fibrinolysis, fertilization, and complement activation during immune responses [**Horl 1989**]. They have also been shown to be associated with many diseases including cancer, arthritis, and emphysema [**Diamandis and Yousef 2002**].The excreted/secreted serine proteases of *Lucilia cuprina* (sheep blowfly) larvae are thought to be involved in wound formation, and the provision of nutrients to the feeding larvae [**Young et al.,1996**]. Similar roles have been established for the same group of proteases secreted by *Chrysomya bezziana* [**Muharsini et al., 2001**].
The present study revealed the activity of both trypsin and chymotrypsin in both excretory/secretory products and homogenates of whole body larvae of third instars larvae of *C. megacephala*. The optimum pH activity for trypsin and chymotrypsin enzymes was at pH 9 in the excretory/secretory products and at pH 7 in homogenates of whole body larvae. and. Also the activity of chymotrypsin was more than that of trypsin in ES while the activity of trypsin was higher in whole larval homogenates than the activity of chymotrypsin. Molecular studies stated that trypsin and chymotrypsin are produced by third instars larvae of *C. megacephala* [**El-Ebiarie and Taha 2012**].
Trypsins are serine proteases that perfentially cleave protein chains on the carboxyl side of basic L-amino acids such as arginine or lysine. Most insect trypsins pH optima are alkaline between 8 and 9, irrespective of the pH in mid-guts from which the trypsins were isolated**.** Chymotrypsins are serine proteases that preferentially cleave protein chains on the carboxyl side of aromatic amino acids. Most insect chymotrypsins pH optima are alkaline between 8 and 9, irrespective of the pH in mid-guts from which the chymotrypsins were isolated [**Terra, and Ferreira, 1994**].
The present study revealed that both ES products and whole larval homogenates of third instar larvae of *C.megacephala* have anti-bacterial activity against *Escherichia coli, Staphylococus aureus,Bacillis subtilis* and no activity against *Pseudomonas aeruginosa.*Also it is clear from minimum inhibitory concentrations studies that both extracts have the same effect against different strains of bacteria except for *Bacillis subtilis* that whole homogenates is more effective than ES products.
Several published studies suggest possible anti-bacterial properties of maggots and their secretions as an explanation for the success observed in the clinic [**Daeschlein et al.,2007**].It was reported by many authors **[Moch et al., 1999]** the mechanism of action of maggot disinfection on wounds and they found that the excretion of maggots exhibited a strong and rapid disinfection action on *S. aureus.* **Kerridge et al.[2005]** performed a zone of inhibition assay showing anti-bacterial activity of native excretory/secretory product against gram-positive bacteria such as *S. aureus and Streptococcus pyogenes*, whereas **Bexfield et al [2004],**using a similar method and found no anti-bacterial activity.Similarly, antibacterial activity against both gram-positive and gram-negative bacteria including *S aureus and E. coli* has further been documented but whole body extracts and the haemolymph were used instead of of ES [**Huberman 2007a**].The whole body extracts and haemolymph fractions from maggots lysed gram positive and gram negative bacteria including *P. aeruginosa,Klebsiella pneumonia and MRSA* isolated from wound.
During feeding, maggots produce a cocktail of proteolytic and anti-microbial substances called ES products of the gut as well as salivary glands origin.In vitro examination of ES products revealed substances including serine proteases chymotrypsin and trypsin –like protease [**Thomas et al., 1999**].
In general, the exact mechanism and components of the maggot's antibacterial activity are still unknown. The action of maggots can increase the microcirculation and probably destroy the very complex structure of a biofilm,

consequentially the bacteria become susceptible to actions of antibiotics and the immune system as well as to actions of maggots. [**Mumcuoglu et al., 2001].** On the other hand, it can be expected that several different antibacterial components, like oligopeptides, disinfectants and low pH act synergistically. [**Bexfield et al., 2004**] Thus, it could be concluded that the different effects of maggot on Gram positive and Gram negative bacteria are mediated by different molecules and mechanisms. [**Van der Plas et al., 2008].** *Proteus* spp. can colonize maggots as well as it is one of the maggots' gut commensals. It is noteworthy that *Providencia rettgeri* (previously known as *Proteus rettgeri*) produces an L-amino acid oxidase which could act on the larval antibacterial oligopeptides. Thus, the persistence of *Proteus* spp. after maggot applications in together with the survival of such organism in wound myiatic cases from which *Lucilia sericata* was isolated may be explained by the little effect of maggots on this bacteria due to either bacterial adaptation,or their symbiotic relationships as members of the *Lucilia sericata* gut flora [**Jaklic et al.,2008].** The failure of MDT in complete eradication of *Pseudomonas* may be related to the biofilm formation [**Van der Plas et al., 2008]** noticed that more ES required to disrupt *P. aeruginosa* biofilms than *S. aureus* biofilms. In addition, it has been shown in vitro that *P. aeruginosa*, but not *S. aureus*, impairs maggot survival [**Andersen et al.,2009**] .Together, these data are in agreement with clinical findings indicating that more maggots should be used for wounds infected with *P. aeruginosa* [**Steenvoorde and Jukema 2004**].

CONCLUSION :

The present study revealed the presence of both trypsin and chymotrypsin in ES and whole homognates of third instar larvae of *C. megacephala* and their antibacterial effect on different bacterial strains. These results support the need for further experiments aimed at validating *C. megacephala* use in larval therapy.

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