KINGDOM OF SAUDI ARABIA

King Saud University

College of Science

Department of Zoology

Purification and Characterization of Antimicrobial Peptides after Immune Bacterial Challenge in Honey Bee *Apis mellifera jementica*

Thesis submitted in partial fulfillment of the requirements for the PhD degree in Entomology and Parasitology at Zoology Department College of Science , King Saud University

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(2016 AD. /1437 H.)

المملكة العربية السعودية
جامعة الملك سعود
كلية العلوم
قسم : علم الحيوان

تنقية وتوصيف الببتيدات المضادة للميكروبات بعد التحفيز البكتيري المناعي في نحل العسل (*Apis mellifera jementica*)

قدمت هذه الرسالة استكمالا لمتطلبات درجة الدكتوراه في العلوم
تخصص(علم الحشرات والطفيليات) كلية العلوم - قسم علم الحيوان

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الدكتورة / نكهت جمال صديقي

الفصل الدراسي الأول 1437هـ/ 2016م


بسم الله الرحمن الرحيم

This Thesis was funded by the National Plan for Science, Technology and Innovation (MAARIFAH) King Abdulaziz City for Science and Technology, Kingdom of Saudi Arabia, Award number (11-AGR2082-02).

Dedication

I dedicate this doctoral thesis; i) to my mother, one of many strong women in my life. She instilled in me a love of nature, a determination to succeed, and a curiosity that continues to drive my aspirations. ii) to my children, for being the best in the world. iii) to my brother and sister for their love, support, and understanding during these years of education .

Acknowledgement

First and foremost, I would like to thank Allah, The Almighty for giving me the strength and for everything I have .

Sometimes it takes the right opportunities and meeting the right people in order to succeed in life, even if you try your hardest. I was fortunate enough to have encountered both. Without meeting these people, I never would have had these opportunities to lead me to where I am today.

I would like to express my deep appreciations to my Supervisor Dr. Tahany Ayaad, Associate Professor of Entomology, Zoology Department, College of Science, King Saud University, for professional supervision, instruction, advice, continuous discussion with moral advising and supporting during all thesis research work. As a teacher, she is passionate about what she teaches. As a Scientist, she shows enthusiasm for her experiments. I have gained so much experience working with her.

I wish also to extend my deep thanks and appreciations to the

cosupervisor, Prof. Dr. Nikhat Siddiqi, Professor of Biochemistry, Biochemistry Department, College of Science, King Saud University, for her guidance, advice, discussion and support in revising this study.

My appreciations are also extended to Dr. Reem Alajmi, Assistant Professor and Deputy of Zoology Department, College of Science, King Saud University, for her guidance and help during this research work.

Thanks and appreciations to Prof. Dr. Abdel-Salam Anwar Mohamed , Bee Research Chair, Department of Plant Protection, College of Food and Agriculture Sciences, King Saud University, For his cooperation and support in the supply of Honey bee samples and providing me with the technical scientific information about laboratory handling of honey bees and revising the scientific writing of Honey bee rearing in laboratory.

Great appreciations to King Saud University for giving me the opportunity to attend the PhD program.

I would like to express my deep thanks to the head of the Zoology Department, Faculty of Science , King Saud University , Dr. Badr Bin Abdullah Dahmash for his great facility offered to me.

Moral appreciations are extended to King Abdulaziz City for Science and Technology and Innovation for funding this research .

Great appreciations are to acknowledge Bee Research Chair, Department of Plant Protection, College of Food and Agriculture Sciences,

King Saud University for their guidance in providing me with all the facilities of practical training and scientific support in Honey bee handling .

My great thanks and belongs are to Umm Al-Qura University for giving me the opportunity to complete my PhD degree and achieve my ambition.

I would like to thank all the staff members of Zoology Department, College of Science, King Saud University, thanks to the researches Mrs Al-Bandari Alrajeh and Miss Zeinab Almasawy, also to my colleagues for their support, guidance and motivation . I am grateful to my best friend Miss Abeer Al-Habash.

I would like to thank Mrs Amani Zain , Researcher in Department of Pharmaceutics , Pharmacy Department , King Saud University , for her support and guidance during the microbiology laboratory work.

Other heartfelt thanks express to my family, thank you for all that you have done for me and for always being supportive to me through all the success and achievements I have gained.

CONTENTS

List of abbreviations

Abbreviation	Term
AC	Anticoagulant
AFB	American foul brood
ALDH	Aldehyde dehydrogenase
AMPs	Antimicrobial peptides
APS	Apis physiological saline
a.s.l	Above sea level
ATCC	American type culture collection
ATP	Adenosine triphosphate
BHI	Brain heart infusion agar
B. subtilis	*Bacillus subtilis*
BLAST	Basic local alignment search tool
cDNA	coding DNA
CBB	Comassie brilliant blue
CFU	colony-forming units
CIP	ciprofloxacin
CLSI	Clinical and laboratory standards institute
csd	complementary sex determiner
DAP	Di amino Pimelic

Abbreviation	Term
DNA	Deoxyribo nucleic acid
DOPA	3.4-DihydroxyPhenylalanine
DWV	Deformed wing virus
E. coli	*Escherichia coli*
EDTA	Ethylen diamine tetraacetic acid
fem	Feminizer
GO	Gene ontology
HEX110	Hexamerin 110
HEX70a	Hexamerin 70a
HEX70b	Hexamerin 70b
HEX70c	Hexamerin 70c
HSP	Heat-shock protein
HWEL	Hen white egg lysozyme
$IAMP_S$	Immune antimicrobial peptides
IMD	Immune deficiency
JAK/STAT	Janus kinases/signal transducers and activators of transcription
LC	liquid-chromatography
LD50	lethal dose 50

Abbreviation	Term
LPS	Lipopolysaccharide
LTQ	Linear trapping quadrupole
MDH	Malate de hydrogenase
MHA	Mueller hinton agar
MIC	Minimum inhibitory concentration
M. luteus	*Micrococcus luteus*
MP	Melanization protease
MRJP	Major royal jelly protein
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS / MS	Tandem mass spectrometry
MW	Molecular weight
NCCLS	National committee for clinical laboratory standards.
NP Y	Neuropeptide y
OBP	Odorant-binding protein
OD	optical density
P. larvae	*Panbacillus larvae*
PBS	Phosphate buffer saline
PG	Peptidoglycan

Abbreviation	Term
PGRP	Peptidoglycan recognition protein
PO	Phenoloxidase
ppm	Parts-per-million
proPO	Prophenoxidase
P. aeruginosa	*Pseudomonas aeruginosa*
r.c.f.	relative centrifugal force
RJ	Royal jelly
RNA	Ribonucleic acid
RNAi	RNA interference
RP-HPLC	reversed-phase high-performance liquid chromatography
S. enteritidis	*Salmonella enteritidis*
SCX	Strong cation exchange
SDS - PAGE	Sodium dodecyl sulfate polyacrylamide gel Electrophoresis
Serpin	Serine protease inhibitors
STAGE	STop and go extraction
St. aureus	*Staphylococcus aureus*
TCA	Tricarboxylic acid
TEP	Thioester-containing Proteins

Abbreviation	Term
α-TNF	α-alpha Tumor necrosis factor
TOF	Time of flight
TP	Total protein
VDV	*Varroa destructor virus*
UV/V Spectrophotometer	Ultraviolet/ Visible spectrophotometer

List of figures

List of tables

Summary

The present study was undertaken to study the effects of immune peptides purified from the induced third larval instar of honey bee native to Saudi Arabia viz., *Apis mellifera jemenitica* (*A. m. jemenitica*). The peptides were studied for their antibacterial activity against *Paenibacillus larvae* (*P. larvae*)(ATCC 10801) and *Salmonella enteritidis*(*S. enteritidis*) (ATCC 13076). *P. larvae* causes American foul brood (AFB), disease affecting honeybees. *S. enteritidis* is the causative organism of Salmonellosis, a human food-borne disease. The antibacterial activity of the peptides were also compared with different standard anaerobic and aerobic bacterial pathogens.

A. m. jemenitica larvae were injected with a standardized dose of LPS (20 ng/larva) and /or 1×10^6cfu/larvae of *P. larvae, S. entritidis, Escherichia coli* (ATCC 10536) or *Micrococus luteus* (National Collection of Type Cultures)(NCTC 2665). Immune responses were measured by estimated standard immunocompetence assays, solid growth inhibition zone assay, nodulation, melanization and cellular phagocytosis of latex beads and lysozyme-like, cecropin-like and phenoloxidase activity assays.

Injection of *A. m. jemenitica* 3rd instar (72h , old) with a standard dose of the pathogenic *P. larvae* or *S. entritidis* produced a cascade of humoral

and cellular immune responses. The humoral immune peptides were shown to exhibit antibacterial activities against the obligate aerobic gram-positive bacteria *M. luteus* and three facultative anaerobic bacteria of which one is the gram positive *P. larvae* and two are gram negative bacteria viz., *S. enteritidis* and *E. coli*.

Injection of *A. m. jemenitica* 3rd instar (72h , old) larvae with *P. larvae* or *S. enteritidis* resulted in maximum nodule formation at 48h post injection. In the larvae injected with 0.9µl carboxylate-modified polystyrene latex beads, the granulocytes showed a noticeable activity with a number of phagosomes containing different phases of lysed latex beads at 24h post-injection.

Lysozyme like activity was measured in the hemolymph plasma of injected larvae using the turbidity assay method. A constitutive level of lysozyme concentration was observed in the unchallenged control larvae. Lysozyme like activity was significantly increased in the hemolymph of challenged larvae at different time intervals post immune challenge ($P<0.05$). The increase in lysozyme like activity was found to be time-independent as there was non significant increase in the enzyme activity at different time intervals post-injection.

Cecropin-like activity was used to estimate the disease resistance (ability of insects to mount an effective humoral immune response) against both *P. larvae* and *S. entritidis*. The antibacterial activity was significantly higher (inhibition zone diameter of 11.3 ± 0.5 mm) in plasma of *A. m. jemenitica* (protein conc. 1.725 ± 0.214) larvae injected with *P. larvae* when compared to identical standard Cecropin B having the same concentration of protein . However at lower protein concentration viz., 5 and 2.5µg/ml the activity was not detected in the control (non injected) larval plasma.

The results of light microscopy and TEM demonstrated that oral infection of *A. m. jemenitica* larvae with *P. larvae* spores resulted in AFB disease. The honey bee larvae crop was severely affected as seen in the histology of the digestive system parts. The response of the gut of honey bee to *P. larvae* infection was that of inflammation and degeneration. The histological picture of the honey bee ventriculus undergoes changes as a result of action of factors as such as toxic substances and bacterial infections, There was generalized separation and detachment of the epithelial cells and increased laceration and vacuole formation. The degeneration of the epithelial cells is being due to secretion of metalloproteases by *P. larvae* that are involved in the larval degradation that occurs after infection.

There was a concomitant increase in the total protein content in the plasma of injected larvae with *S. enteritidis* and *P. larvae* bacteria. The increase in the protein content was highest in the larvae injected with *S. enteritidis* and *P. larvae* (1.799 ± 0.152, 1.725 ± 0.214) followed by that of LPS and *M. luteus* (1.54 ± 0.40, 1.54 ± 0.41) compared with that of uninfected hemolymph (1.24 ± 0.32).

SDS-PAGE showed that the hemolymph of injected *A. m. jemenitica* larvae with *P. larvae* or *S. enteritidis* displayed over expression of polyphenol oxidase which exhibited molecular mass of 70 kDa. The protein with lysozyme like activity exhibited a molecular mass of 15 kDa and bands below 11 kDa which may correspond to bee AMPs like defensins or cercropin.

Induced immune peptides were isolated from the hemolymph of challenged larvae using Reverse-Phase High Performance Chromatography (RP-HPLC). Detectable antibacterial activities with variable minimum inhibitory concentration (MICs) against *S. enteritidis* (ATCC 13076; Basingstoke, UK), *E. coli* (ATCC 10536), *M. luteus* (NCTC 2665; Sigma, UK), *St. aureus* (ATCC 6538) , *B. subtilus* (ATCC

6051), *P. aeruginosa* (ATCC 9027) and *P. larvae* (ATCC 10801) were observed using broth micro dilution assay.

The purified peptide fractions (coded A, B, C and D) exhibited antibacterial potency similar to the ciprofloxacin, a reference antibiotic against the gram negative *E. coli* and gram positive *M. luteus*. However *P. larvae* strain was found to be the most resistant organism followed by *S. enteritidis* then *P. aeruginosa*, against all the tested purified peptides. The peptide fraction (B) showed the lowest MIC (32µg/ml) towards *S. enteritidis*.

The antimicrobial susceptibility of *S. enteritidis* and *P. larvae* to the six purified peptide fractions were determined by Kirby-Bauer disk diffusion method. Peptide fraction (B) showed the highest antibacterial activity with inhibition zone diameter around 21 ± 0 (mm) and 20.5 ± 0.71 (mm) against *S. enteritidis* and *P. larvae* respectively when compared to ciprofloxacin antibiotic (as reference). *In vitro* evaluation of these isolated purified peptide fractions (A, B and C) or (D, E and F) from the hemolymph plasma of larvae injected with *P. larvae* or *S. enteritidis* respectively showed decreased attachment of *S. enteritidis* to human intestinal epithelial cells.

The purified peptide fractions (A, B, C and D) also showed decreased adhesion (19.5±0.71, 21±0, 18.5±0.71, 19±1.41mm) (P<0.05) of *S. enteritidis* to human epithelial cells *in vitro*. Peptide fractions (A and B) showed significant inhibition of (P<0.05) *S. enteritidis* adhesion to human epithelial cells.

Conclusion

Results of the present study showed that natural peptides from *A. m. jemenitica larva* 3rd instar injected with different bacteria possess antibacterial activity against a panel of bacterial pathogens viz., *E.coli, M. lutues, B. subtilis and P. aeruginosa.* Moreover, RP-HPLC purified AMPs from induced plasma of *A. m. jemenitica 3rd* instar larvae were shown to have potent efficiency against the *A. m. jemenitica* pathogenic bacterium, *P. larvae*.

Antiadhesive effect of certain purified peptide fractions towards eukaryotic epithelial cells and *S. entritidis* the causative organism of human Salmonellosis supports their role as a potent bactericidal agent.

This study is a step towards identifying immune factor(s) in *A. m. jemenitica* as candidate insect-derived antibiotic(s) against human and honeybees food-borne diseases which may encourage the beekeeping industry and vertical farming as well as public health in Saudi Arabia.

INTRODUCTION

1 INTRODUCTION

1.1 Overview of honey bees

Humans' fascination with bees began even before recorded history, as seen from cave paintings depicting bee hives (Clark *et al*., 1999), and bees being worshipped as goddesses by the ancient Greeks (Cook, 1895). Medicinal use of bee products, known as apitherapy, has been practiced globally (Clark *et al.,* 1999) and is still a topic of active research. Based on fossil records, it appears that honey bees have been a separate species for more than 80 million years (Michener *et al.,* 1988). As a testament of their importance to the ecosystem, not only have they evaded extinction, but are important agents of pollination (Aizen *et al.,* 2008).

Honey bees are eusocial insects, placing them in the highest level of social organization where they are joined only by wasps, ants, termites. The definition of eusociality features the presence of overlapping generations in a single colony, caring cooperatively for the young, division of labour among the sterile members, with reproductive tasks limited to a subset of individuals (Wilson and Holldobler., 2005). Honey bees, *Apis mellifera*, occur naturally over vast and varied geographical areas, extending from Scandinavia in the north to the Cape of Good Hope in the south, and from Dakar in the west to Oman in the east. Different

populations are adapted to a very wide range of climatic conditions (e.g., Ruttner *et al.,* 1978).

Apis mellifera colonies are found up to 1,000 m above sea level (a.s.l.) in temperate zones and up to 3,700 m a.s.l. in the tropics. They survive in the hot and arid zones of Oman at 200 m a.s.l. (Dutton *et al.,* 1981). A distribution over vast areas with extremely different climates and geographical isolation has led to the diversification of honey bee morphology, behavior, and resulting in many lineages and subspecies of *A. mellifera.*

The division of labour in bees gives rise to three castes in each hive: queen, drone, and worker (Figure 1). Reproduction is limited to the queens and drones. Drones are males with the sole purpose of mating with The queens, which are females with organs for sperm storage. Under normal conditions, the queen is the single fertile female within the hive. At a very early stage of her adult life, the queen mates with typically one to two dozen males, the drones, after which they die. The queen lays up to 2000 eggs per day for the next one to three years, until she dies or her sperm stores are depleted (Winston, 1987). The queen can control the gender of her offspring, with females arising from diploid eggs and males from unfertilized, haploid eggs: a sex-determination system called haplodiploidy (Beukeboom, 1995) (Figure 2). The males, having 16

chromosomes, are entirely hemizygous for every gene while the females have 32 chromosomes.

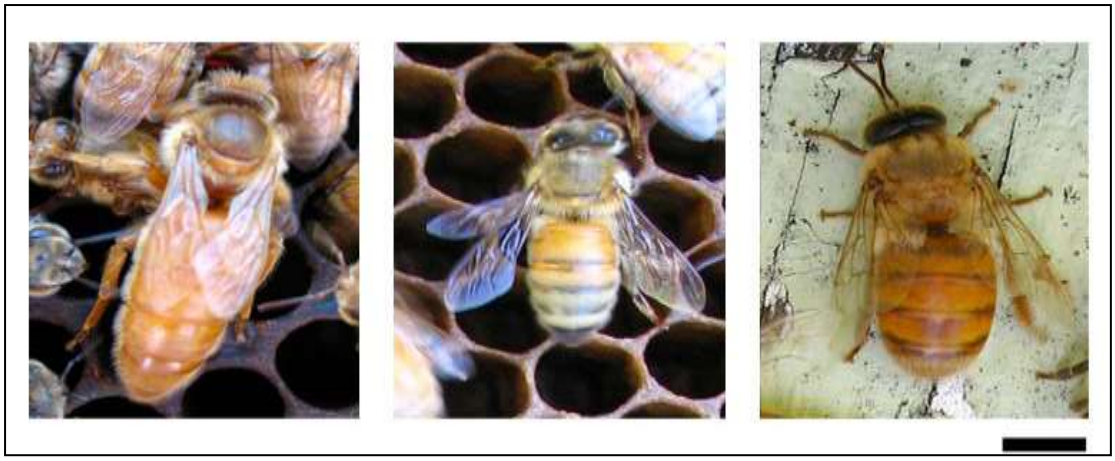


Figure (1): The three castes of adult honey bees (Chan, Q.W. and Foster, L.J. 2009) are shown: queen (left), worker (middle), drone (right). (Scale bar = 5mm.) Workers are the smallest caste member. Queens, after mating, are characterized by their long, enlarged abdomen where the ovaries take up the majority of the space within the abdomen cavity. Drones are larger than workers and have a proportionally wider abdomen; drones are most easily recognized by their eyes, which are larger than the other castes.

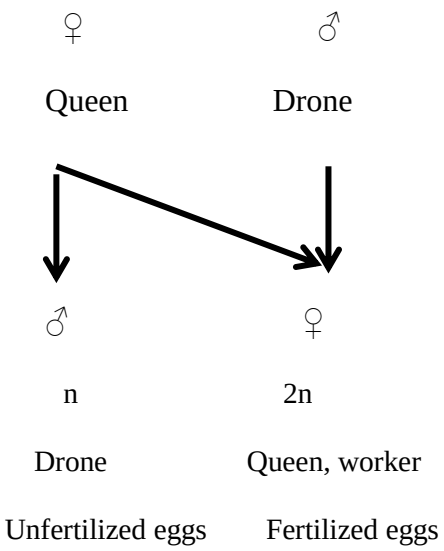


Figure (2): Haplodiploid sex determination (Beukeboom, 1995).
Haplodiploidy dictates that the sex is determined by the number of sets of chromosomes in an individual. Drones receive only one set of chromosomes (n), araising from unfertilized eggs and are therefore male. Those that receive two sets of chromosomes (2n) are females, arising from diploid eggs. In a laboratory setting where highly inbred bees can be produced, diploid males are possible, indicating that sex determination is more complex than is outlined by haplodiploidy (Dzierzon, 1845).

Unmated queens, with unfertilized eggs, are capable of producing only males (Dzierzon, 1845) . Sex determination in bees as understood today, however, involves at least two genes located at the sex determination locus vis., complementary sex determiner (csd) and feminizer (fem). Through a series of RNAi experiments, it has been shown that knockdown of csd and fem in females results in sex transformation, causing the production of male organs. It is now known that the csd gene induces the female pathway early in embryogenesis, initiating the transcription of fem, which mediates its own positive feedback system that maintains the female phenotype throughout development (Gempe *et al.*, 2009). Workers, all of which are females, arise from fertilized eggs. The eggs proceed through three developmental stages, spanning about three weeks in total, before reaching adulthood: three days as an egg, about one week as a larva during which its soft exoskeleton moults to accommodate its increasing size in stages called instars at the rate of about once per day, and the remaining time as a pupa during which metamorphosis occurs within a sealed hexagonal wax cell. Adult workers perform all the necessary tasks involved in maintaining and protecting the hive, as well as providing food for all its members. These are all sterile female bees with very small ovaries compared to the queen; in rare situations when a colony becomes queenless, these sterile individuals are

capable of laying unfertilized, haploid eggs that all become drones. Adult workers themselves can never become queens, so to replace a lost or under-performing queen a young female larva is moved to a special cup within the colony and fed a protein-rich diet of royal jelly (RJ). Workers secrete RJ when it is needed and its feeding to the young larva triggers a developmental switch that causes the larva to develop into a queen (Winston, 1987).

1.2 Life cycle of honey bees

The life stages of a honeybee are egg, larva, pupa and adult. Development from egg to adult takes 21 days. The length of these stages is set out in the table below.

Table 1: Duration of worker bee life stages

Worker Bee Life Cycle Timetable	
Hatching of egg:	**3 days**
Larva stage (Feeding):	**5 days**
Pupa stage:	**13 days**
Total:	**21 days**

Egg

The queen lays an egg in a cell of the honeycomb. The egg is cylindrical, about 1.6 mm long and 0.4 mm in diameter. When first laid, it is vertical, on the second day it bends over and on the third day it lies on its side. On the fourth day it hatches into a white legless larva.

Larva

The larva lies coiled at the bottom of the cell. It comprises of a head, thoracic and abdominal segments. The larva eats, grows bigger and sheds its shell 5 times between hatching and emerging. During the first three days the larva is fed by the nurse bees on a diet of royal jelly, a protein-rich whitish milky fluid, produced from the head glands of worker bees. After the third day, the larva is fed beebread, a mixture of honey and pollen prepared by worker bees. Six days after hatching, the curled-up larva is fully-grown, and can't feed any more (Fig. 3). At this stage the cell is sealed over with a porous covering of wax and pollen, and starts to change into a pupa.

Pupa

This is the non-feeding stage, during which the larva is transformed into an adult. On the10thday, the larva spins a cocoon (protective covering) for itself and becomes a pupa. During pupation, gradually the adult body structures forms. First the head and the thorax of the pupa develop and

change color followed by the abdomen, and finally the wings develop before emergence. Once the pupa has transformed into an adult, it is ready to emerge.

Adult

On the twenty-first day of laying the egg, the adult bee bites its way out of the capping. These eusocial insects display temporal polyethism, which is an age-driven division of labor. Younger adult workers remain in the hive and tend to develop brood, while older adult bees forage for pollen and nectar to feed the colony (Ahmad, 2010). After a bee hatches, it cleans cell and it's the neighboring cells. A newly emerged bee is light in color (greyish colored). The newly hatched bee remains in the hive apart from orientation flights for about 21 days performing various duties, as illustrated in (Table 2). When it is 22 days old, the bee becomes a forager (field bee) and leaves the hive to visit flowers. The life span of an adult worker bee varies with the time at the year. During the spring and summer, when the colony is active the workers bee may live for 5-6 weeks. During winter when the colony is selectively inactive the life span and the worker bee is 5 months or more .

Table 2: Period of worker tasks serving as a household bee

Period (days)	Tasks
1-2	Cleans cells and warm the brood nest
3-5	Feeds older larvae with honey and pollen
6-11	Feeds young larvae with royal jelly
12-17	Produces wax and constructs comb, ripens honey
18-21	Guard the hive entrance and ventilate the hive
22+	Start forage outside the hive for nectar, pollen, propolis and water

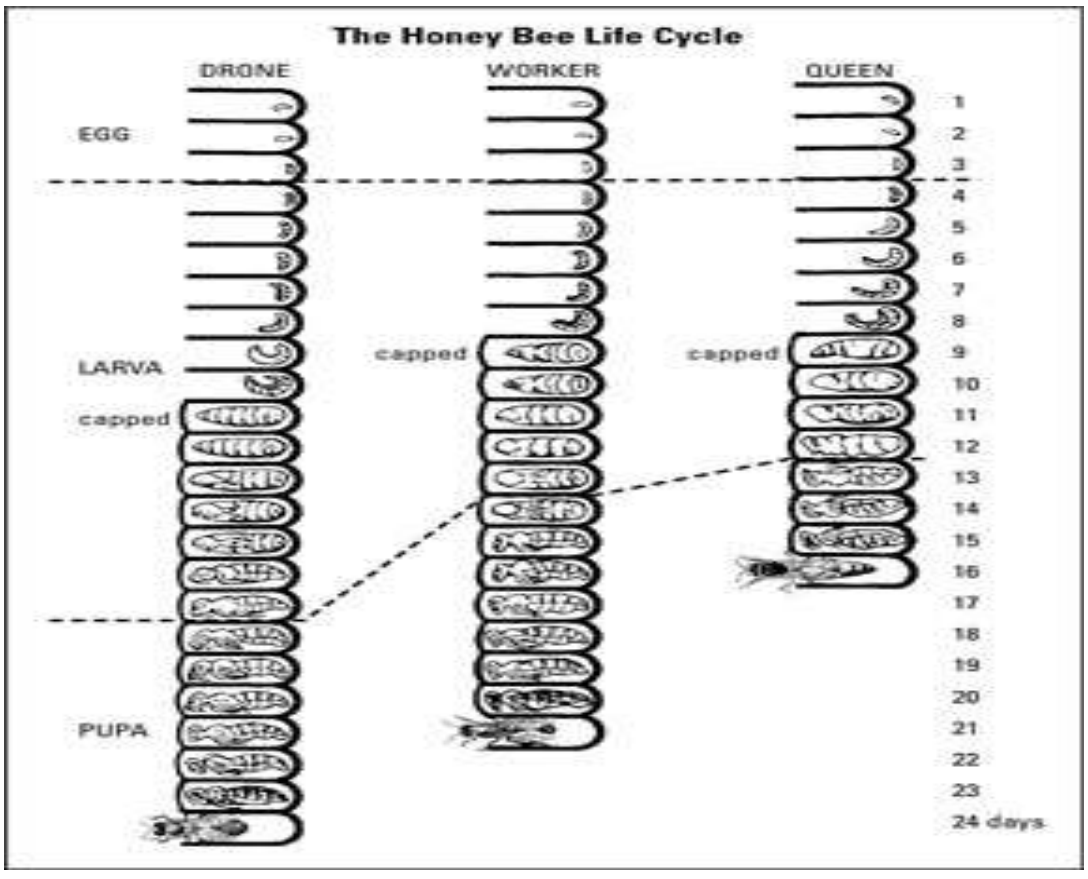


Figure (3): Chart showing the daily development cycle of all three castes, from egg to adult (Wilson and Holldobler, 2005).

1.3 Economic importance of *Apis mellifera*

Honeybees (*Apis mellifera*) are one of the well-known economic beneficial insects known to man from time immemorial . These eusocial animals display temporal polyethism, an age-driven division of labor. Younger adult bees remain in the hive and tend to develop brood, while older adult bees forage for pollen and nectar to feed the colony (Ahmad, 2010).

In Saudi Arabia Beekeeping contributes to the traditional economic development of the country by providing sustainable additional income through self employment (Al-Ghamdi and Nuru, 2013a; Al-Ghamdi and Nuru, 2013b).

1.4 Geographical distribution of *A. m . jemenitica*

The natural distribution of honey bees is extremely large, extending for 4,500 km from the Arabian Peninsula to West Africa. Different populations of *A. m. jemenitica* exhibit a high degree of morphometric variation. Different names, including *A. m. nubi*, *A. m. sudanensis*, and *A. m. bandasii* have been applied to different populations of *A. m. jemenitica.* Although African and Asian *A. m. jemenitica* are reported to be morphometrically similar, genetic data has shown African *A. m.*

jemenitica to be genetically closer to the Asian *A. m. jemenitica* than to adjacent, contiguous African subspecies like *A. m. litorea*, *A. m. adansonii* and *A. m. scutellata*, which exchange genes continuously. The African and Asian groups differ in migratory, aggressive, and brood-rearing behaviors. The present homelands of *A. m. jemenitica* (near East Asia and East Africa) have been suggested as the geographic origin of *A. mellifera*. Ruttner (1988) recognized the presence of several different populations of *A. m. jemenitica* in the Asian region. These populations vary considerably in many characters related to body size, hair length and intensity of colour (Table 2). El-Sarrag *et al.* (1992) reported the presence of two different races in Sudan vis., *A. m. sudanensis* and *A. m. nubica*.

The identity of the honey bees of Saudi Arabia is also being further studied under investigation which may prove to be a distinct subspecies, or an ecotype of the *A. m. jemenitica* found in Oman and Yemen. As the density of sampling across Africa and the Arabian Peninsula increases, it is likely there will continue to be changes and improvements in our assessment of the identity and distribution of *A. mellifera* subspecies.

Table (3): Morphometric values (mean ± standard deviation) of five populations of *A. m. jemenitica* (from Ruttner, 1988)

Population	No.	Length of tergites 3 & 4 (mm)	Proboscis length (mm)	Forewing length (mm)	Hind leg length (mm)	Hair length (mm)	Cubital vein 1 (mm)	Angle J 16 (degrees)	Tergite 4 colour
Saudi Arabia	6	3.748 ± 0.153	5.277 ± 0.210	7.868 ± 0.224	6.916 ± 0.259	0.172 ± 0.021	2.28 ± 0.25	89.94 ± 2.90	4.60 ± 0.99
Yemen & Oman	30	3.937 ± 0.137	5.481 ± 0.132	8.135 ± 0.192	7.120 ± 0.219	0.195 ± 0.020	2.20 ± 0.40	91.09 ± 4.16	4.52 ± 1.27
Somalia	9	3.981 ± 0.121	5.552 ± 0.120	8.214 ± 0.179	7.207 ± 0.203	0.213 ± 0.017	2.27 ± 0.36	99.33 ± 8.03	7.75 ± 1.03
Sudan	9	3.965 ± 0.180	5.450 ± 0.187	8.219 ± 0.214	7.214 ± 0.245	0.193 ± 0.033	2.45 ± 0.42	92.60 ± 3.49	6.38 ± 1.15
Chad	8	3.914 ± 0.121	5.356 ± 0.187	8.136 ± 0.141	7.175 ± 0.265	0.211 ± 0.019	2.39 ± 0.38	95.90 ± 3.96	5.36 ± 1.11

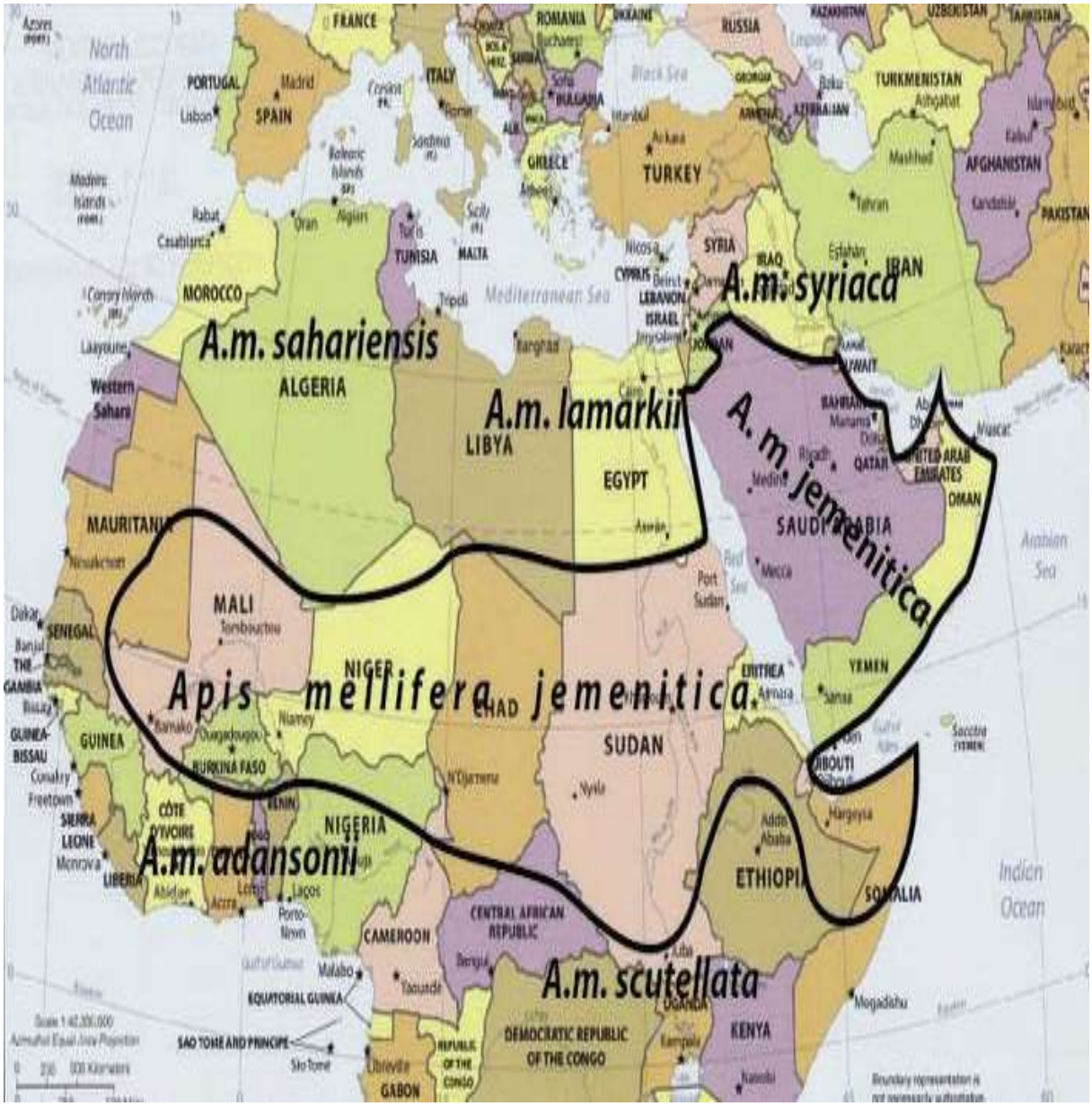


Figure (4): The distribution of *A. m. jementica* in Africa and Asia (all areas within bold line) based on published reports (Ruttner, 1988; Hepburn and Radloff, 1998).

There are two honey bee races commonly found in the Kingdom of Saudi Arabia; the indigenous bees (*A. m. jemenitica*) and the Carniolan bees (*A. m. carnica*).The indigenous race is widely spread in many parts of Saudi Arabia, in particular the west, south west and south parts while most of the Carniolan race is found in the north, east and center parts of the kingdom. The majority of Carniolan race is imported from Egypt. Most of the beekeepers in Saudi Arabia prefer to raise the indigenous bees in traditional hives (called Aoud). The Ministry of Agriculture in Saudi Arabia conducted a survey in 2009 on type of the hives use in the beekeeping, traditional and modern hives, and the ratio between the indigenous and European races. The survey results revealed that the indigenous race which was raised in traditional hives represented about 92.43% and Carniolan race which was raised in Lang stroth (modern) hives represented about 7.57%. It was also found that traditional hive yielded about 1.8 kg/colony/year. Lang stroth hive yielded, however, about 3.3 kg/colony/year. Many beekeepers have been started transferring their indigenous bees from traditional hives into Lang stroth hives to improve their characteristics and production features. (Ruttner, 1988 , Al-Ghamdi., *et al* 2013).

1.5 American Foulbrood and other bacterial diseases

Most common bacterial infections which is lethal to *A. mellifera* larval stage. American foulbrood (AFB), caused by the endospore-forming, Gram-positive bacteria *Paenibacillus larvae* (*P. larvae*), (Shimanuki, 1997), was first noted to be widespread in the United States in the beginning of the last century (White, 1906). The infectious spores, which contaminate the food stores in the hive, cannot be completely cleaned by honey bees or human intervention. Consequently, infected hives must be incinerated as the only practical means of bacteria removal, and to prevent the disease from spreading to neighboring hives. The less pervasive and less severe European foulbrood is caused by *Mellisococcus pluton* (Govan *et al.*, 1998, Sanford, 2003) and has a similar effect on the honey bee host. Due to its economic and agricultural importance, American foulbrood is an active area of research. Despite significant ongoing efforts for more than 100 years only recently the mechanism of infection has stated to be elucidated. The full picture, especially at the molecular biology level, remains far from complete. In the larval gut, the spores germinate into a rod-shaped vegetative state. It was initially thought that they breach the gut epithelium by phagocytosis (Davidson, 1973; Gregorc and Bowen,1998), and proliferate in bulk hemolymph, causing death by systemic infection (Bailey and Ball, 1991). However,

Yue *et al.* (2008) showed that proliferation occurs in the midgut and bacteria enter the hemocoel by a para cellular route, between cells of the midgut epithelium. Dancer and Chantawannakul, (1997) and Antunez *et al.* (2009) carried out and screening for proteinaceous virulence factors secreted by *P. larvae* that are used to degrade host epithelial cell-cell junctions .However there has been no clear evidence that these factors are the definitive players. Despite the lethality of infection by *P. larvae,* bees are almost completely resistant to its effects for most of their lives, except for an approximately 48-hour after egg-hatching. The LD50 for susceptible larvae is as low as 8.49 spores (Brødsgaard *et al.*, 1998), while older larvae and adults are resistant (Shimanuki, 1997, Brødsgaard *et al.*, 1998).Various theories implicating physiological and biochemical parameters have been postulated to explain this phenomenon. For example, there is an age-dependent increase in thickness of the peritrophic matrix that envelops incoming food which may help contain the bacteria in the mid gut (Bamrick, 1964). Substances extracted from larval homogenates have the ability to inhibit the growth of *P. larvae*, suggesting that the resistance is excited by chemical or biochemical, rather than physical means. Interestingly, homogenates of larvae that are 2-5 days old were found to be most inhibitory against bacterial growth

(Wedenig *et al.*, 2003), roughly mirroring the emergence of *P. larvae* resistance in the natural environment.

A qualitative investigation of infected bee larval hemocytes (blood cells) found shifts in the numbers and percentages of cells with certain morphologies, suggesting a cellular response to the infection (Zakaria, 2007). Two gene expression studies from the same authors, each exploring different ages and infection methods, show that in young larvae, a very small number of genes respond to *P. larvae* challenge. However, the results were not consistent between the studies (i.e. opposite regulation trends for a gene encoding for abaecin, an antimicrobial peptide), and most of the genes with putative roles in immunity showed a reduction in gene expression (Evans, 2004, Evans et al., 2006). Furthermore, it is not known whether the transcript levels correlate with biosynthesis of the corresponding enzymes. How *P. larvae* infect a bee within short time to induce a fatal consequence remains an unanswered question.

1.6 Developmental biology of honey bee larvae

There are four stages of development in the life of a honey bee: egg, larva, pupa, and adult. The pre-adult stages are sometimes collectively called “brood”. Apart from feeding and expanding in size, the larva does

little else. On the molecular level, however, several major changes that take place imply the presence of intricate biochemical controls at work. For example, dietary levels of protein in the form of major royal jelly proteins (MRJPs) within two days of eclosion determine whether a larva is destined to be a queen or a worker, due to epigenetic controls (Elango *et al.*, 2009). Huge metabolic demands and a corresponding increase in efficiency of protein synthesis allow a body mass to increase 900-fold (in the case of workers). Within this five- to six-day-long developmental stage to the larvae increase about 140 mg, and growing from a 2.7 mm egg to nearly its adult size of 1.7 cm (Winston, 1987).The overall anatomy of larvae does not change drastically over this five- to six day long phase, despite dramatic size changes. It has a simple body structure, containing mostly the organs essential for feeding and digestion, namely the mouth and mid gut, with the latter taking up the majority of space within the body cavity. Excretory tubules and a hindgut (rectum) are present but are not connected to the mid gut until the end of larval development, which is about the time when feeding stops. They have an exoskeleton, which is white and soft relative to that of adults, and is shed about once a day during this stage of rapid growth to reflect its increasing size. With the matured silk glands, bees begin to spin a cocoon in the last half of larval development. Despite having no legs, they are able to

wiggle and uncurl from the C-shaped position taken up earlier in larval life, so that their heads point towards the cap of their hexagonal wax cells. By the fifth or sixth day from eclosion, their cell is sealed with wax by an adult worker bee (Sulaimon and Kitchell, 2003) .

1.7 Nutrition

The basic building blocks of honey bees are derived from nectar and pollen, providing carbohydrates and proteins, respectively. Workers take up nectar in their foregut (crop) and redeposit the solution into an individual cell in the honeycomb. In doing so, they also add enzymes from their hypo pharyngeal glands. These enzymes are either involved in the breakdown of sugars or confer anti-bacterial properties. Pollen grains, which are the male germ plasm of plants, are treated with phytocidal acid before storage to prevent germination and bacterial spoilage. Bees, which are not equipped with teeth, are presumed to have enzymes in their gut for breakdown of the pollen wall. Larvae, having no legs or other means of transport, are completely dependent on adults for food. One report stated that, on average over the larval period, each larva is inspected 1926 times and fed during 143 visits (Lindauer, 1952); another group observed 7200 visits and 1140 feedings. The frequency of contact between bees is a major factor for disease transmission (Winston, 1987). Larvae are semi-

immersed in a milky white, slightly viscous fluid, which is deposited by workers. This is a mixture containing clear secretion from the hypo pharyngeal glands and a milky white substance from the mandibular gland of adult nurse bees. As the larva approaches the third instar, the white component decreases, and the food is directly mixed with increasing amounts of honey and pollen. Contents of glandular secretions have not been well documented, but it presumably contains vital growth factors. A formulation containing vitamins, minerals, and free amino acids supplemented with Royal jelly (RJ) extracts is able to support the development of normal-appearing adults (Shuel and Dixon,1986). RJ is the milky white substance rich in proteins and lipids that is fed to honey bees during larval development. Larvae, prior to the third instar, can become a queen or worker depending on the amount of RJ fed: higher amounts will generate queens and lower amounts will produce workers. It is also used to feed adult foragers (Crailsheim, 2004). RJ has been shown to have many different enzymatic activities like ascorbin oxidase , amylase, inverters, catalase, acid phosphatase etc. (Albert *et al*., 1999). Its reputation as a health food product comes from numerous reports, with data to indicate that it decreases blood pressure (Sultana *et al.*, 2008), lowers triglyceride and very-low-density lipoprotein levels (Guo *et al.*, 2007), and inhibits or induces T-cell proliferation depending on the

concentration or component used (Gasic *et al.*, 2007). Due to the commercial value of this bee product, it is not surprising that RJ was the topic of the first bee proteomics publication (Scarselli *et al.*, 2005) and several subsequent studies (reviewed in (Chan *et al.*, 2006)). Despite this research, the value of human consumption of RJ is still not clear. For the bees, the RJ is thought to provide building blocks for growth and protect them from infections.

1.8 Proteins in developing larvae

1.8.1 Juvenile hormone processing proteins

Juvenile hormone is a small molecule produced in many organs found on the sides of the esophagus in larva and adults (Winston, 1987). Of the several subtypes found in insects, the honey bee expresses only juvenile hormone III (Hagenguth and Remboldt, 1978). As the name suggests, it maintains larvae in the juvenile state by preventing metamorphosis. Degradation of juvenile hormone with juvenile hormone esterase or juvenile hormone epoxide hydrolase causes hormone levels drop below the threshold causing larva to transform into a pupa. This temporal gradient of the hormone controls the level of proteins and biological activities associated with larval maturation.

As with all insects, blood of bees called hemolymph which is distributed throughout the body in an open-circulatory system. Like mammalian blood, this fluid contains various nutrients, proteins, hormones and waste products from the organs and their individual cells. The protein content of hemolymph varies drastically according to age, sex, and season. The study of hemolymph provides on insight into the physiological state of the individual (Antunez *et al* ., 2009).

1.8.2 Insect storage proteins

During the honey bee pupal stage, adults seal the cells containing these developing individuals using wax. Despite the increased metabolic demands due to development growing legs, wings, eyes, and all other adult organs (many of which are non-existent in the larval form), the pupa are not fed during this time. The increased requirement and biomolecules is meet by large amounts of lipid accumulated during the larval stage. The amino acids necessary for this massive biosynthetic activity are stored in proteins called hexamerins (Levenbook and Bauer ,1984), which are composed of six identical. The fat body, which is scattered throughout the body, is the major source of hexamerins. This organ has two functional phases during larval development: first, hexamerin synthesis and subsequent release into the hemolymph, and second, reuptake of this

protein by receptor-mediated endocytosis before transition into the pupal stage (Rao *et al*., 2004).

Before protein sequencing became commonplace, hexamerins were named according to their molecular weight as observed in polyacrylamide gel electrophoresis experiments. The nomenclature of these subunits was more unified after it was discovered that several distinct bands were actually derived from the same transcript. There are four known genes encoding hexamerin subunits: hexamerin110 (HEX110) (Bitondi *et al*., 2006), hexamerin 70a (HEX70a) (Martins *et al.,* 2008), *hexamerin 70b* (HEX70b), and hexamerin 70c (HEX70c) (less studied). Subunits HEX70b and HEX110 are highly abundant during larval development and are significantly less abundant in pupa and adults. They are most widely studied due to their abundance and easy detection abundant. The less understood HEX70a and HEX70c are generally expected to have similar expression patterns. Of these, HEX70a is the only subunit documented to be prevalent in adult hemolymph also suggesting that they have a more specialized role.

Hexamerin expression is highly regulated: their levels in the hemolymph increase sharply in the last two days of larval development, and decrease quickly almost immediately upon entry into the pupa stage. This cessation of hexamerin expression is linked with a decrease of juvenile

hormone, an event that signals the larva-pupa transition (Martins and Bitondi,2012).

Twenty-two proteins of the whole larval proteome have been quantified with respect to age, reporting up-regulation of proteins for carbohydrate metabolism, down-regulation of heat shock proteins, and an increase of an imaginal disc growth factor (Li *et al.*, 2007).

1.8.3 Major royal jelly proteins

Royal jelly contains a number of different biological activities. 90% of its protein content is made up of the MRJP protein family. They are produced mainly in the hypo pharyngeal glands of young adult workers as a form of nutrient to feed immature bees. All of them have N-terminal regions that direct their secretion. Currently there are eight known MRJPs, with MRJP1-4 being the most abundant, and therefore better studied; MRJP5-8 are relatively recent discoveries. Three of these in particular – MRJP1, MRJP2, MRJP5 – have large amounts of the ten essential amino acids (Schmitzova *et al.*, 1998). Though they have a role in nutrition, their genes are also expressed in the brain (Garcia *et al.*, 2009).

Two-dimensional gel electrophoresis patterns of RJ and the subsequent proteomic identification suggests that MRJPs are highly phosphorylated and glycosylated (Furusawa *et al.*, 2008).

REVIEW OF LITERATURE

2 REVIEW OF LITERATURE

2.1 Insect Immunity

Insects have diverse immune mechanisms to overcome infection, *via* the innate and/or induced immune responses. Immunity in insects involves cellular and humoral immune responses

(Brey and Hultmark, 1998). Phagocytosis or nodule formation and encapsulation of macro organisms, such as parasites, constitute the main cellular response mechanisms mediated by hemocytes (Ratcliffe and Rowley, 1979; Lackie, 1988; Kotthoff *et al*., 2011). These immune responses are always immediate and nonspecific (Townson and Chaithong 1991; Dimopoulos *et al.*, 2001). Humoral immunity in insects involves induction of antimicrobial peptides for fighting against invading pathogens. They are synthesized mainly in insect fat bodies and in certain hemocytes as a response to microbial infection (Irving *et al.*, 2005; Amaral and Neto, 2010). Upon microbial infection, these peptides are rapidly released into hemolymph where they act against the invading microorganisms (Hetru *et al*., 1998; Kavanagh and Reeves, 2004; Gatschenberger *et al*., 2013).

Insects are capable of producing different antibacterial peptides depending on the type of invading bacteria (Boman and Hultmark 1987; Boman, 1991 & 1995). In *Drosophila* for example, Gram-negative

bacteria elicit diptericin, drosocin, cecropin and attacin , while Gram-positive bacteria elicit defensin, and fungi produce drosomycin (Tzou *et al.*, 2002; Irving *et al.*, 2004).

The immune antimicrobial humoral response of insect induces antimicrobial peptides which play a crucial role in innate immunity against a wide range of invading microorganisms. These Immune antimicrobial peptides (IAMPs) are capable of killing a wide range of microorganisms (Boman and Hultmark 1987; Hoffmann, 1995). Thus, studying the insect IAMPs gives an insight into the innate immunity of invertebrates and produces templates for designing novel, broad-spectrum antibiotics that would function in overcoming antibiotic resistance in humans (Kopacek *et al*., 1995; Giles *et al*., 2002). AMPs have shown promising effects against skin infection (Nizet *et al*., 2001), enterococcal infection (Montecalvo, 2003) and some human diseases like cancer (Chen *et al*., 1997; Lu and Chen, 2010).

Insect IAMPs have emerged as the most promising group of candidates for the development of a new class of antibiotics (Hancock, 1997b; Silva, 2004; Lu and Chen, 2010). Hence, the analysis of their potential pharmacological properties and drug discovery have recently been undertaken (Cherniak, 2010; Rajanbabu and Chen, 2011; Ratcliffe *et al*., 2011). Promising examples of AMP-derived antibiotics have already

been provided for clinical purposes (Nizet *et al*., 2001; Chen *et al*., 2009). Expression of these peptides in bacteria is, by far, the simplest and most inexpensive means to produce large amounts of these products (Valore and Ganz, 1997; Rao *et al*., 2004 ; Niu *et al*., 2008).

2.2 Major honey bee diseases

The honey bee is an excellent model of disease control in high population densities, where individuals are extremely social and have frequent transfer of body fluids. Tropholaxis (mouth-to-mouth food sharing) occurs frequently between adults. Adults feed glandular secretions of food to immature bees in the larval stage. Because of their unique hive environment, honey bees are susceptible to a number of species-specific pathogens, many of which are asymptomatic unless the host is already under stress from other diseases (Brødsgaard *et al.,* 2000). This is particularly true of viral infections, with Israeli acute paralysis virus being an excellent example: This virus has been linked to Colony Collapse Disorder (Chen and Siede, 2007; Cox-Foster *et al.,* 2007). There is mounting evidence that fungal infection by *Nosema ceranae* may also be related to this disease (Higes *et al.,* 2008). In addition the bees are also exposed to possible threats from bacteria, parasites, viruses, insecticides, and artificial bee food (Cox-Foster *et al.,* 2007; Oldroyd, 2007; Stokstad,

2007; Aliouane *et al.*, 2008; Higes *et al.*,2008). Other common viruses include sacbrood virus, deformed wing virus (DWV), and Kashmir bee virus, all of which have positive-sense, single-stranded RNA genomes (Chen *et al.*, 2006).

Significant losses have been attributed to the parasitic mite *Varroa destructor*, which feeds on the hemolymph of immature bees (Sammataro *et al.*, 2000), and to a lesser extent to *Acarapis woodi* (McMullan and Brown, 2006), which resides the trachea of adult bees and fungi like *N. ceranae*. As honey bees mature, the types of pathogens that infect them change. Therefore, the pathogen pressure may affect bees differently throughout their lifespan. The high population density in a bee colony implies a high rate of disease transmission among individuals. Bees have been predicted to express only two-thirds of immunity genes as solitary insects, e.g., mosquito or fruit fly (Tzou *et al.*, 2002). Though the immune response in bees is subdued in favor of social immunity, some specific immune factors are up-regulated in response to infection (Evans, 2004). Bees are constantly under threat due to combined damage from bacteria, parasites, viruses, pesticides, and artificial bee food (Cox-Foster *et al.*, 2007; Oldroyd, 2007; Stokstad, 2007;Aliouane *et al.*, 2008; Higes *et al.*, 2008) this is no more evident than in the case of Colony Collapse Disorder which resulted in United States beekeeping operations losing an

average of 40% of their colonies, and in extreme cases as much as 90% (Cox-Foster *et al.*, 2007). Bee research is necessary to help ensure that bees remain available to help us. Furthermore, there is still much to learn about their unique behavior and biology.

Insect IAMPs are active against both bacterial types of different Gram-staining patterns, with minimal inhibitory concentrations (MICs) (Casteels *et al.*, 1989),as well as affecting DNA and protein synthesis (Bulet *et al*., 1999; Matsuzaki, 1999). Most of the known AMPs cause microbial membrane disintegration (Yeaman and Yount, 2003). Other antibacterial peptides can kill bacterial cells by causing protein precipitation in the cytoplasm (Brogden *et al*., 2003; Yeaman and Yount, 2003). It has been shown that microbial resistance is generally less likely to occur with insect antibacterial peptides than with conventional antibiotics (Steinberg *et al*., 1997; Zhang *et al*., 2005). This is because insects produce different antibacterial peptides simultaneously, which work synergistically against the different invading bacteria (Tzou *et al*., 2002). Therefore, these peptides alternatives for the classical antibiotics because they are characterized by selectivity, fast killing, broad antimicrobial spectra, and development of little resistance against them (Zasloff, 1992; Huang, 2000). Ratcliffe *et al*.(2011) reported that the site of action of antibacterial peptides is the bacterial outer membrane, (killing

occurring within seconds) making quick re-configuration of this membrane by bacteria difficult.

Honeybees produce defensin, abaecin and hymenoptaecin as humoral antimicrobial immune peptides (Casteels *et al*., 1989; 1990; 1993; Casteels, 1998; Evans, 2004). Apidaecins are proline-rich antibacterial peptides are also produced in honeybees (Evans, 2004; Evans *et al*., 2013).

The innate immune strength has been found to vary between bees' developmental stages (Wilson-Rich *et al*., 2008; Laughton *et al*., 2011). Different sexes and life stages have been found to alter their immune system management based on the combined factors of disease risk and life history (Schmid *et al*., 2008; Laughton *et al*., 2011; Siede *et al* ., 2012).

The immune peptides from the honeybees, *Apis florae* and *Apis carnica* have been isolated ,and showed antibacterial activities against the human pathogenic bacterium, *Klebsiella pneumoniae*, strain (ATCC 11678) that causes urinary tract infections (Ayaad *et al*., 2012). Based on previous work and that of others (Evans, 2004; Evans *et al*., 2006 ; Chen *et al*., 2009; Janesch *et al*.,2015). (Shipman *et al*.,1987; Alippi *et al*.,2005; Honeybee Genome Sequencing Consortium, 2006;Yue *et al*. ,2008). (Evans *et al*., 2006) have described the isolation of bacteria *P. larvae* that

infected honey bee larvae and caused disease with symptoms of AFB. A growth inhibition zone was described by (Hultmark ,1998 ; Mundo *et al.* ,2004).

lysozyme-like activity against Gram positive bacteria and cecropin-like activity against Gram negative bacteria are demonstrated, because they possess antimicrobial responses. These responses are known to be good estimates of disease resistance (ability of insects to mount an effective immune response) (Schneider, 1985; Rantala *et al*., 2002; Adamo, 2004). Hence, they are important components of the insect immune system.

PO involved in cuticular sclerotization in newly hatched larvae, performs different immunological functions such as melanin biosynthesis (for melanization), wound healing, nodule formation (nodulation), humoral encapsulation, and phagocytosis (Cerenius and Soderhall, 2004). PO activity is also an indicator investigated of insect resistance as to a particular entomopathogenic agent (Saul and Sugumaran ,1988 ; Kopacek *et al.* ,1995; Nigam *et al*.,1997;Da-Silva *et al*., 2000; Adamo *et al* .,2001; Adamo, 2004 ;Lourenco *et al*.,2005; Laughton and Siva-Jothy, 2010 ; Laughton *et al*., 2011).

Nodulation is an insect cellular immune response that is activated by bacterial, fungal and some viral infections. The number of nodules

formed reflects the extent of the nodulation response (Da Silva *et al* .,2000; Niu *et al.*, 2008).

2.3 Biochemistry of honey bee immunity

Insect innate immunity can be divided into cellular and humoral responses, though the categorization is only an artificial one, since the regulation of both are tightly linked. Encapsulation, nodulation, and phagocytosis are considered part of the cellular response. These aspects are generally less well understood than humoral responses, which include antimicrobial peptides, complement, and humoral melanization. This is especially true with regards to antimicrobial peptides, was expression is regulated by the Toll and Immune Deficiency pathways. Most of these pathways have been elucidated in Drosophila (extensively reviewed in (Bulet *et al.*,1993), and are believed to exist in the honey bee as well (Evans *et al.*, 2006).

Bees, like other insects, are equipped with an innate immune system. They have no adaptive immunity and therefore lack lymphocytes, or any of their subtypes such as eosinophils and neutrophils and they cannot synthesize antibodies. As a result, it is not suitable to treat mammalian

and insect immunity as entirely analogous, although some aspects are similar, e.g., Toll and Toll-like receptors. The honey bee colony presents an interesting opportunity to study disease progression in a community environment, in contrast for example to *D. melanogaster* which are solitary species. However, the social nature of bees and their high population density within a hive presents complications for research: genes for hygienic behaviour (Lapidge *et al.*, 2002) and queen bee mating promiscuity (Seeley and Tarpy, 2007) have been linked to disease resistance, factors which must be considered in experiment design. Given the high pathogen load conferred by their eusocial lifestyle, one might expect that bees have more immunity genes than solitary insects. Comparative genomics tells us that bees have significantly fewer of these genes than Anopheles and Drosophila (Evans *et al.,* 2006; Chen *et al.*,2013). Bees' unique living environment, relatively clean food sources such as honey (Mundo *et al.*, 2004) mean that they may face fewer and more niche-specific pathogens. Thus they require fewer defenses in contrast this to the fruit fly, which feeds notoriously on rotting food and

other wastes. *A. m. jemenitica* has a wide distribution (4500 km from East to West) in tropical Africa, and in the hot desert of the Arabian Peninsula, having been recorded from Chad, Ethiopia, Somalia, Sudan, Oman, Yemen, and Saudi Arabia. It is found in the areas of highest seasonal temperature as well as the zone of lowest and most irregular rainfall, regions where other honey bee races have not been able to persist (Alqarni *et al*., 2011). Although the native Saudi honey bee is recognized as *Apis mellifera jemenitica* , it has some significant morphological and biological differences from its conspecifics, as well as some other populations of this same race in Africa . It has been noted that the Saudi honey bee samples were clearly distinct (i.e., smaller, more slender, shorter setae, and more yellow in coloration), while adding that there was more homogeneity between Yemeni and Sudanese bees (Ruttner ,1988). This led him to be the first to consider the Saudi populations as a distinct ecotype of *A. m. jemenitica.* Most Saudi honey bee samples have been clustered with *A. m. jemenitica* reference group, but others were more similar to *Apis mellifera litorea* reference group (Alqarni *et al*., 2011) .

2.4 Salmonellosis

Food poisoning is becoming a global concern that recognized as a major health concern in the Kingdom of Saudi Arabia (KSA). *Salmonella* food poisoning is mostly a result of consuming inadequately cooked meat or contaminated meat products, poultry, dairy products and raw eggs (Pui *et al*., 2011). In KSA over the last 30 years, rapid socio economic development and civilization have contributed in a major way to dissemination of food borne pathogens. Also, expansion of fast food business established by poorly educated personnel, who do not possess adequate training in food safety as improper handling of food stuffs and unhygienic cooking conditions has problem contributed to this situation (Cetinkaya *et al.,* 2008). As early as 1998, Al-Ahamadi *et al.* (1998) studied an outbreak of food poisoning associated with restaurant-made mayonnaise in Abha, Saudi Arabia. They found *Salmonella enterica* was isolated from 124 (84%) of the 159 persons with symptoms of food poisoning, and 91 (73%) belonged to serogroup Enteritidis. Salmonellosis is characterized by acute onset of fever, abdominal pain, diarrhea, nausea and sometimes vomiting. The onset of disease symptoms occurs 72 hours after ingestion of *salmonella*, and illness lasts 2-7 days (Pui *et al.*, 2011).

The appearance of multiple drug resistance to quinolones by *Salmonella* poses a serious therapeutic problem and it is a matter of great disquiet for physicians and microbiologists (Nakaya *et al.*, 2003). This is due to the use of quinolones in animal food which has lead to an increase in resistance (Panhotra *et al.*, 2004). The emergence of antibiotic-resistant pathogenic bacteria has given rise to the needs of antimicrobial agents that are derived from natural products which are less likely to cause pathogen resistance. The most profound and traditional example of the antimicrobial from the nature is antimicrobial peptides (AMPs) that serve as natural innate barriers limiting microbial infection or act as an integral component in response to inflammation or microbial infection.

Bacterial adhesion to host mucosal surfaces is often a first step in the establishment of an infection via the interaction of bacterial surface components with distinct host receptors. Many bacterial pathogens use subcellular surface appendages such as pili (fimbriae) and flagella that radiate from the bacterial surface for initial adherence (Allen-Vercoe and Woodward 1999). After oral infection, *Salmonella* colonizes the intestinal tract followed by adhesion to and invasion of the intestinal epithelium which may be followed by the development of enteritis in susceptible mammalian hosts (Setta *et al*., 2012). Some times *Salmonella* has the ability to colonize intestinal tract without causing clinical symptoms of

disease (Bearson *et al.,* 2013), this is the main reasons for difficulty in controlling the pathogen.

2.5 Aims of the study

In this study the immune strength of honey bees has been quantified using standard immunocompetence assays viz., solid growth inhibition zone assay, melanization against latex beads, lysozyme-like and cecropin-like activities assays and phenoloxidase activity.

Moreover purification and characterization of antibacterial peptides from the hemolymph plasma of bacterial immunized larvae of the Saudi native honeybee *A. m. jemenitica* against pathogenic bacteria viz., honeybee brood pathogenic bacteria, *Paenibacillus larvae* (*P. larvae*) and the human pathogenic bacteria ,*Salmonella enteritidis* (*S. enteritidis*) were investigated . The identification and characterization of these antibacterial peptides may help to identify a potential natural candidate antibiotic against bacterial diseases of human and honey bees that may further provide a boast to bee keeping industry in KSA.

The current work aims primarily at exploring the humoral immune responses of the larval stages of the native honeybee, *A. m. jemenitica* , in response to bacterial challenge. Moreover, the evaluation of the resultant

effective antibacterial immune peptides against *P. larvae* and *S. enteritidis in vivo* and *in vitro*. And also purification of candidate antibacterial immune peptide(s) from *A. m. jemenitica* 3rd instar to apply them against diseases of bees caused by *P. larvae* and to explore and highlight the diverse natural purified peptides that have a potential antimicrobial effect and to evaluate the ability of the isolated purified peptide fractions to prevent the attachment of *S. enteritidis* (the causative agent for food poisoning) to human epithelial cells *in vitro*.

MATERIALS AND METHODS

3 MATERIALS AND METHODS

Insect materials and rearing

3.1 Royal Diet preparation

The larval diet consisting of 50% natural royal jelly (RJ) (w/v) in distilled water was used to feed the larvae in vitro. The fresh RJ was obtained from a beekeeper, divided into 5g aliquots and kept at -18°C. Prior to any treatment, the required amount of diet was prepared and kept at 5°C during the feeding period of the experiment. The total estimated amount of diet was about 160 µl during larval development (Chan *et al.* ,2009). The diet was pre-warmed to 35°C in an incubator prior to each feeding.

3.2 Grafting of *A. m. jemenitica* larvae

The method of Chan *et al.* (2009) with some modifications was used to obtain the larvae of similar ages (3^{rd} instars,72 h). Ten healthy honeybee colonies, each headed with young mated queen, were chosen for these experiments. The queen in each colony was confined to a fully drawn empty comb in a cage (46 x 24 x 6 cm) made of queen excluder material as described by Pierce *et al*. (1997). The comb was placed in the center of brood nest and after 36 hours the queen was released. The comb was again returned to the cage to prevent the queen from laying new eggs.

The ages of the laid eggs were about 24h; as previously reported, the queens need 6- 12h to adapt themselves to the new confinement conditions before eggs laying Chan *et al.* (2009). On the fourth day hatching was started and the worker bees were allowed to move in and out the exclude cage, to feed the larvae after eggs hatching.

Hand-made trays containing 48-queen cell starter cups (Figure 5A)were fixed with modeling clay and supplied with 50µl of the pre-warmed diet (50% royal jelly (v/v), and 50 % autoclaved aqueous solution of D-glucose (12% w/v) and D-fructose (12% w/v) for grafting the larvae. One larva was reared per well and the exact amount of diet that a larva consumes daily was administered. The total estimated amount of diet was 160 µl during larval development (Chan *et al.* 2009).

Grafting larvae was done using a grafting needle, to queen cell starter cups fixed in the plastic tray according to Aupinel *et al.* (2005). Larvae representing the 2nd to the 3rd stages were grafted from the worker cells to the hand made cups for further experimentation of immune induction at 4,18,24 and 48h post injections (Figure 5B) .

Figure (5): A) Hand-made trays containing 48-queen cell starter cups. Cups were fixed with modeling clay and supplied with 50µl of the pre-warmed diet (50% (w/v) natural royal jelly (RJ), and 50% distilled water) for the grafting larvae. B) One larva are reared per well and the exact amount of diet that a larva consumes daily are administered.

Figure (6): Manual investigation of brood in their combs, for detection of any signs of AFB . (A and B). C: Diagnostic kit used in the field. D: workers and larval stages in their breeding cells.

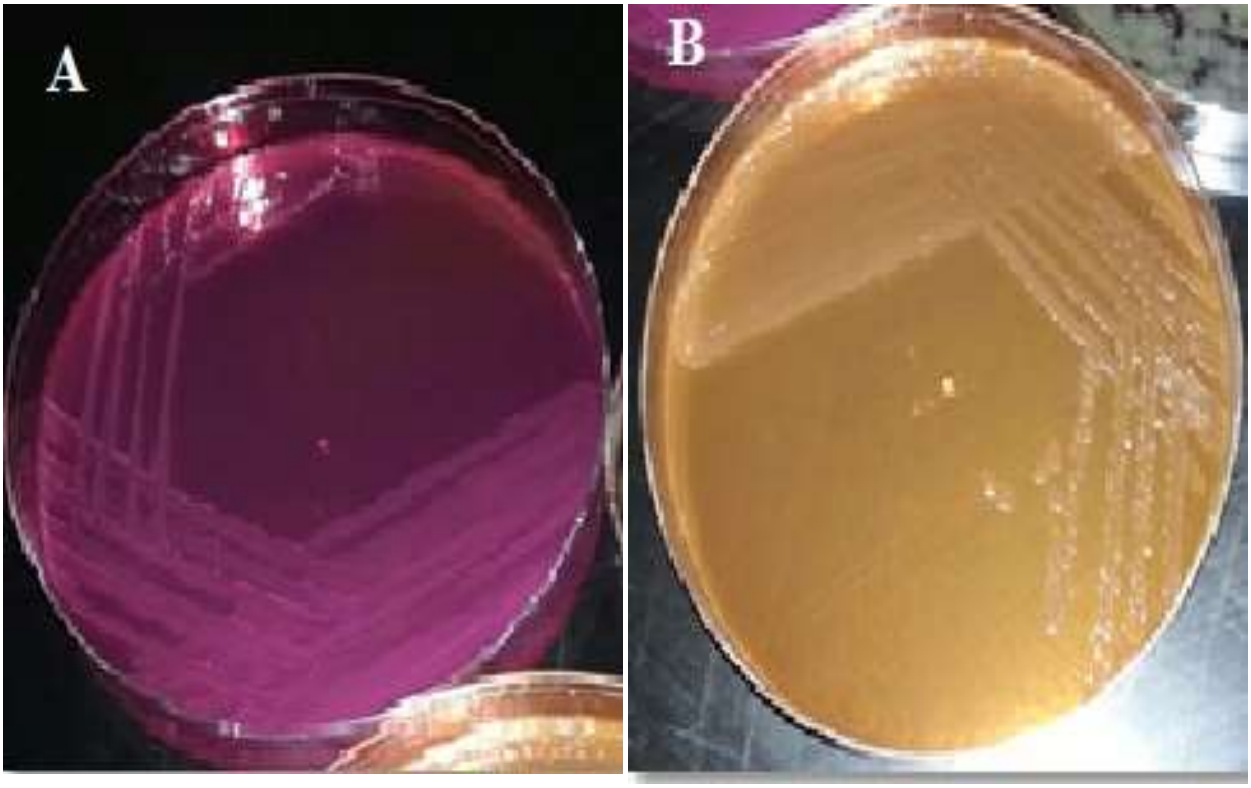
3.3 Maintenance of experimental bacteria and growth conditions

Experiments were carried out for maintaining the experimental microorganisms used in the present work. Standard human pathogenic bacteria *Salmonella enteritidis* (ATCC 13076; Basingstoke, UK) and reference bacteria, *Escherichia coli* (ATCC 10536) *Micrococcus luteus* , National Collection of Type Cultures NCTC 2665; Sigma, UK) , *Staphylococcus aureus* (ATCC 6538) , *Bacillus subtilus* (ATCC 6051) , *Pseudomonas aeruginosa* (ATCC 9027) were purchased from (Sigma, UK) and maintained at – 20°C in brain heart infusion broth with 20% (v/v) glycerol for other experiments .

AFB has not been detected in Apiaries of targeted locality in Saudi Arabia during the survey (February , 2013 -2014) (Figure 6).

Paenibacillus larvae (*P. larvae*) (American Type Culture Collection (ATCC) 10801, White) the causative organism for AFB, were purchased from(Sigma, UK). VA 20110 USA. *P. larvae* were grown in brain heart infusion agar. BHIT(brain heart infusion medium supplemented with thiamine) agar suspended 47 g brain heart infusion agar (Oxoid) in 1 liter of distilled water. Autoclave at 121 c for 15 min. Cooling to 50ºC add sterile solution of thiamine hydrochloride to obtain a final concentration of 1mg per liter. Bacterial suspensions from overnight cultures of *P.*

larvae and / or *S. enteritidis* were prepared and standardized to have a turbidity equivalent in density to no. 0.5 McFarland standards approximately 1.5×10^8 colony-forming units (CFU)/ml then diluted to reach $1x10^6$ CFU /ml and spread on BHI agar plates (Figure 7).The plates were incubated at 35° C for 24 h and BHI medium alone was used as a control (Alippi *et al*., 2005). The gram negative bacteria are identified using growth off Gram-negative bacteria on MacConkey Agar Plates(Figure 7).



Figure(7): Growth of Gram-negative bacteria on MacConkey Agar Plates. A: *E.coli* gives pink colour colonies (lactose fermenter). B: Salmonella gives colourless colonies (non lactose fermenter).

3.3.1 Preparation of the McFarland standard

0.5 ml of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2$.$2H_2O$) was added to 99.5 ml of 0.18 M H_2SO_2 (1% v/v) with constant stirring. They were distributed into screw cap tubes of the same sizes and volume as those used in growing the broth cultures. The tubes were tightly sealed to prevent evaporation. The tubes were protected from light and kept at room temperature. Before use turbid tubes are mixed by vortex. Standards can be used for 6 months (NCCLS; 2003).

3.4 Immune induction of *A. m. jemenitica* 3rd instar larvae using LPS and different reference and pathogenic bacteria

Bacterial injections were carried out as described by Ahmad and Hoopingarner (2002) and Laughton *et al*. (2011). Control larvae (3rd instars,72 h old) were immobilized on ice. Sterilized with 70% (v/v) ethanol and then injected with LPS (20 ng/larva), *P. larvae* , *S. entritidis* , *E. coli* and *M. luteus* (1×10^6cfu/larva) using a Hamilton microsyringe. Larvae (10,each) were then maintained for recovery in standard suitable rearing conditions . Active larvae were chilled for 5 min on ice, surface sterilized with 70% (v/v) ethanol and used for hemolymph plasma and hemocyte separation.

3.5 Hemolymph plasma and hemocyte separation

Clear hemolymph samples were withdrawn 4,18,24 and 48h post- larval injection for isolation of hemolymph plasma and hemocytes as described (Zufelatoa *et al*., 2004; Laughton *et al*., 2011). 3rd instar larvae (72 h-old) (n=10) were washed in 10 ml of 70% (v/v) ethanol to reduce contamination. An incision was made on the left side of each larvae .The oozing milky white clear hemolymph was withdrawn with a glass capillary. larval hemolymph was kept in ice chilled microfuge tubes containing equal volume of anticoagulant (AC) (98 mM NaOH, 186 mM NaCl, 17 mM EDTA and 41 mM citric acid, pH 4.5) with EDTA-free Protease Inhibitor Cocktail . The micro centrifuge tubes were spun at 1500 rpm in a micro centrifuge (Biofugefresco, Heraeus, and D- 3752)for 10 min at 4°C to pellet hemocytes from larval hemolymph only which were kept for further cellular reactions (Latex beads phagocytosis). Clear plasma were decanted into1.5 ml Eppendorf tubes and kept at -80° C for further experiments.

3.5.1 Protein estimation

Total protein concentration of clarified plasma of 3rd larval instar and /or adult workers (hemocyte - free) were estimated by the method of Bradford (1976) using Coomassie Blue Protein Assay Reagent (ICI

Americas, Inc.) according to the manufacturer's instructions . For the calibration curve bovine serum albumin was used .

3.5.2 (Solid growth inhibition zone assay) (Diffusion assay, CLSI , 2010)

Plates were prepared by dispensing 25ml of a sterile Diagnostic Sensitivity Test agar (DST; Oxoid) into 100mm sterile Petri dishes. Growth inhibition zone assay was carried out as described by Yamauchi (2001) and Ayaad *et al*. (2012). The solidified agar plates were streaked uniformly with each of cultured bacteria : Gram-positive *M. luteus* (NCTC 2665), *P. larvae* (ATCC 10801) and Gram-negative bacteria *E. coli* (ATCC 10536), *S. enteritidis* (ATCC 13076) , separately . Four equidistant wells, with a diameter of 5 mm, were made in the agar . Each well was filled with 50 µl of diluted plasma (estimated protein concentration) of 3rd instar larvae either injected with *P. larvae* or *S. enteritidis* compared to controls (non injected) . All the plates were left for 1 h at room temperature for proper diffusion after which they were incubated at 37 °C for 24 h. The diameter of inhibition zones was measured in millimeters (mm). Assay was carried out in triplicate. The presence of definite inhibition zones around each well indicated

antibacterial activity. Positive control using Ciprafoxacin (discs) antibiotic was also used for comparison (Hultmark ,1998;Mundo *et al*.,2004).

3.5.3 Antibacterial activity of plasma obtained from injected *A. m. jemenitica* 3rd instar larvae

Groups of larvae (third instar) (100 larvae each) were injected with *P. larvae* or *S. enteritidis* as well as *M. luteus* or *E. coli* as described previously. Hemolymph was collected at 24 h post-injection in each case and was subjected to inhibition zone assay against *P. larvae* or *S. enteritidis*, or *M. luteus* and *E. coli*. An inhibition zone assay was carried out in the same manner as described previously. Any potentially active hemolymph against *P. larvae* and/or *S. enteritidis* was used for peptide purification and performing the next phases of the study.

3.6 Purification of *A. m. jemenitica* 3rd instar larvae immune peptides by reversed-phase high-performance liquid chromatography (RP-HPLC)

The crude peptides obtained from each larval injection , separately were dissolved in 10% acetic acid and purified by a reversed-phase high-

performance liquid chromatography (RP-HPLC) on a preparative (15 µm, 10 × 250 mm) C18 Bondapak column using a water-acetonitrile gradient [30 - 70% / 30 min] containing 0.05% trifluoroacetic acid (TFA) Purity of the purified peptides was checked by a RP- HPLC on an analytical (10 µm, 4.6 × 150mm) C18 Pepmap column using a water-acetonitrile gradient [30 - 70% / 30 min] containing 0.05% trifluoroacetic acid (TFA). according to the method described by Xiao *et al.* (2007) with some modifications. The purified peptide fractions from each injection were , separately hydrolyzed with 6 N HCl at 110 ^{o}C for 24 h, and lyophilized and stored till used .

3.6.1 Sodium dodecyle sulphate polyacrylamide gel Electrophoresis (SDS- PAGE)

For the isolation of AMP from the plasma of larva *A. m. jemenitica*. SDS-PAGE of crude plasma and/or purified antimicrobial peptides was carried out by the discontinuous buffer system as described by Laemmili (1970) and Bearson *et al*. (2013) with some modifications. Electrophoresis was carried out at a constant voltage of 200 V for 90 min. and 12% polyacrylamide gel, under denaturing conditions. Samples were denaturated with 2% SDS containing 5% β mercaptoethanol by boiling for 3 min. Treated samples were centrifuged at 14000 g for 5 min before

being loaded on to the gels. The gels were calibrated with standard molecular weight proteins (97, 66 , 43 , 31, 20 and 14 kDa). Protein bands were visualized by Commassie Brilliant Blue dye staining (CBB). Molecular weight (MW) calculations were determined by regression analysis using the manufacturers' soft-ware.

3.6.2 Minimal inhibitory concentration of the purified peptide fractions of injected *A. m. jementica* 3rd instar larvae

The anti-microbial activity of the purified peptides isolated from injected *A. m. jemenitica* larvae (3rd instar,72h) were examined by determining the Minimal Inhibitory Concentration (MIC) using the micro dilution broth technique. Bacterial inoculate were prepared and standardized to have a turbidity equivalent in density to 0.5 McFarland standards approximately 1.5×10^8 colony-forming units (CFU)/ml then diluted to reach $1x10^6$ CFU /ml. The method used by **(**CLSI, 2010**)** is based on the protocol of Allen-Vercoe and Woodward (1999) with some modifications.

The MIC values of the purified peptide fractions isolated from plasma either of post – injection with *P. larvae* (A, B, C) or *S. enteritidis* (D, E and F) bacteria were determined by micro titer dilution plate method. For

broth micro dilution, susceptibility panel in 96-well micro titer plates (Fisher Scientific, Illinois, IL) were prepared by dispensing 100µl of the isolated purified peptides with the highest concentrations into the first column wells. Then, two-fold serial dilutions of peptide solutions were made by drawing 50 µl of peptide solution from the first column wells into the second column and then move on to the next columns to achieve the final concentrations. 50 µl aliquots of the bacterial suspensions in Mueller-Hinton broth were inoculated into wells of the micro titer plates to obtain a final volume of 100 µl in each well of the plate. The last two wells were positive and negative controls, respectively. The positive control was inoculated with bacterial suspension only, while the negative well was left as a blank without inoculation. The antibiotic Ciprofloxacin was used as a reference. The 96-microwell plates were sealed using a perforated plate seal (TREK Diagnostic Systems Inc., Cleveland, OH) and incubated at 37^{o}C for 24 h. The MICs were recorded as the lowest concentration where no viability was observed in 96-microwell plates after incubation for 24 h. Experiment was conducted in triplicate.

3.6.3 Determination of susceptibility of *S. enteritidis* to purified peptide fractions of injected *A. m. jemenitica* 3rd instar larvae by disk diffusion method .

The susceptibility of the bacteria *S. enteritidis* to the antimicrobial peptide fractions either of *P. larvae* (A, B, C) or *S. enteritidis*(D, E and F) injections was determined using disk diffusion method (the Kirby-Bauer technique) according to National Committee for Clinical Laboratory Standards (NCCLS; 2003).

All tests were performed on Müeller -Hinton agar (Oxoid, U.K.). The dried surface of a Müeller-Hinton agar plate was inoculated by streaking the suspensions of *S. enteritidis* ATCC 13076 ($1x10^6$ CFU/ml) using swab stick. The inoculums spread evenly over the entire surface of the agar plate by inoculating in three directions.

Stock peptide solutions containing 5 mg/ml for each peptide (A, B, C, D, E and F) were prepared. 5 µl (25 µg) of the stock solution was added onto each sterile disk and disks were in a sterile dried laminar flow cabinet .

The disks which were impregnated in purified peptide fractions were placed on the agar with sterile forceps. Each disk was pressed down to ensure complete contact with the agar surface. Inoculated plates were incubated within 15 min of disk application inverted at 37 ^{0}C for 24

hours. On the next day, plates were read from the back against a black background illuminated with reflected light by taking measurement of inhibition zone (in millimeters) and interpreted according to Clinical and Laboratory Standards Institute (CLSI, 2010). Ciprofloxacin disk was placed on to the surface of inoculated agar plates as reference.

3.6.4 *In vitro* adherence assays

3.6.4.1 Bacteria preparation for adherence assays

The cells were harvested from cultures of *S. enteritidis* ATCC 13076 at the exponential phase of growth by centrifugation (4,000 g at 4°C for 30 min), washed twice with phosphate buffered saline at pH 7.2 (PBS) and resuspended in PBS to give a bacterial suspension of 1.5 x 10^{6} cells/ml, and used immediately for the adhesion assays.

3.6.4.2 Preparation of epithelial cells

Human epithelial cells were used in this assay. The urine samples were obtained in the morning from healthy pregnant woman and the cells were harvested by centrifugation at 1000 g for 10 min. The cell sediments were

washed twice with PBS at pH 7.2 and resuspended in 2ml PBS then standardized to a concentration of approximately 10^5 cells/ ml using hemocytometer.

3.6.4.3 Effect of purified peptide fractions of *A. m. jemenitica* 3rd instar larvae on *S. enteritidis* bacteria adhesion to epithelial cells .

The anti- adhesive effect of peptide fractions on the bacteria adhering to epithelial cells was determined by microscopic analysis using crystal violet method (Vesterlund *et al*., 2005). Approximately150µl of *S. enteritidis* ATCC 13076 was added into microtiter plate wells coated with 150 µl of epithelial cells in absence and presence of purified peptides in concentration ½ MIC and ¼ MIC. The mixture was incubated at 37 °C for 1 hour. The non-adherent bacteria were removed by washing the wells three times with 250 µl of PBS at pH 7.2. The adherent bacteria were fixed at 60 °C for 20 min and stained with crystal violet (100 µl/ well, 0.1% solution) for 45 min. Wells were subsequently washed five times with PBS to remove excess stain. Finally, 100µl of citrate buffer (20 mmol/L, pH 4.3) was used to lyse the cells for 45 minutes at RT and optical density was measured at 570 nm.

3.6.4.4 Count method

Plates were co-cultured with *S. enteritidis* in total volume in absence and presence of peptides in concentration ½ MIC. Cells were incubated at 37°C for 1 hour, washed 3 times with PBS, fixed at 60°C for 30 min, stained with crystal violet for 45 minutes and washed 5 times with PBS. Adherent bacterial cells were counted under microscope (x1000 magnification) and images were taken. All experiments were repeated three times (n=3).

3.7 *In vivo* nodule formation against injected bacteria

Nodulation is an insect cellular immune response that is activated by bacterial infection. The number of nodules formed reflect the extent of the nodulation response (Miyagi *et al* .,2000). Nodule formation was assessed in larvae (3^{rd} instars,72 h old) . and newly emerged adult workers at selected times (24h) .

Insects were injected with $1x10^6$ viable *P. larvae* and /or *S. enteritidis* bacteria. Before analysis, adult bees were submerged in (PBS, pH: 7.2) . The dorsal abdominal tergits of adult individuals either control (non injected) or injected were carefully removed and the melanized nodules were counted under a stereomicroscope (Olympus SZX7, Hamburg,

Germany). Larvae were transferred onto a sheet of parafilm on top of a layer of PBS and fixed dorsally with two needles. The ventral side was cut from the cranial to the caudal end (avoiding injury of the gut) and the larval skins were then turned over and fixed with four additional needles before counting the visible nodules in the hemocoel.

3.8 *In vivo* phagocytosis of Latex® beads

Latex® beads (Aldrich, USA) were used for investigating melanization as well as *in vivo* phagocytosis responses (Ahmed, 2005; Li and Paskewitz, 2006; Paskewitz *et al.,* 2006). *A. m. jemenitica* larva were injected with 0.5µl of *P. larvae* (1×10^6) and /or carboxylate-modified polystyrene latex beads (0.9µm diameter) with a micro syringe (20 - gauge needle).

The larvae were then incubated in the rearing incubator at 34˚C for 24h. Only active larvae were used for dissection, 24 h post-injection (Koella and Sørensen 2002). Each larva which showed any degree of melanization was dissected and nodules of different sizes were counted (10 larvae from each group) were replicated five times (N=5) for statistical analysis. More over after 24h of injection with latex beads larval hemocytes were collected and processed for Electron microscopy as described by Giulianini., *et al* (2003) ; Marringa *et al*., (2014). An amount of 100µl hemolymph samples were drawn from the dorsal vessel

of each larva in a microsyringe filled with 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 and the hemocytes pelleted by 1000 g centrifugation for 10 min. The pellets obtained from pooled hemolymph of two larvae were then post-fixed in 1% osmium tetroxide in the same buffer, serially dehydrated in ethanol and embedded, via propylene oxide, in Embed 812/Araldite (Electron Microscopy Sciences, Fort Washington, PA).

Seven differential cell counts were made by two different from 2 µm semithin transverse sections of the full pellet thickness stained with toluidine blue. A total of 1050 –1572 cells were scored from three slides per pellet. Parallel controls were made with non-injected larvae (n=10).

3.9 Determination of phenoloxidase activity in plasma of injected *A. m. jemenitica* 3rd instar larvae

The PO activity was measured in the plasma 24h post-injection of *A. m. jemenitica* 3rd instar larvae with the pathogenic *P .larvae* according to the method described by Laughton *et al*. (2011). Briefly, 20µl aliquots of plasma, from *A. m. jemenitica* 3rd instar larvae injected or non injected control with *P. larvae* bacteria were mixed with 20 µl of (135 ml distilled water + 20 ml PBS, (pH 6.5) + 5µl trypsin (0.5 mg ml^{-1}) in an ice pre-chilled 96-well plate. The reaction mixture was incubated at 27 ^{o}C ±

2^{o}C for 5 min. This was followed by addition of 20µl of pre-filtered L-dopa (saturated, between 2.88 mM and 5.75 mM). Absorbance was measured at 490 nm every 15 seconds for 1h. The change in enzyme activity was estimated as the maximum linear rate of substrate conversion. PO activity was expressed as change in absorbance/ mg protein/ min. Experiments were replicated five times (n=5) for statistical analysis.

3.10 Determination of lysozyme activity in plasma of injected *A. m. jemenitica* 3rd instar larvae

The previously separated plasma of *A. m. jemenitica* 3rd instar larvae (3rd instars,72 h old) that were injected with *P. larvae* bacteria (1×10^{6} cfu/larvae) / or control (non injected) was subjected to lysozyme-like activity assay against the *P. larvae* as described by Brogden *et al*. (2003). 50 µl of plasma. 50 µl PBS (phosphate buffered solution: 150 mM NaCl, 10 mM Na_2HPO4, pH: 6.5). was added to 0.9 ml of an *M. luteus* cell wall suspension 12.5 mg/25 ml PBS, (pH: 6.5). The cuvettes containing mixture were incubated at room temperature. The change in absorbance at 450 nm was measured every 5 min for 30 min. The results were compared with the absorbance observed in the control cuvettes (i.e. those

with PBS only). Experiments were replicated five times (n=5) for statistical analysis.

3.11 Determination of cecropin-like activity in plasma of injected *A. m. jemenitica* 3rd instar larvae

Plasma (7.0 µg) either from control (non injected) or *P. larvae* – injected *A. m. jemenitica* 3rd instar larvae (n=10) was used (7.0 µg). Cecropin B (Sigma–Aldrich, UK) were used as standard . Briefly the previously prepared samples were loaded into wells in the *E. coli*-seeded agar plates and growth inhibition zones were detected as described by (Lee *et al.,* 2000) with some modifications. Measurements are carried out as described previously in the solid growth inhibition zone assay.

3.12 Oral infection of *A. m. jemenitica* 3rd instar larvae with pathogenic *P. larvae*

The third instar of *A. m. jementica* 3rd instar larvae were infected with *P. larvae* in the following manner.0.5 µl of the solution containing 20 µl (4 X 10^6 CFU/ml) of *P. larvae* was added to 100 ml of sterilized distilled water was mixed with 50 µl of pre-warmed diet (50% royal jelly V/V). It

was mixed with 50% autoclaved aqueous solution of D-glucose (12%V/V) and D-fructose (12%W/V). One larva was grafted per well as shown in figure 6. The amount of diet consumed by the larva daily was determined. Healthy *A. m. jementica* 3rd instar larvae 72 hours old larvae were used as non infected control. The digestive system of each group of larvae was dissected out after 4 and 24 hours of treatment. It was separated into three parts viz., fore gut (honey crop), mid gut (ventriculus) and hind gut (intestine). All the parts of the digestive system of control and infected larvae were immersed in 0.1 M cacodylate buffer, pH 7.3 containing 2% glutaraldehyde.

3.12.1 Transmission electron microscopy (TEM) of digestive system sections of orally infected *A. m. jementica* 3rd instar larvae

The dissected parts of the digestive system of orally infected larvae were separately fixed in 1% osmium tetroxide, serially dehydrated in ethanol and embedded with propylene oxide in Embed 812/Araldine (Electron Microscopy Sciences For Washington, PA). For TEM, silver/gold-coloured sections were cut on an ultra microtome (Publish Top Ultra 150) and collected on 200-mesh nickel grids. Sections were stained with

uranyl acetate and lead citrate for 5 min each, were observed with a TEM Philips, EM 208. Negative slides were digitized with an Epson Photo Perfection scanner at 1200 dpi (optical resolution) and saved as a Tagged Image Format file (Giulianini *et al* ., 2003).

3.12.2 Light microscopy of digestive system of orally infected *A. m. jemenitica* 3rd instar larvae

For light microscopy, different parts of orally infected *A. m. jementica* 3rd instar larvae digestive system about 2 µm thick were loaded on slides and kept for 5 min at -80 ˚C and stained with 0.5% toluidine blue in 0.1% carbonate solution at pH 11.1 and the same temperature. Image analysis for light microscopy of selected areas was observed under Olympus BX50 microscope, and images were recorded with an Olympus DP11 photo camera at a resolution of 1712 × 1368 pixels (Santos and Serrao , 2006).

3.12 Statistical analysis

Experiments were done in triplicate (n=3), and statistical analysis was done by Graph Pad prism 5. Mean, standard deviation and significance were calculated.

Statistical analyses were done using Minitab version 21 software (King Saud University). Data were tested for normality and for variances homogeneity before further analysis with suitable tests. For non-parametric data, the Kruskal–Wallis test was used to determine the overall effects of treatment, before individual comparisons using the non-parametric Mann–Whitney U test. For parametric data ANOVA and\ or t – test were used.

RESULTS

4. RESULTS

4.1 Total protein (TP) estimation and SDS-PAGE of *A. m. jemenitica* larval plasma

The data presented in figure (8) shows that total protein content was highest in the plasma of *A. m. jemenitica* 3rd instar larvae injected with *S. enteritidis* followed by *P. larvae* bacteria. However those injected with LPS and *M. luteus* showed non significant increase when compared to that of control values. Similar alterations in the total protein content was observed in all the diluted samples .

SDS-PAGE of plasma protein of control , *P. larvae* and/or *S. enteritidis* injected *A. m. jemenitica* 3rd instar larvae is shown in figure (9). Visible bands were observed indicating the presence of proteins with MWs of about 14, 66-70 KDa which we speculate to be lysozyme and PO respectively. Bands exhibiting MW of less than14 KDa may be AMPs like defensins or cecropin figure (9).

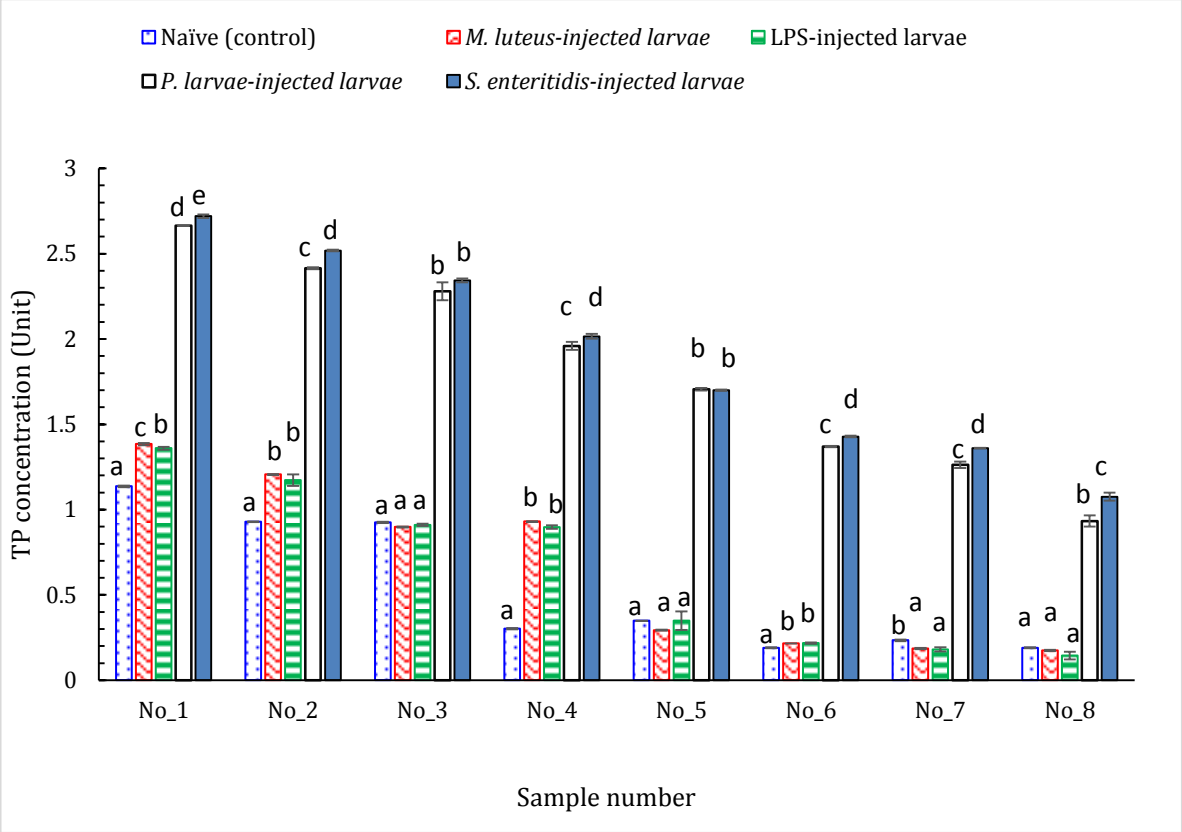


Figure (8): TP levels of serial plasma dilutions of *A. m. jemenitica* 3rd instar larval injected with different bacteria or/LPS. Bars marked with similar letters are similar (P>0.05) whereas those marked with different letters are significantly different (P<0.05). No 1- 1: fold dilution , No 2- 2: fold dilution, No 3- 3: fold dilution, No 4- 4: fold dilution, No 5- 5: fold dilution, No 6- 6: fold dilution, No 7- 7: fold dilution, No 1- 8: fold dilution (n=5).

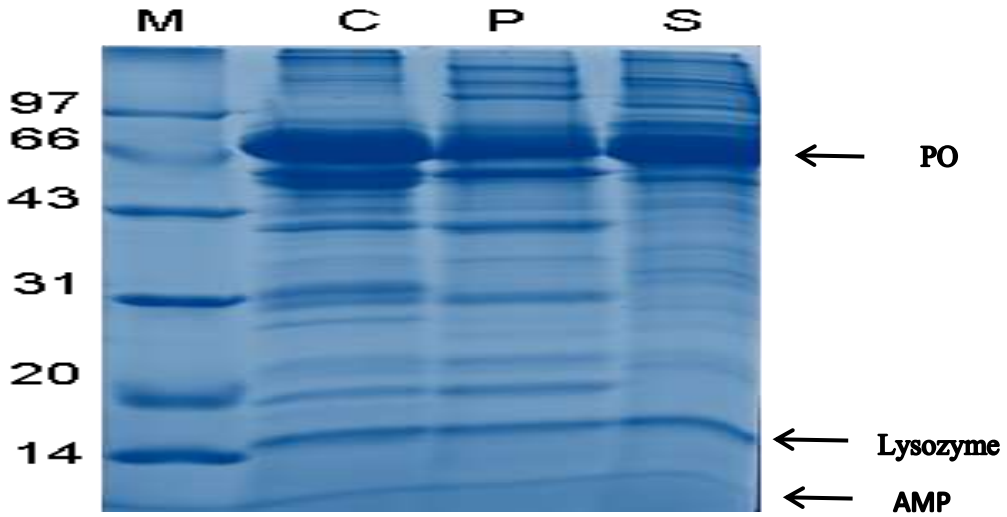


Figure (9): SDS-PAGE separation of plasma of *A. m. jemenitica* 3rd instar. M):standard molecular weight markers, C): naïve, P): *P. larvae* and S): *S. enteritidis* injected. Each lane contains a 2.5 µl of plasma proteins .

4.2 RP- HPLC and SDS-PAGE profile of induced antimicrobial peptides of *A. m. jemenitica* 3rd instar larvae

Major antimicrobial peptides isolated and partially purified from plasma of *A. m. jementica 3rd instar* larvae either injected with *P. larvae or S. entritidis* were fractionated by RP- HPLC by using 30-70% (v/v) acetonitrile containing 0.05%v/v trifluoroacetic acid. Three independent peaks were observed (Figure 10) for each treatment. Gel electrophoresis indicated apparent homogeneity and approximate MW of 10.22 , 11.47 and 11.80 KDa for the peaks corresponding A, B and C, respectively and 11.52, 11.52 , 11.52 KDa for peaks D, E and F , respectively (Figure 11)

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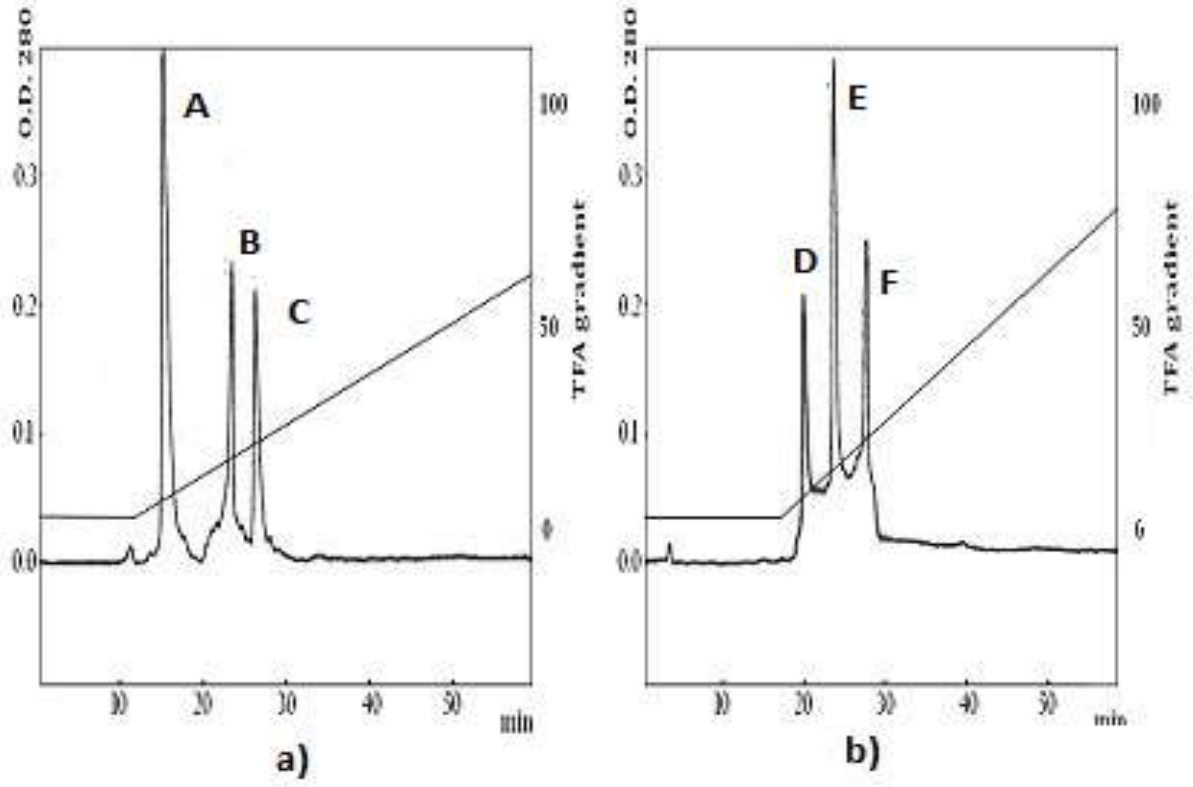


Figure(10): RP-HPLC Patrial purification profile of antimicrobial peptide fractions from plasma of *A. m. jemenitica* 3rd instar **larvae induced with *P. larvae* and /*S. entritidis* bacteria** . a) and b) eluted fractionation of plasma of *P. larvae* and /*S. entritidis* injected larvae, respectively. Heat treated plasma was fractionated, separately, on a C18 Bondapak and finally on C18 Pepmap columns. Fractions A, B, C, D, E and F developed using a water-acetonitrile gradient [30 - 70% / 30 min] containing 0.05% trifluoroacetic acid (TFA).

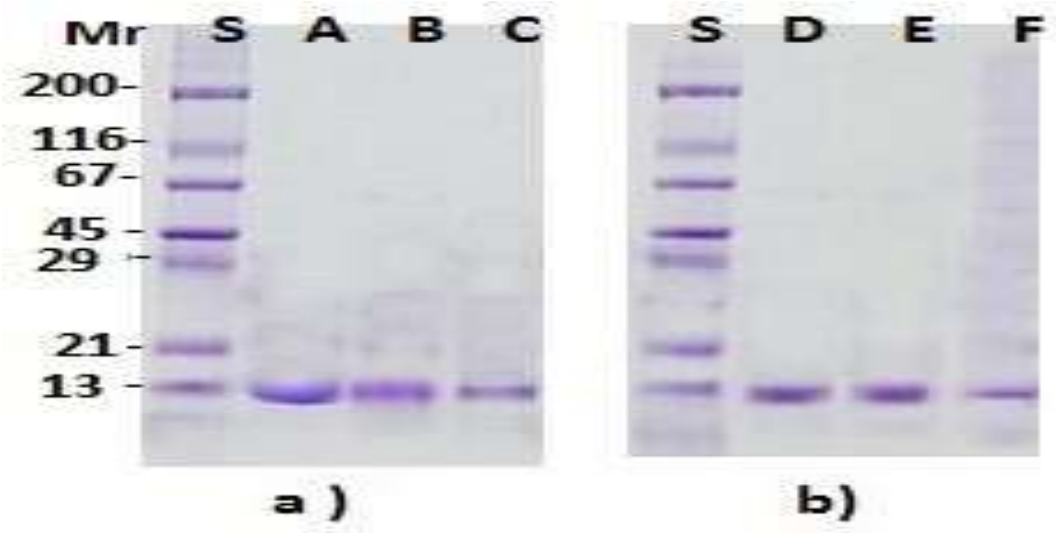


Figure (11): SDS-PAGE profile of purified fractions of *A. m. jemenitica* 3rd instar larvae, a) injected with *P. larvae* lanes (A , B and C) and b) injected with *S. entritidis* lanes (D, E and F) . (lane S) high and low molecular weights of standards are shown in as Mr ($x10^3$) in the left side of the figure. Commassie brilliant blue (CBB) stained.

4.3 Anti- bacterial activity of plasma from *A. m. jemenitica* 3rd instar larvae 24h post injection with *P. larvae* and /or *S. enteritidis* against different bacteria

4.3.1 Humoral Immunity

4.3.1.1 Inhibition zone diameters of plasma total protein from *A. m. jemenitica* 3rd instar larvae injected with *P. larva* and /or *S. enteritidis* against various bacteria

Antibacterial activity of *A. m. jementica* 3rd instar larvae plasma 24h post injection with *P. larvae* and /or *S. enteritidis* against different gram (-ve) and gram (+ve) bacteria revealed non significant differences up to a plasma concentration of 2.5µg/ml (P=0.7). The highest antibacterial activity of larval plasma was obtained towards *M. luteus* and *P .larvae* with inhibition zone diameter (mm) of 12 ± 0.88 followed by *E. coli* and *S. enteritidis* that were 11± 0.88 (mm) and 10 ± 0.88 (mm), respectively at the highest concentration used (20 µg/ml) (Figures12, 13 and tables 4, 5). This was still effective uptill 2.5µg/m

Table (4): Inhibition zone diameters of plasma from injected *A. m. jemenitica* 3rd instar larvae with *P. larvae* against various bacteria

Conc. of total protein plasma (µg/ml) / Type of bacteria	20	10	5	2.5	P-value
M. luteus	12 ± 0.5mm	9 ± 1.1mm	7 ± 0.41mm	3 ± 0.22mm	0.01
E. coli	11 ± 0.7mm	6 ± 1.1mm	4 ± 0.12mm	2 ± 0.41mm	0.01
S. enteritidis	10 ± 1.4mm	7 ± 1.4mm	3 ± 0.41mm	1 ± 0.12mm	0.1
P. larvae	12 ± 1.5mm	7 ± 1.7mm	5 ± 0.57mm	3 ± 0.22mm	0.5

Values represent the diameter(mm) of the inhibition zone (mean ± S.D).

P-value between all groups indicates that there is significant result between groups at 0.05 level

Table (5): Inhibition zone diameters of plasma total protein from injected *A. m. jemenitica* 3rd instar larvae with *S. enteritidis* towards different bacteria

Conc. of plasma total protein (µg/ml) / Type of bacteria	*20*	*10*	*5*	*2.5*	P-value
M. luteus	10 ± 0.53mm	7 ± 1.05mm	5 ± 1.3mm	2 ± 1.8mm	0.01
E. coli	12 ± 0.41mm	9 ± 0.6mm	7 ± 1.05mm	4 ± 1.7mm	0.01
S. enteritidis	8 ± 1.53mm	7 ± 1.1mm	4 ± 1.7mm	1 ± 1.9mm	0.1
P. larvae	7 ± 1.05mm	6 ± 1.88mm	4 ± 1.7mm	2 ± 1.8mm	0.5

Values represent the diameter(mm) of the inhibition zone (mean ± S.D).

P-value between all groups indicates that there is significant result between groups at 0.05 level.

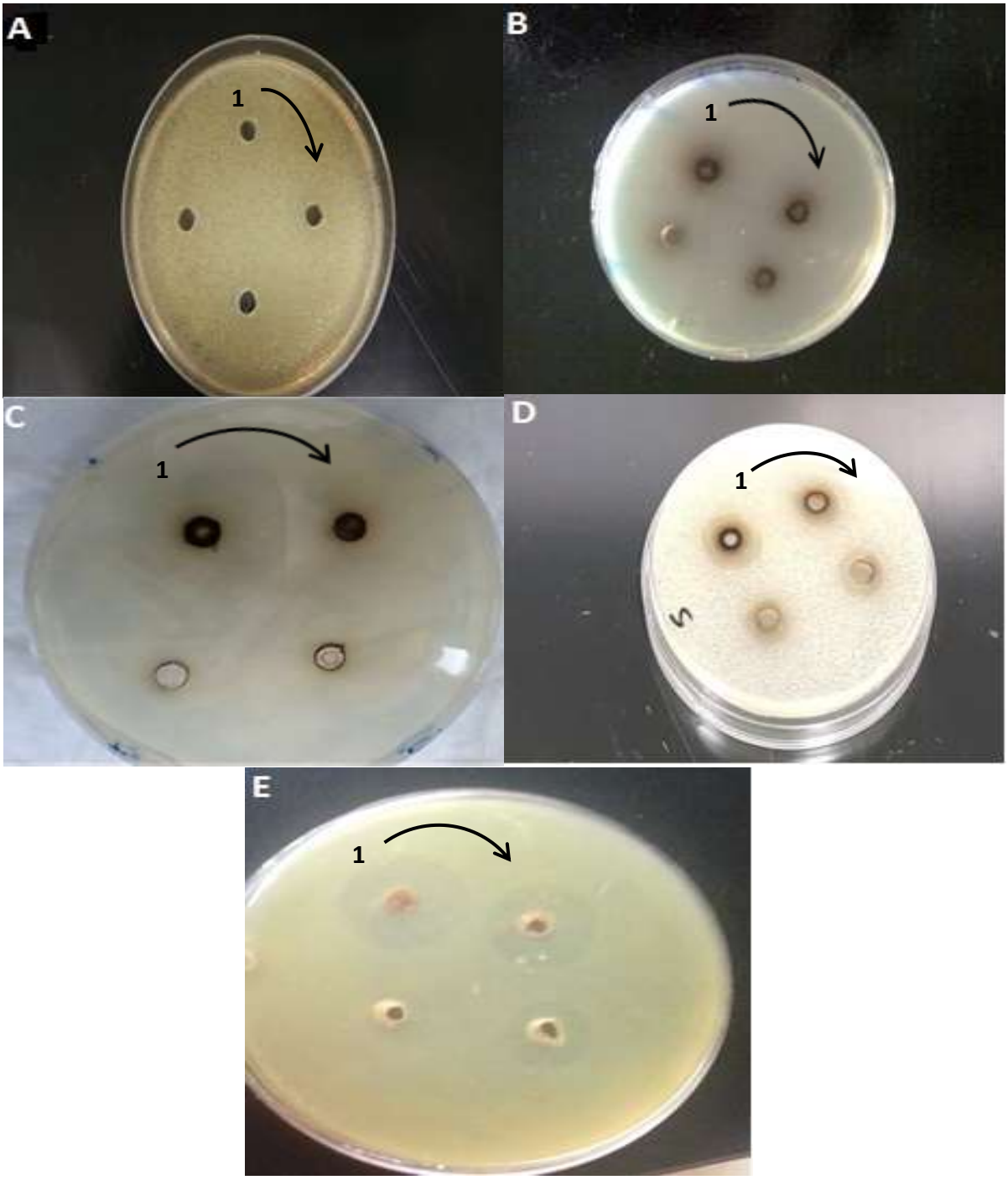


Figure (12): Inhibition zone diameters of plasma of *A. m. jemenitica* 3rd instar larvae injected with *P. larvae* . A :Control , B: *M. luteus* , C: *E. coli* , D: *S. enteritidis* and E: *P. larvae*

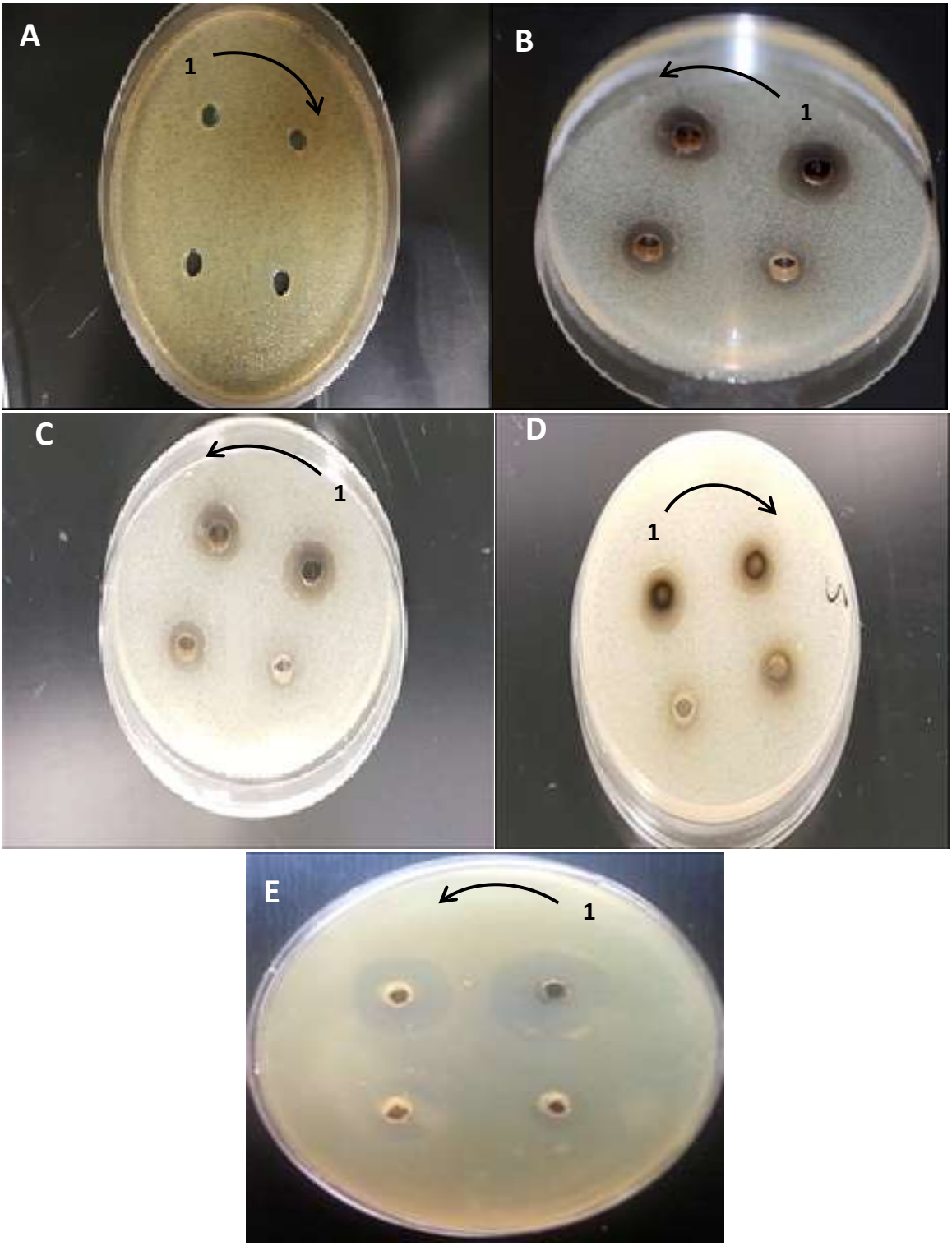


Figure (13): Inhibition zone diameters of *A. m. jemenitica* 3rd instar larvae plasma total proteins of larvae injected with *S. enteritidis*. A: Control , B: *M. luteus* , C: *E. coli* , D: *S. enteritidis* and E: *P. larvae*.

4.3.1.2 Lysozyme – like activity of different plasma concentrations from *A. m. jemenitica* 3rd instar larvae were investigated 24 h post-injection with *P. larvae*

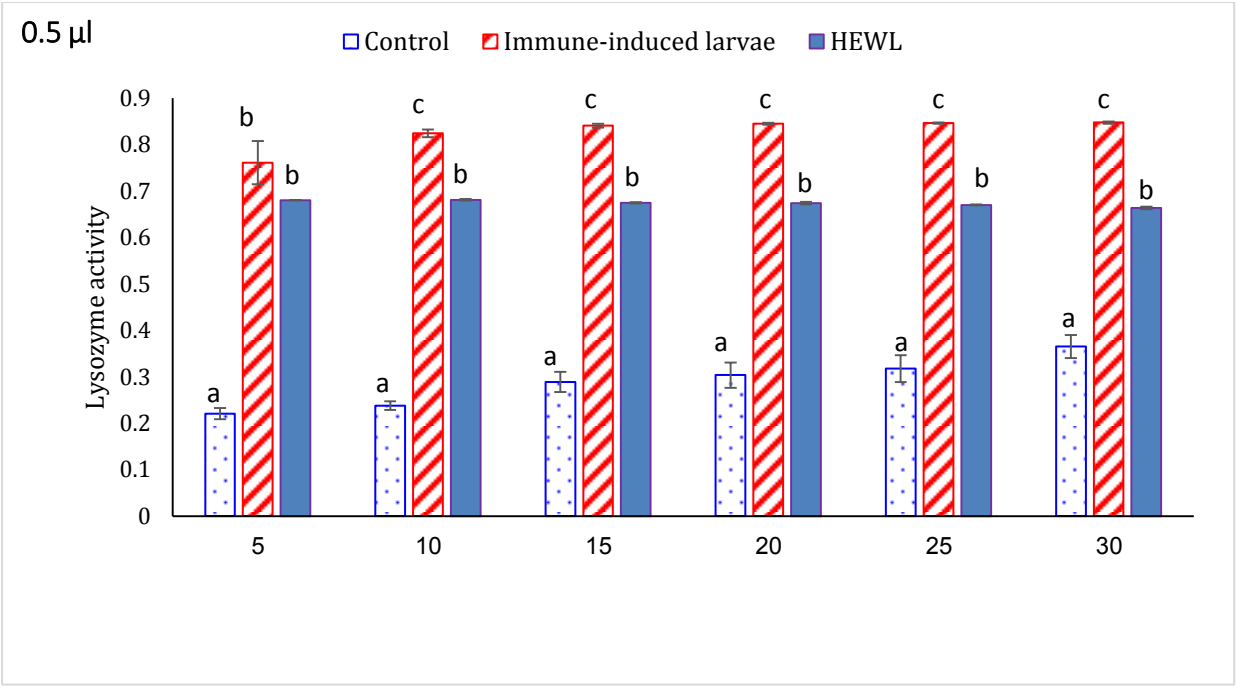
Table 6 and Figure 14 show significant increase $P < 0.01$ in lysozyme like activity in plasma of *A. m. jemenitica* 3rd instar larvae of *P. larvae* injected and control (non injected) when compared to lysozyme standard groups containing similar concentration of protein during all times post injection from 5 up to 30 min of incubation.

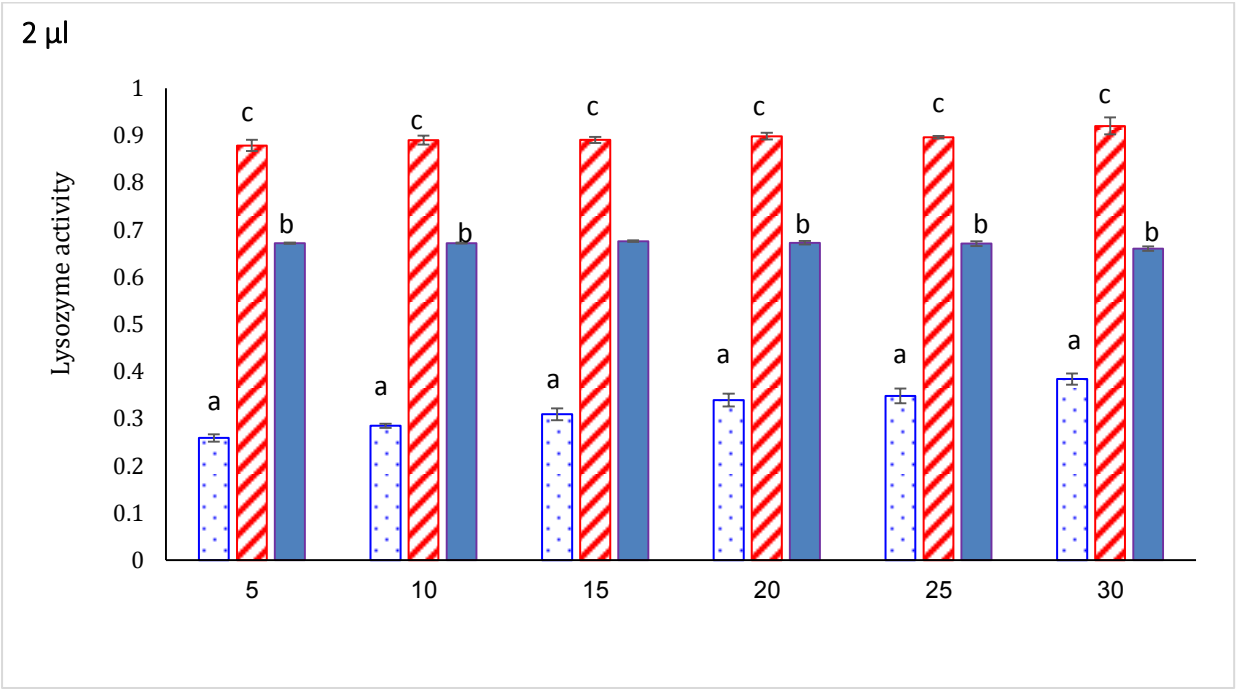
Table(6): Lysozyme- like activity of plasma from *A. m. jemenitica* 3rd instar larvae post-injection with *P. larvae*.

Time (post.incubation (min)	Total protein Conc.(µg/ml)								
	5	20	40	5	20	40	5	20	40
	Plasma of control larvae			Plasma of treated larvae			lysozyme standard		
5	3.3 ±0.016	4.0 ± 0.016	3.1±0.015	4.5±0.013	5.2±0.012	5.2 ±0.012	0.68 ±0.004	0.67 ± 0.003	0.716 ± 0.008
10	3.8 ± 0.016	4.3 ± 0.016	4.7±0.016	4.8±0.013	5.2±0.012	5.3 ±0.013	0.68 ± 0.004	0.67 ± 0.003	0.714 ± 0.008
15	4.7 ±0.017	4.8 ± 0.017	5.1±0.017	4.9±0.013	5.3±0.012	5.4 ± 0.013	0.67 ± 0. 003	0.67 ± 0.003	0.714 ± 0.008
20	5.0 ±0.017	5.1 ± 0.017	5.4±0.017	5.0±0.014	5.4±0.013	5.6 ± 0.014	0.67 ± 0. 003	0.67 ± 0.003	0.719 ± 0.009
25	5.2 ±0.017	5.4 ± 0.017	5.7±0.017	5.1±0.014	5.5±0.013	5.8 ± 0.014	0.67 ± 0. 003	0.67 ± 0.003	0.722 ± 0.012
30	5.6 ± 0.017	5.5 ± 0.017	5.8±0.018	5.3±0.014	6.1±0.015	6.1 ± 0.015	0.66 ± 0. 001	0.66 ± 0.001	0.717 ± 0.009

Values are expressed as mean ± S.E (n=3)

Enzyme activity is expressed as change in absorbance /mg protein/mi


0.5 µl
Control
Immune-induced larvae
HEWL
Lysozyme activity
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
25
30


2 µl
Lysozyme activity
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
25
30

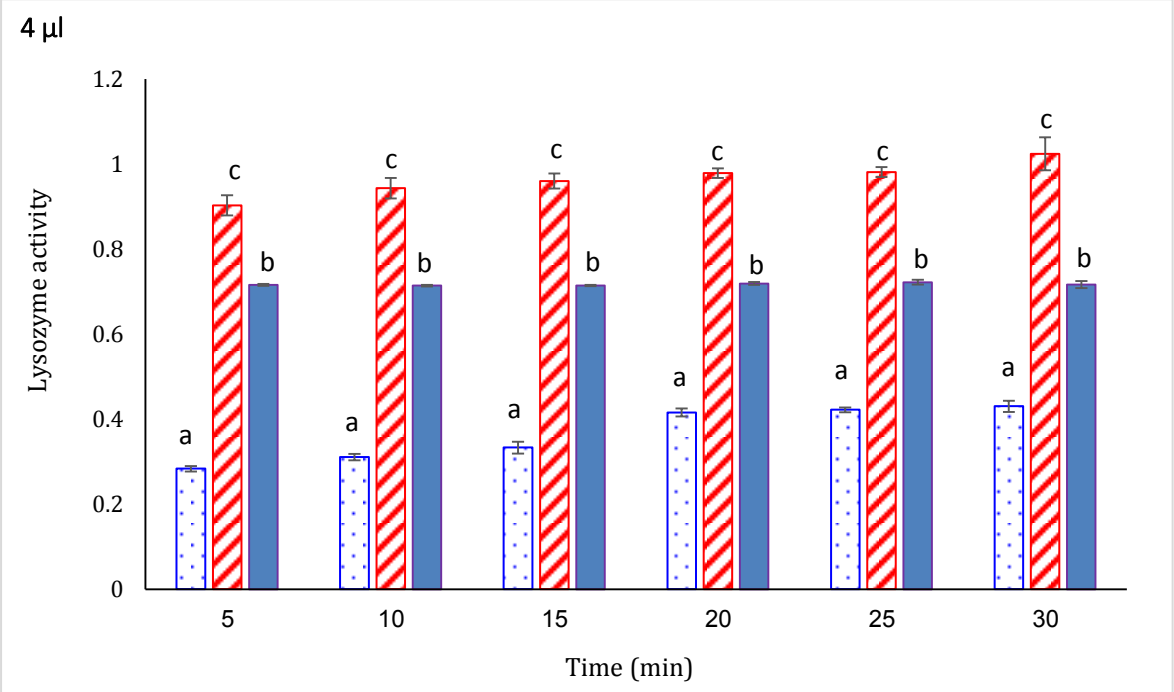


Figure (14): Lysozyme- like activity of plasma from *A. m. jemenitica* 3rd instar larvae injected with *P. larvae* bacteria. At any corresponding time, bars marked with similar letters are non significant (P>0.05) whereas those marked with different letters are significantly different (P<0.05).

4.3.1.3 Cecropin-like antibacterial activity of plasma of *A. m. jemenitica* 3rd instar larvae injected with *P. larvae* bacteria

Cecropin - like activity of *A. m. jemenitica* 3rd instar larvae plasma post-injection with *P. larvae* bacteria was detected by Inhibition Zone Assay cup agar diffusion method where activity was observed towards the gram negative *E. coli*. After 24h of incubation, each plate was examined. The diameters of the zones of complete inhibition were measured, including the diameter of the disk, using a ruler. Depicted results show that, plasma of *A. m. jemenitica* 3rd instar larvae injected with *P. larvae* have Cecropin – like activity in a concentration of plasma protein as low as 2.5 µg/ml .Control and injection with *P. larvae* have the same antibacterial potency compared with Cecropin B as reference. The antibacterial activity was significantly higher (inhibition zone diameter of 11.3 ± 0.5 mm) in *P. larvae* injected plasma when compared to identical standard Cecropin B having the same concentration of protein . However at lower protein concentration viz., 5 and 2.5µg/ml the activity was not detected in the control (non injected) larval plasma Figs (15,16) .

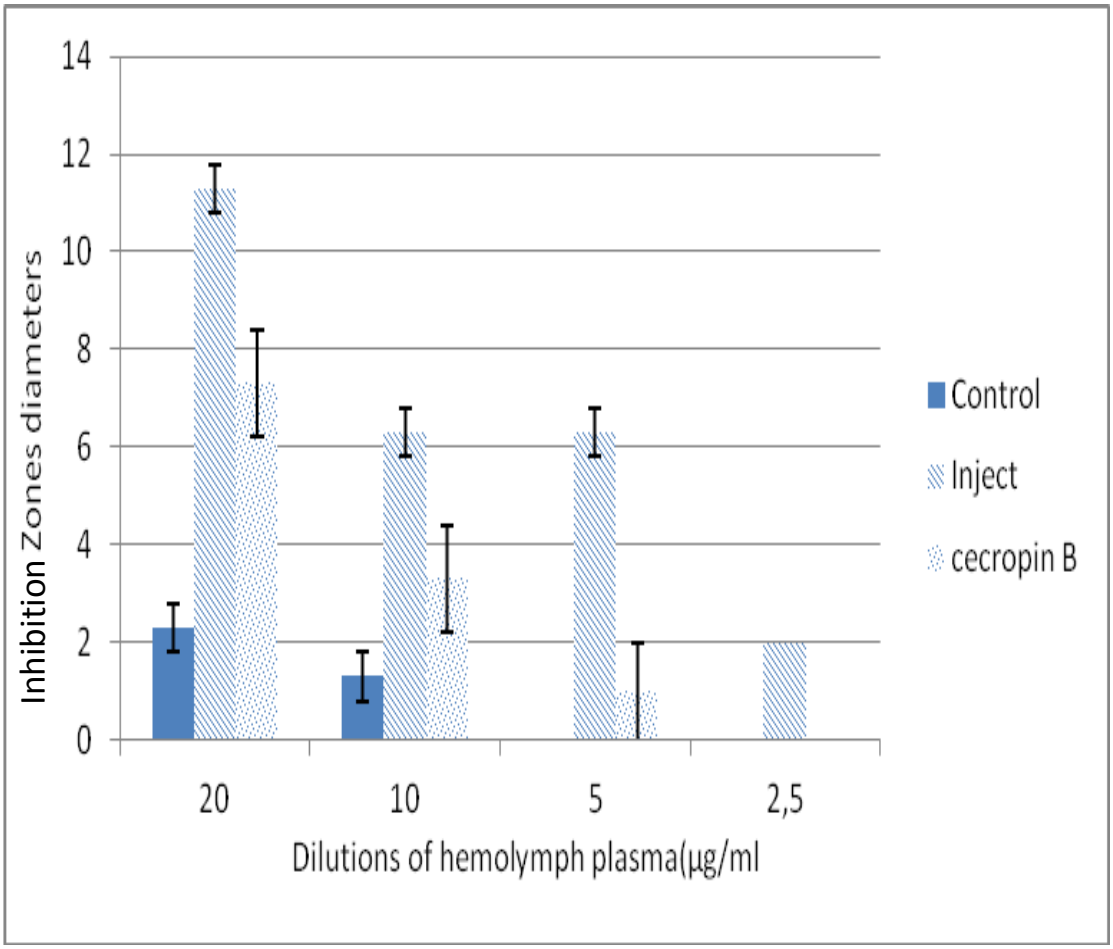
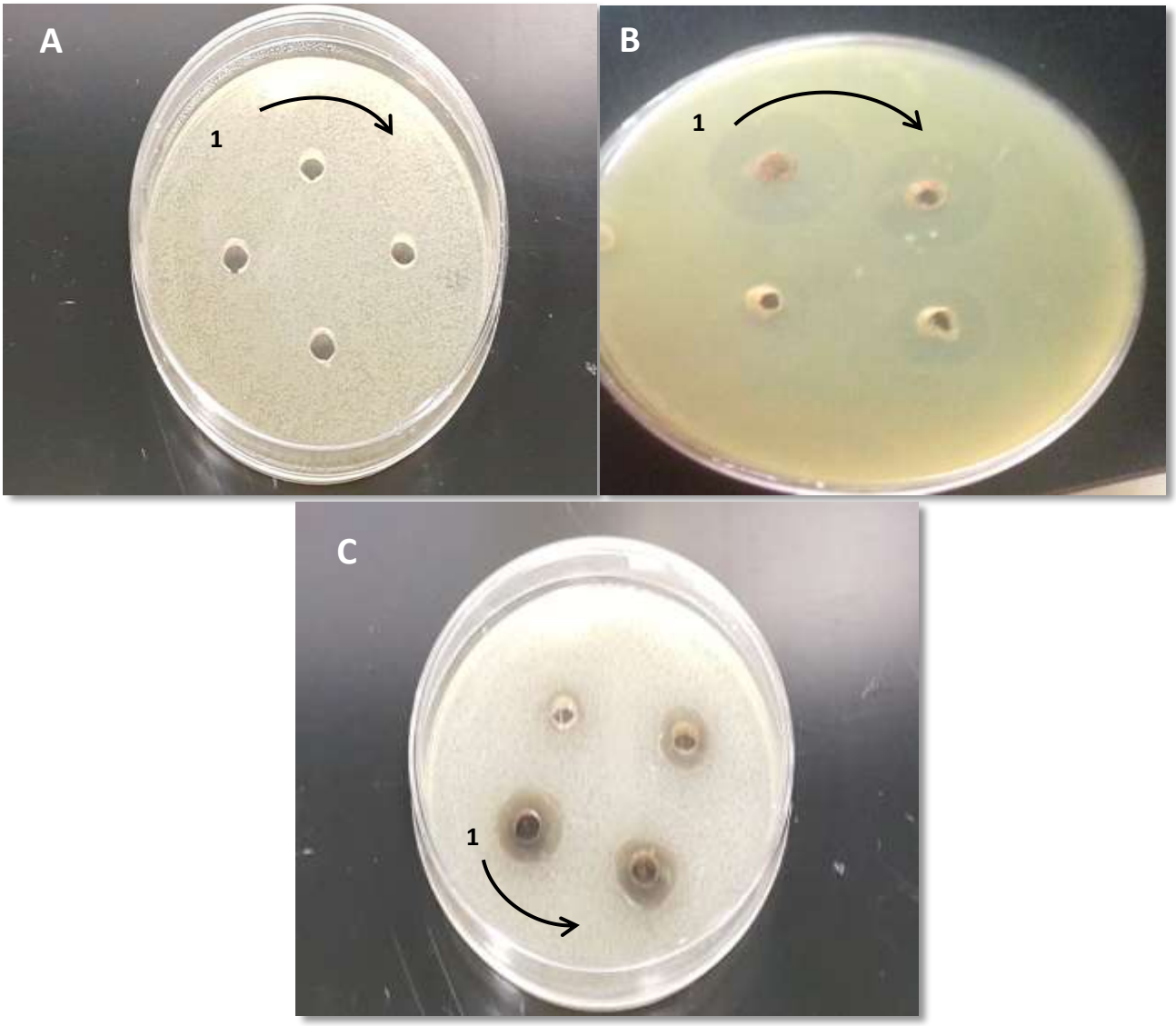


Figure (15): Cecropin – like activity of plasma of *A. m. jemenitica* 3rd instar larvae injected with *P. larvae* towards *E. coli* using Cecropin B as reference.



Figure(16): Mueller Hinton agar plate presenting inhibition zone Assay showing antibacterial activity of *A. m. Jemenitica* 3rd instar larvae plasma towards *E. coli* : (A) Control , (B) Injected with *P. larvae* and (C) Cecropin B reference.

4.3.1.4 Phenoloxidase activity in plasma of *A. m. jemenitica* 3rd instar 24h - post injection with *P. larvae*

PO activity was estimated in plasma of *A. m. jementica* (3rd instar) at 24h- post injection with *P. larvae* using L- Dopa as substrate. Results (table 8) indicate that initially at zero time the plasma of (control non injected +trypsin)as well as that of *P. larvae* injected groups with trypsin showed identical enzyme activity which were significant higher compared to control without trypsin treatment (0.07±0.005). However the plasma of *P. larvae* injected group + trypsin treatment displayed significant higher activity(0.63±0.032) when compared to control+ trypsin and *P. larvae* injected without trypsin. With the progression of time of injected larvae showed a significant increase in PO activity with or without trypsin treatment up to one hour of incubation .

This fluctuation in the rate of PO enzyme activity was due to the stimulation of production of PO from its inactive zymogen as a result of the immune receptor protein scattered on the blood cell surfaces as well as that of the hemolymph plasma (Figure17).

Table(7): Phenoloxidase activity of *A. m. jemenitica* 3rd instar larvae plasma post-injection with *P. larvae* bacteria.

Time Post incubation (min)	Treated witout Trypsin	Control without Trypsin	Treated with Trypsin	Control with Trypsin	P-value
0	0.32 ± 0.000	0.07 ± 0.005	0.63 ± 0.032	0.32 ± 0.003	0[a]
15	0.38 ± 0.003	0.08 ± 001	0.67 ± 0.004	0.33 ± 0.002	0.002[b]
30	0.45 ± 0.003	0.10 ± 001	0.71 ± 0.008	0.36 ± 0.002	0.01[b]
45	0.45 ± 0.003	0.10 ± 001	0.71 ± 0.008	0.36 ± 0.002	0.001[b]
60	0.59 ± 0.003	0.12 ± 003	0.81±0.009	0.38 ± 0.001	0[a]

Activity of phenoloxidase measured as OD /mg protein /min using L- dopa as a substrate , at 490 nm. [a] P-value between all groups

[b] there is significant between all groups at 0.05 level.

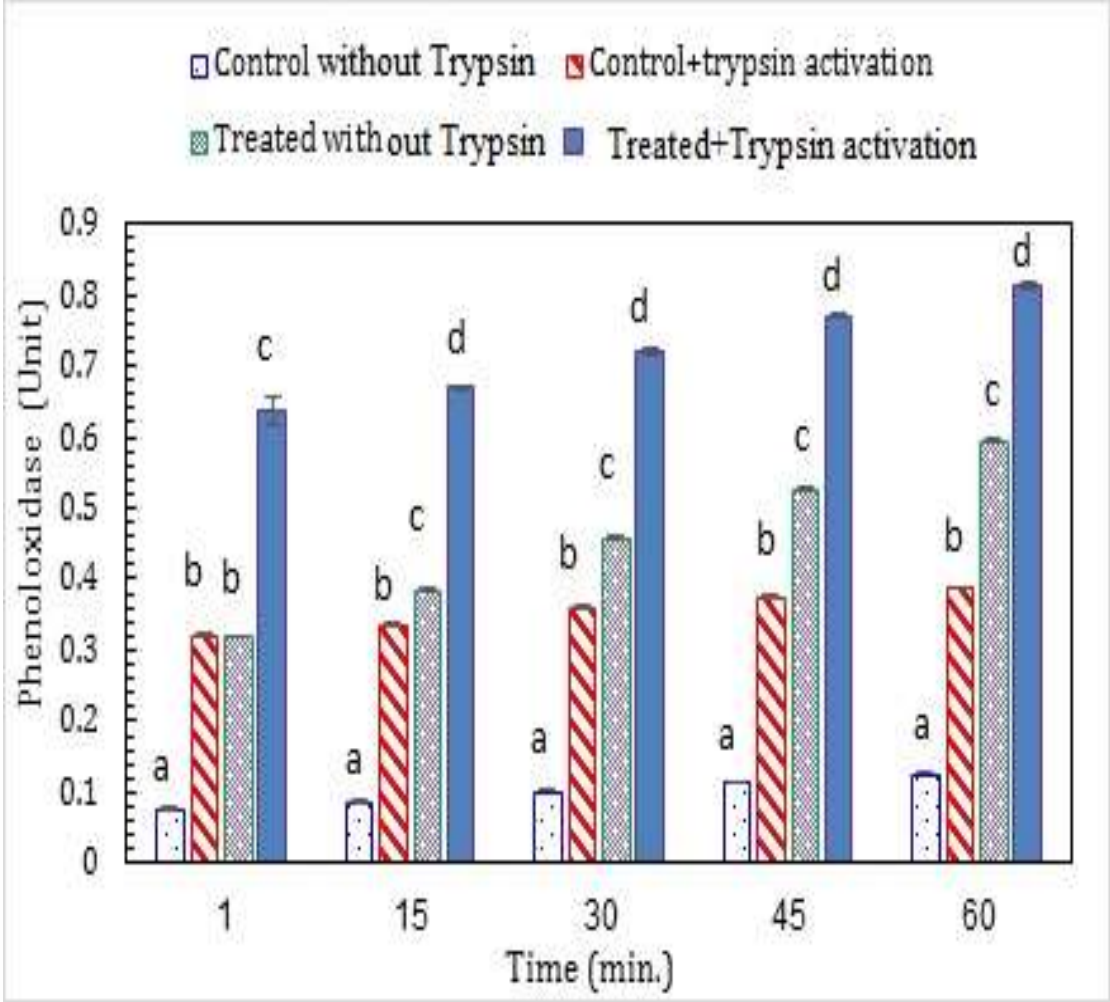
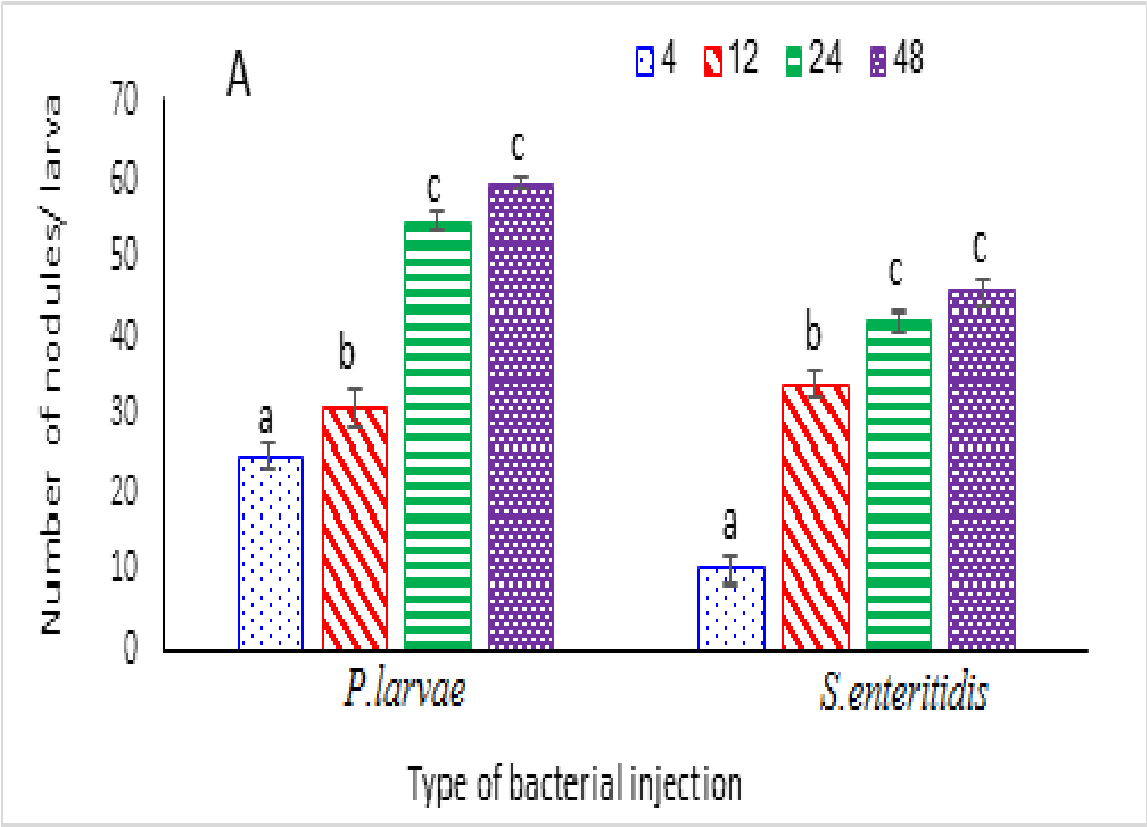


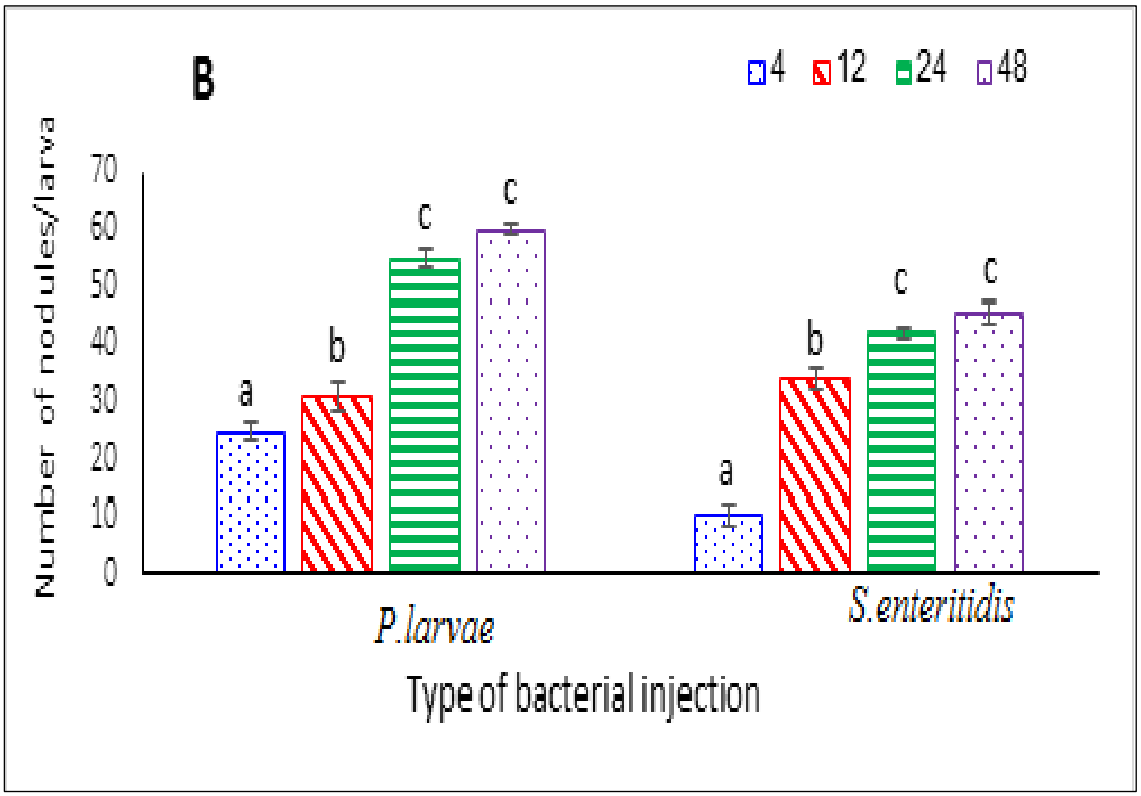
Figure (17): Phenoloxidase activity in plasma of *A. m. jemenitica* 3rd instar larvae post-injection with *P. larvae*. At any corresponding time, columns marked with the same letters are non significant (P>0.05) whereas those marked with different letters are significantly different (P<0.05).

4.3.2 Cellular Immunity

4.3.2.1 Cellular nodules in *A. m. jemenitica* 3rd instar post-injected with *P. larvae* and/or *S. enteritidis* at different time intervals

Figure 18 shows the number of different sized nodules formed in 3rd instar larval of *A. m. jemenitica* in response to bacterial injection at different time intervals. Injection with *P. larvae* lead to nodules formation 4h post injection. The total number of formed nodules increased with time where the highest number being observed at 48 h post injection (13 nodules/ larva) compared to the control ones which showed no nodules. Similarly, injection with *S. enteritidis* also resulted in the appearance of nodulation 4 h post injection with a maximum number being observed 48 h after injection (9 nodules/ larva). However, the appearance of large nodules was observed only after 24h of injection in contrast to *P. larvae* where the large nodule formations was observed 4h post injection . Nodules produced in bacterial challenged larvae showed significant variation in size (Table 7). Small sized nodules (5-10µm) ranged from approximately 40-66% of the total nodule counts while medium and large sized nodules formed ~23-67% (10-30µm) and ~ 0-16% (40-80µm) respectively of the total nodules .


A
4
12
24
48
Number of nodules/ larva
70
60
50
40
30
20
10
0
a
b
c
c
a
b
c
c
P.larvae
S.enteritidis
Type of bacterial injection


B
4
12
24
48
Number of nodules/larva
70
60
50
40
30
20
10
0
a
b
c
c
a
b
c
c
P.larvae
S.enteritidis
Type of bacterial injection

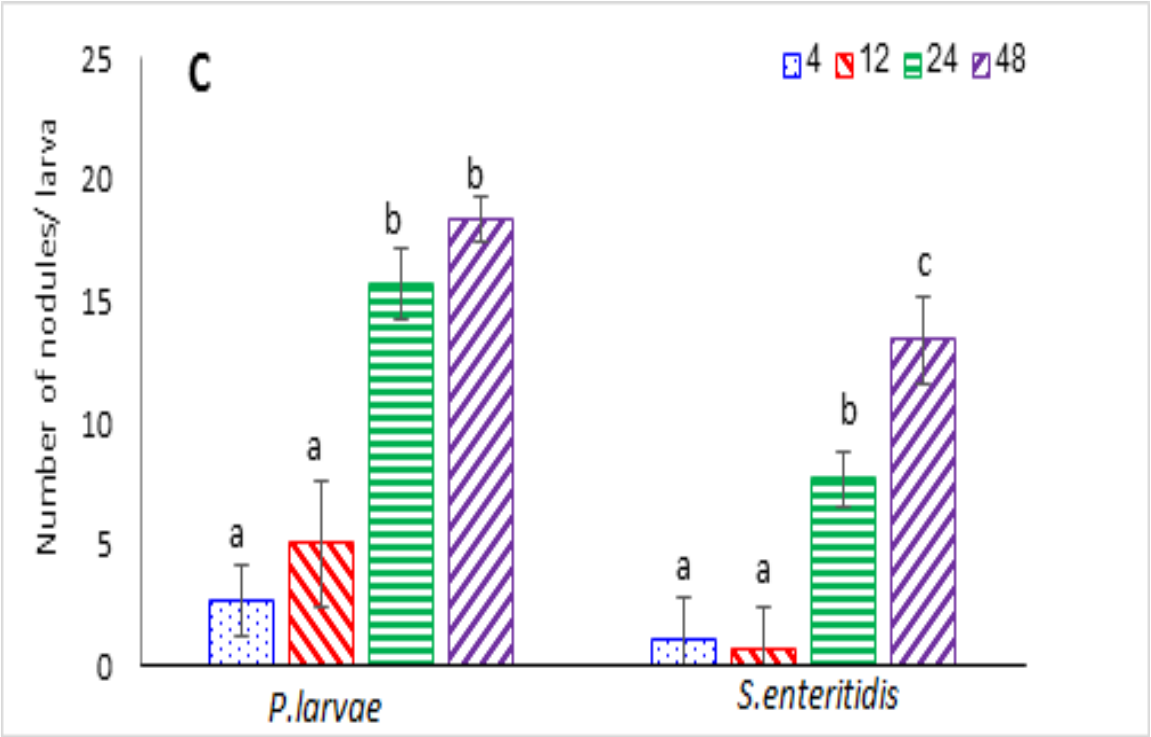


Figure (18): Number of cellular nodules post injection with *P. larvae* /or *S. enteritidis* . A) small nodule size (5-10 µm). B) medium nodule size (10-30 µm). C) large nodule size (40-80 µm). Bars marked with similar letters are non significant different (P>0.05) whereas those marked with different letters are significantly different (P<0.05).

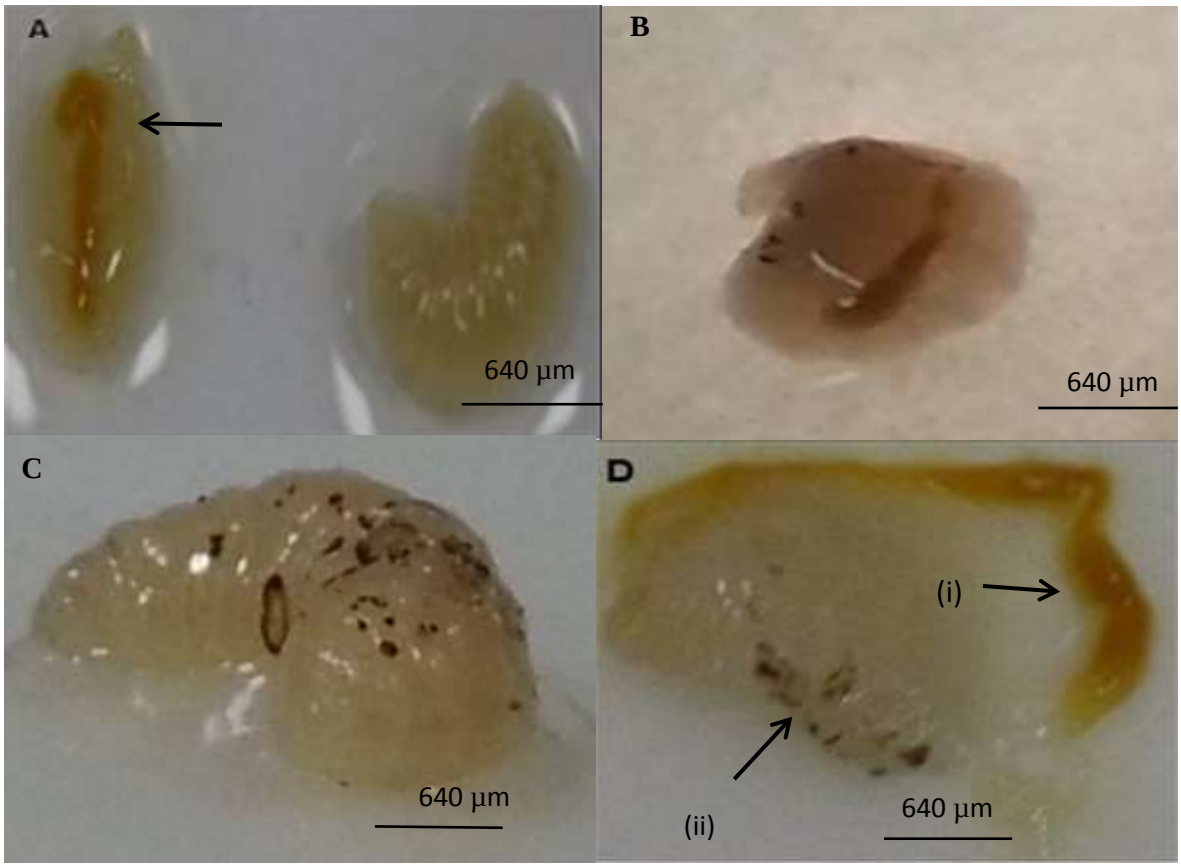


Figure (19a): Light microscopic images of *A. m. jemenitica* 3rd instar larvae injected with *P. larvae*. A) control larvae (Arrow indicates dissected larvae). B) Dissected larva showing the nodules 4h post injection. C) Whole larva showing different sized nodules 24 h post injection. D) Dissected larva 24 h post injection showing alimentary canal (i) and nodules around circulatory system(ii); Canon camera X 80.

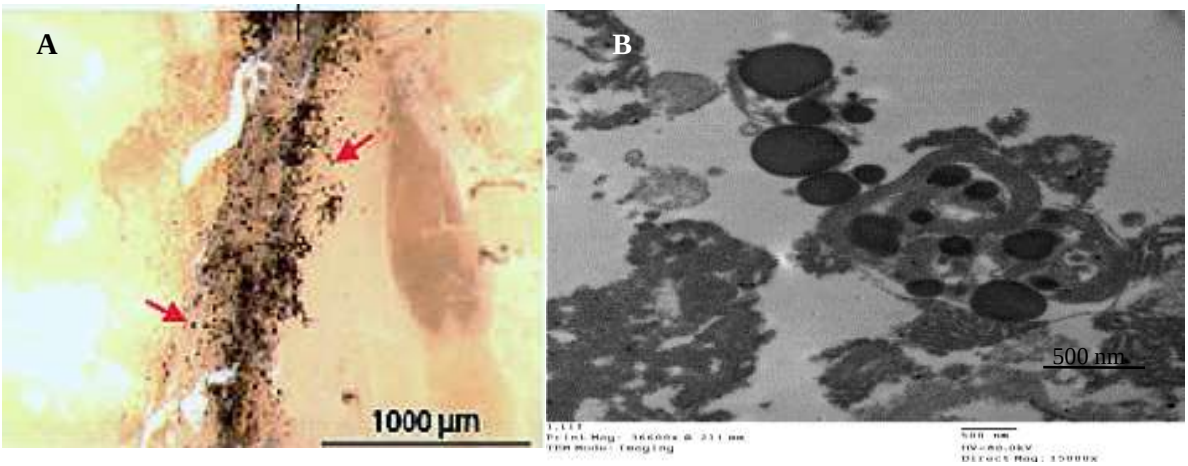


Figure (19b): Light and TEM images of *A. m. jemenitica* 3rd instar larvae injected with *P. larvae*. A) nodules surrounding the heart 24 h of injection . B) With higher magnification (X 10000).

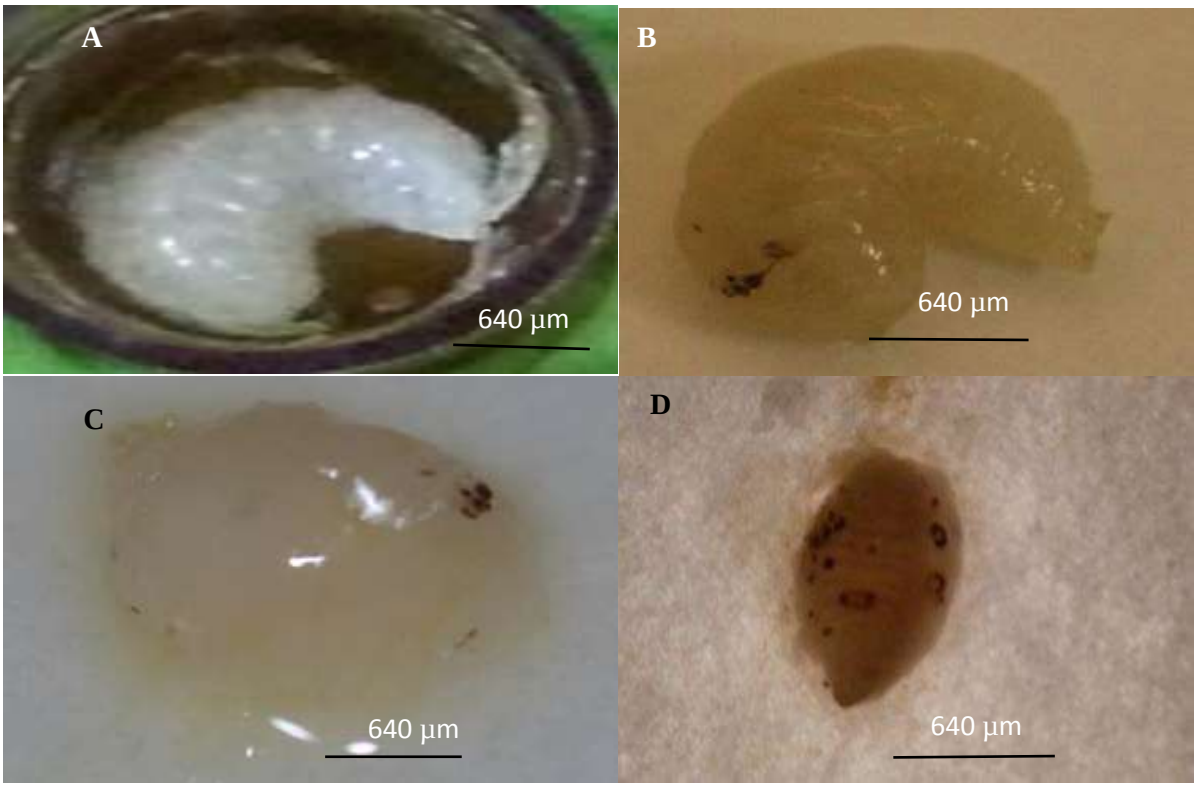


Figure (20a): Light microscopic images of *A. m. jemenitica* 3rd instar larvae injected with *S .enteritidis* .A) Control larva . B) Whole larva 4 h post injection. C) Dissected larva 24 h post injection. D) Whole larva 24 h post injection; Canon camera(X 80).

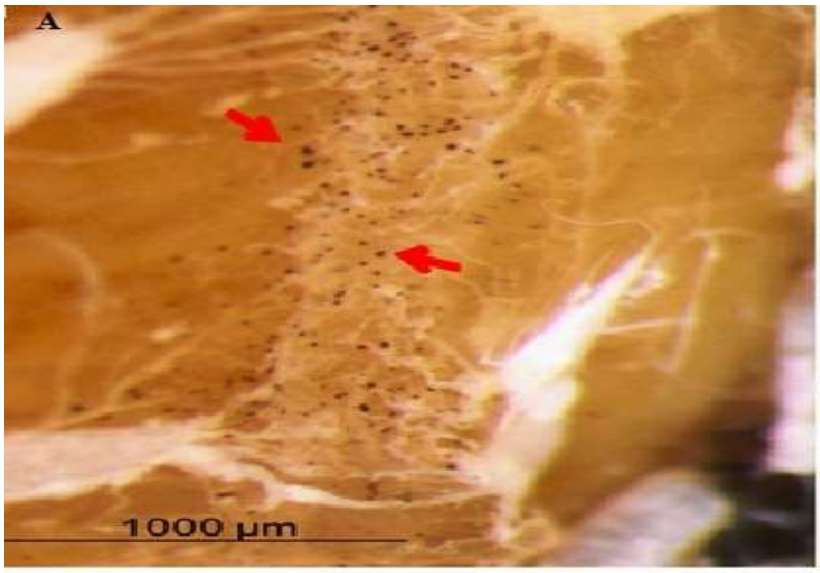


Figure (20b): Stereo microscope image of *S. enteritidis* injected larva. A) Dissected larva 24 post injection showing cellular nodules surrounding the heart (X 80).

Table (8): Number of different sized nodules in 3rd instar larvae of *A. m. jemenitica* injected with *P. larvae* or *S. enteritidis* at different time intervals.

Times post injection (h)	Bacteria	The number of cellular nodules depending on the size (µm)			Total count/10 larvae
		Small	**medium**	**Large**	
4	***P. larvae***	27 (64.29%)	13 (30.95%)	2 (4.76%)	42
	S. enteritides	13 (43.33%)	17 (56.67%)	0 (0%)	30
12	***P. larvae***	32 (40.51%)	42 (53.16%)	5 (6.33%)	79
	S. enteritides	37 (66.07%)	19 (33.93%)	0 (0%)	56
24	***P. larvae***	57 (60.64%)	22 (23.40%)	15 (15.96%)	94
	S. enteritides	44 (61.97%)	20 (28.17%)	7 (9.86%)	71
48	***P. larvae***	60 (46.51%)	49 (37.98%)	20 (15.50%)	129
	S. enteritides	49 (56.98%)	24 (27.91%)	13 (15.12%)	86

Nodule size: small (5-10 µm), medium (10-30 µm), large (40-80 µm).Each point was represented as percentage of the total nodule counts/10 larvae. Control groups (non injected larvae) showed no nodule formations at all time intervals.

4.3.2.2 Light and TEM examination of nodules in dissected *A. m. jemnitica* 3rd instar larvae post-injection with *P. larvae*

Figure (19a) shows nodules formed in *A. m. jemenitica* 3rd instar larvae after injection with *P. larvae*. A) clear milky white larvae with no nodulation (arrow indicates dissected larva). B) Dissected larvae observed with nodules formed 4 h post injection. C) Whole larva showing different sized nodules 24h post injection. D) Dissected larvae 24h post injection showing alimentary canal (i) and nodules surrounding dorsal blood vessel (ii). The same pattern was also observed post injection with *S. entritidis* (figure 20a) .

Light and TEM images showing nodules surrounding the dorsal blood vessel after 24h of injection with *P. larvae* or *S. entritidis* A and B (figure 19b ,20b).

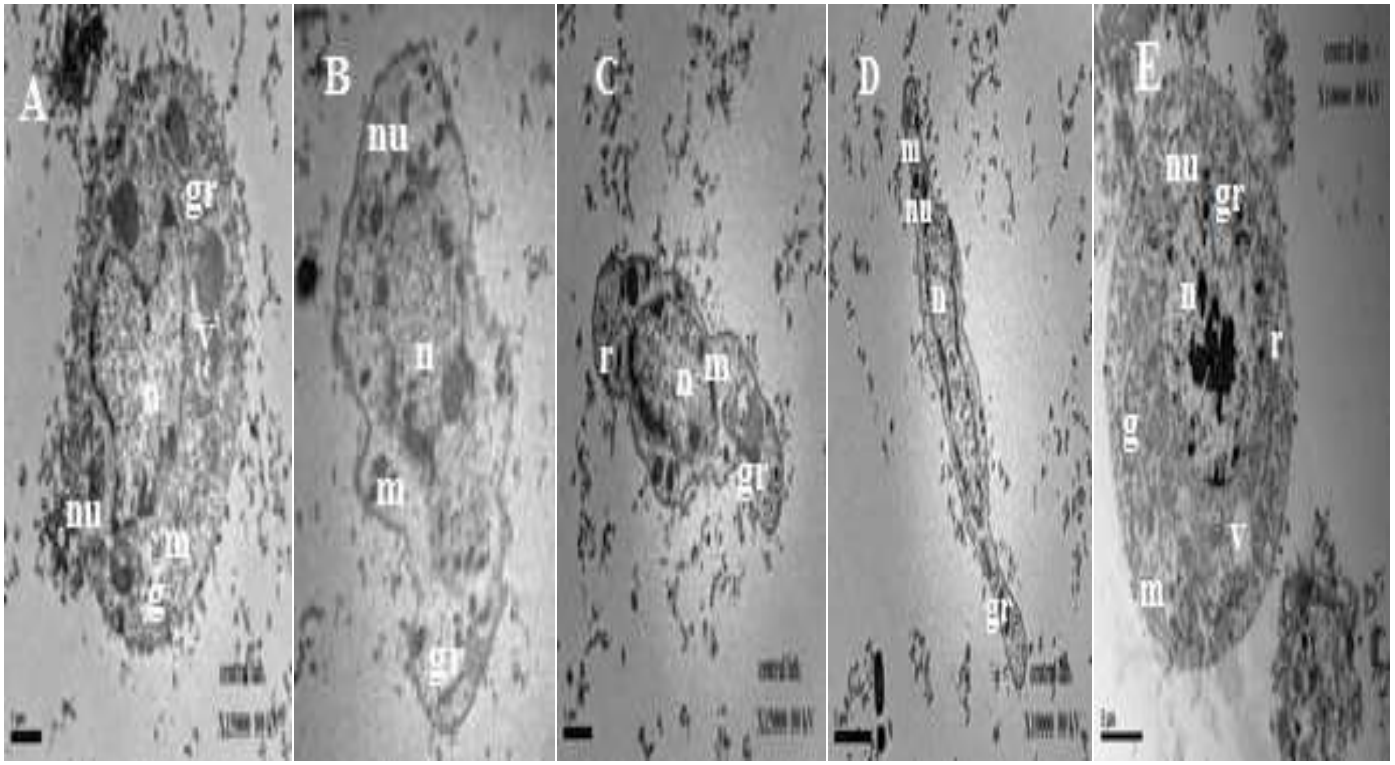
The time course of nodulation in bee larvae showed that maximum number of nodules/h were produced 48 h with *P. larvae* bacterial injection. The pattern generally shows increased number of nodules with increased incubation time, up to about 8h.

4.3.2.3 Transmission electron microscopy of *A. m. jemenitica* 3rd instar larvae hemocyte phagocytosis *in vivo*

Note is non injected cells in their natural state that granulocytes cells are round in shape containing many granules(Figure21A). Plasmatocytes cells look oval (spindle-shaped) pointed parties have become larger and thicker and be false feet (Pseudopodia) and feet Plate (Lamellipodia) and the nucleus being large round in the center of the cell (Figure21B,C,D) a. Spherule cells become rounded to an oval shaped containing numerous spherules. As oenocytoids become semi-transparent with an eccentric side nucleus (Figure21E).

Twenty four hour post injection of *A. m. jemenitica* 3rd instar larvae with 0.9µl carboxylate-modified polystyrene latex beads suspension, the granulocytes showed a noticeable activity with a number of phagosomes containing the latex beads and often with the nucleus pushed towards the outer edge of the cytoplasm (Figure22B,C). Vacuoles with lysed latex beads inside the phagosomes were observed. The granules fuse with the phagosomes and discharge their content inside them making the space around the latex beads more electron dense (Figure22E). Multi vesicular bodies were also present (Figure22A). Granulocytes that presented conspicuous phagocytic activity showed their cytoplasm filled with latex

beads, lacked granules and plasma membrane ruptures (Figure22E). Activated plasmatocytes showed irregular shape varying from round to very elongated ,and their nuclei was deeply indented (Figure23D). A remarkable effect of *P. larvae* injection was the presence of variable numbers (up to 20) of round to irregular vesicles (Figure 24 A,C). The phagocytizing granulocyte and plasmatocyte containing latex beads particles inside the phagosomes and vesicles were also observed (Figure 24 C,E). Activated oenocytoids of *P. larvae* induced *A. m. jementica* 3rd instar larvae engulfed latex beads, each inside a single phagosome; the surface of the plasma membrane remained smooth with only few short filopodia present (Figure 24H). Phagocytosis of a single latex bead engulfed by short large filopodiawas observed (Figure23A,Band 24D,E,G). Granulocyte showing many granules, lobated nucleus, rim cytoplasm and central large nucleus with few granules. All cell types were featureless, degranulated, with large vaculation bubbling and complete lysis. Activated spherule cells showed changes in shape which became very irregular. The electron density of the cytoplasm and the number of granules varies from cell to cell. Ingested latex beads were not observed in spherule cells.



Figure(21): TEM of *A. m. jemenitica* 3rd instar larvae hemocytes 24h control. A,E) granulocyte with many typical granules (*gr*), lobated nucleus (*n*) with an evident nucleolus (*nu*), rough endoplasmic reticulum (*r*), mitochondria (*m*),Golgi complex (*g*),round vesicles (*asterisks*) and electron dense vesicles (*v*). B,C,D) plasmatocyte oval shaped with few granules (*gr*), nucleus (*n*) with evident nucleolus (*nu*), mitochondria (*m*).

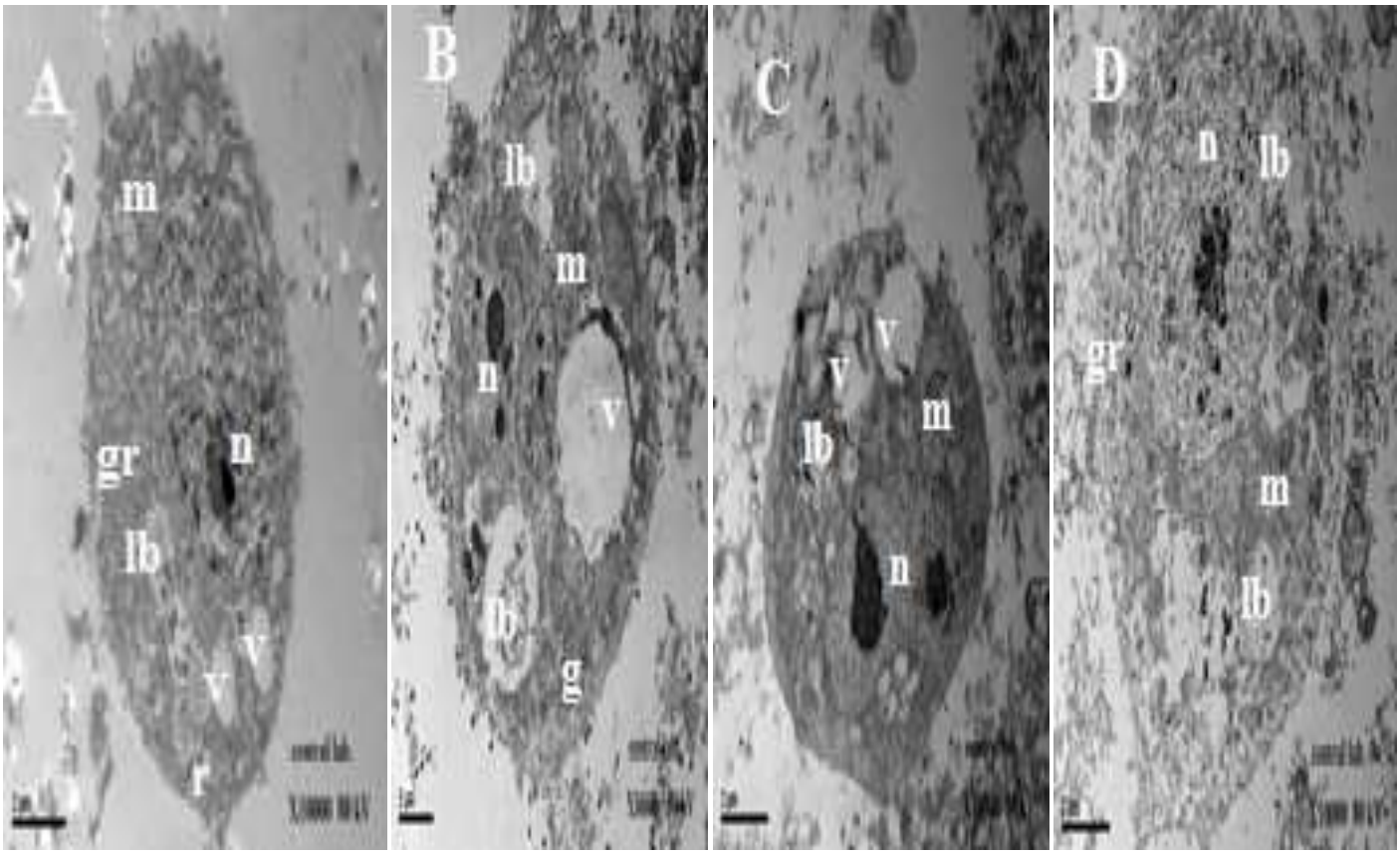


Figure (22): TEM of *A. m. jemenitica* 3rd instar larvae hemocytes 24 h post- injection with (0.9 µl) latex beads suspension. A) Oenocytoid showing large nucleus, many Golgi complex (g), the cytoplasm with large vesicles (*v*), mitochondria (*m*). B) Plasmatocyte showing bubbling and featureless of cell membrane with phagosomes containing lysed latex beads(*lb*),an eochromatic nucleus (*n*) and mitochondria (*m*). C,D) Granulocyte (degranulated), nucleus (*n*), mitochondria (*m*) and Golgi complexes (*g*).

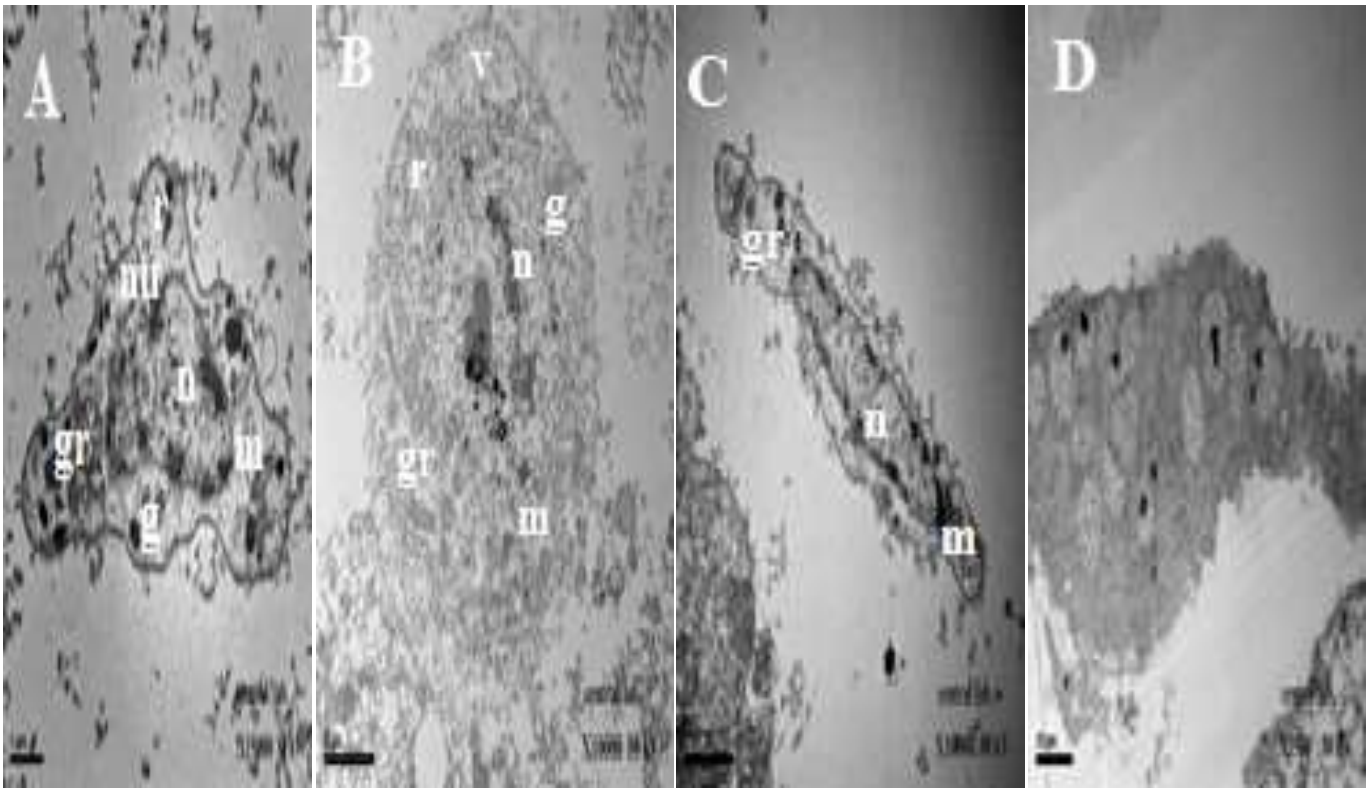


Figure (23):TEM of *A. m. jemenitica* 3rd instar larvae hemocytes 24 h post- injection with (0.5µl from 1x10^{6}/ml) of *P. larvae*. A,C) plasmatocyte with eccentric nucleus (*n*) filopodia projection (*f*), rough endoplasmic reticulum (*r*) and mitochondria (*m*). D) coagulocytes with a heterochromatic nucleus (*n*) presenting characteristic perinuclear spaces formed by parting inner and outer nuclear membranes detaching themselves (*ps*) cytoplasm presents multi vesicular bodies. B). oenocytoid showing the characteristic round shape and the homogeneous, medium electron dense and finely granular cytoplasm with a central nucleus (*n*). Few lemon-like granules (*g*), small mitochondria (*m*), round electron lucent vesicles and rough endoplasmic reticulum (*r*) are present. The plasma membrane presents few short filo podia (*f*) on its surface. Granulocyte showing many granules(*gr*) with central nucleus(*n*), filopodia projection (*f*) and round vesicles (*asterisks*).

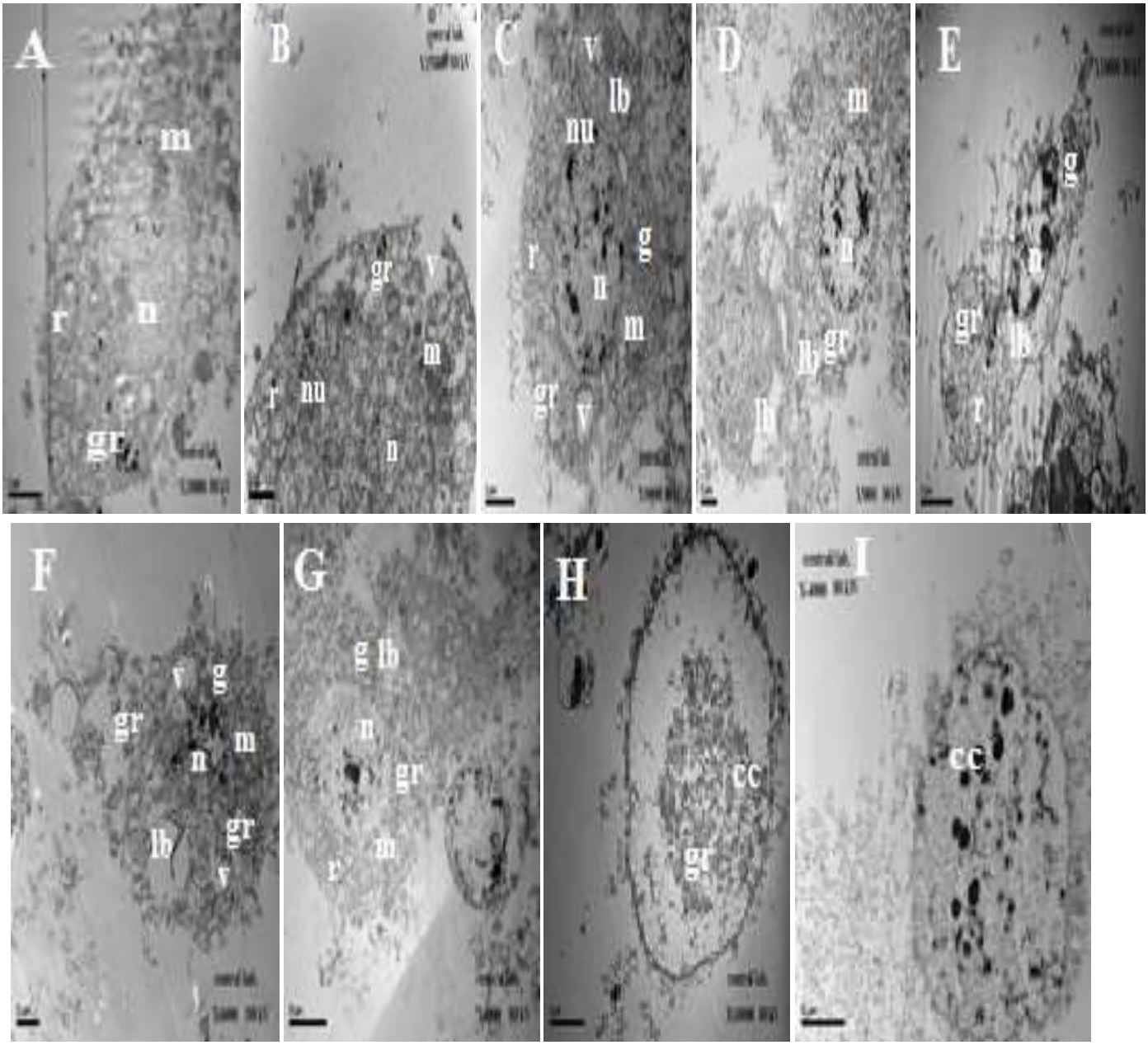


Figure (24): TEM of *A. m. jemenitica* 3rd instar larvae hemocytes 24 h post- injection with (0.5µl from 1x10^{6}/ml) of *P. larvae* and (0.9 µl) latex beads. A,B) Oenocytoid showing elongated shape and the homogeneous. E) Plasmatocyte showing many elongated Golgi apparatus (g), eccentric nucleus (*n*) with nucleolus (*nu*), mitochondria (*m*), rough endoplasmic reticulum (*r*) and phagosomes containing lysed latex beads(*lb*), . C,D,F,G,H and I) Granulocyte showing many granules (*gr*), lobated nucleus (*n*), rough endoplasmic reticulum (*r*), mitochondria (*m*) , rim cytoplasm and central large nucleus with few granules H,I) showing crystal cells(cc). All cell types are Featureless, degranulated, with large vaculation bubbling and complete lysis.

4.3.2.4 *In vivo* melanization of *A. m. jemenitica* 3rd instar post injections with *P. larvae / S. entritidis* compared to *M. luteus*

Figures 25 and 26 showed complete blackening , melanization , PO activation followed by increment of percentage mortality 48h post injection of *A. m. jemenitica* 3rd instar with *M. luteus* as well as *P. larvae* compared to *S. entritidis* that showed no signs of blackening or melaniztion or mortality as indicated in figure (27).

Figure (25):Photograph *A. m. jemenitica* larvae (3rd instar). (A) control larvae ,(B,C,D,E) 4,18,24 and 48 h post- injection with *M. luteus*, respectively (E) complete melanization treated larvae , (X80) Canon camera.

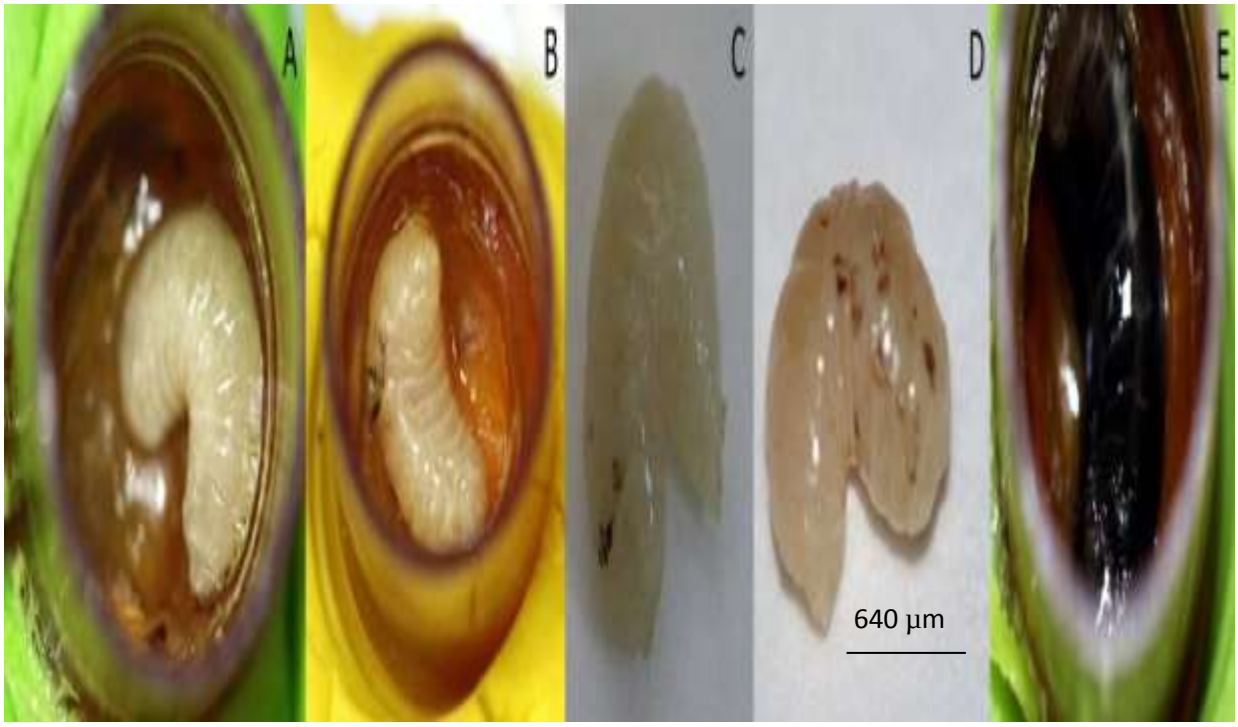


Figure (26):Photograph *A. m. jemenitica* larvae (3rd instar). (A) control larvae ,(B,C,D,E) 4,18,24 and 48 h post- injection with *P. larvae*, respectively (E) complete melanization treated larvae , (X80) Canon camera.

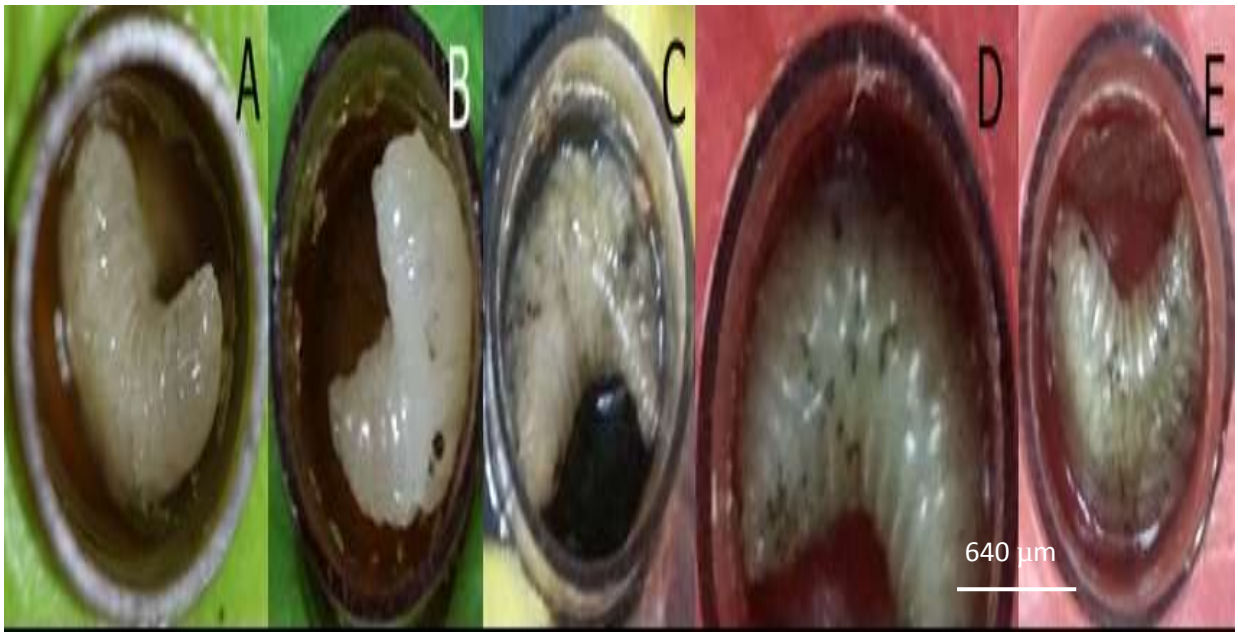


Figure (27):Photograph *A. m. jemenitica* larvae(3rd instar). (A) control larvae ,(B,C,D,E) 4,18,24and 48 h post- injection with *S. enteritidis* , respectively , (X80) Canon camera.

4.4 Light microscopy and transmission electron microscopy (TEM) of digestive system of orally infected *A. m. jementica* 3rd instar

4.4.1 Stomach of *A. m. jemenitica* 3rd instar

The results demonstrate that infection of *A. m. jemenitica* 3rd instar larvae with *P. larvae* spores resulted in AFB disease. The honey bee crop was severely affected as seen in the histology of the digestive system parts.

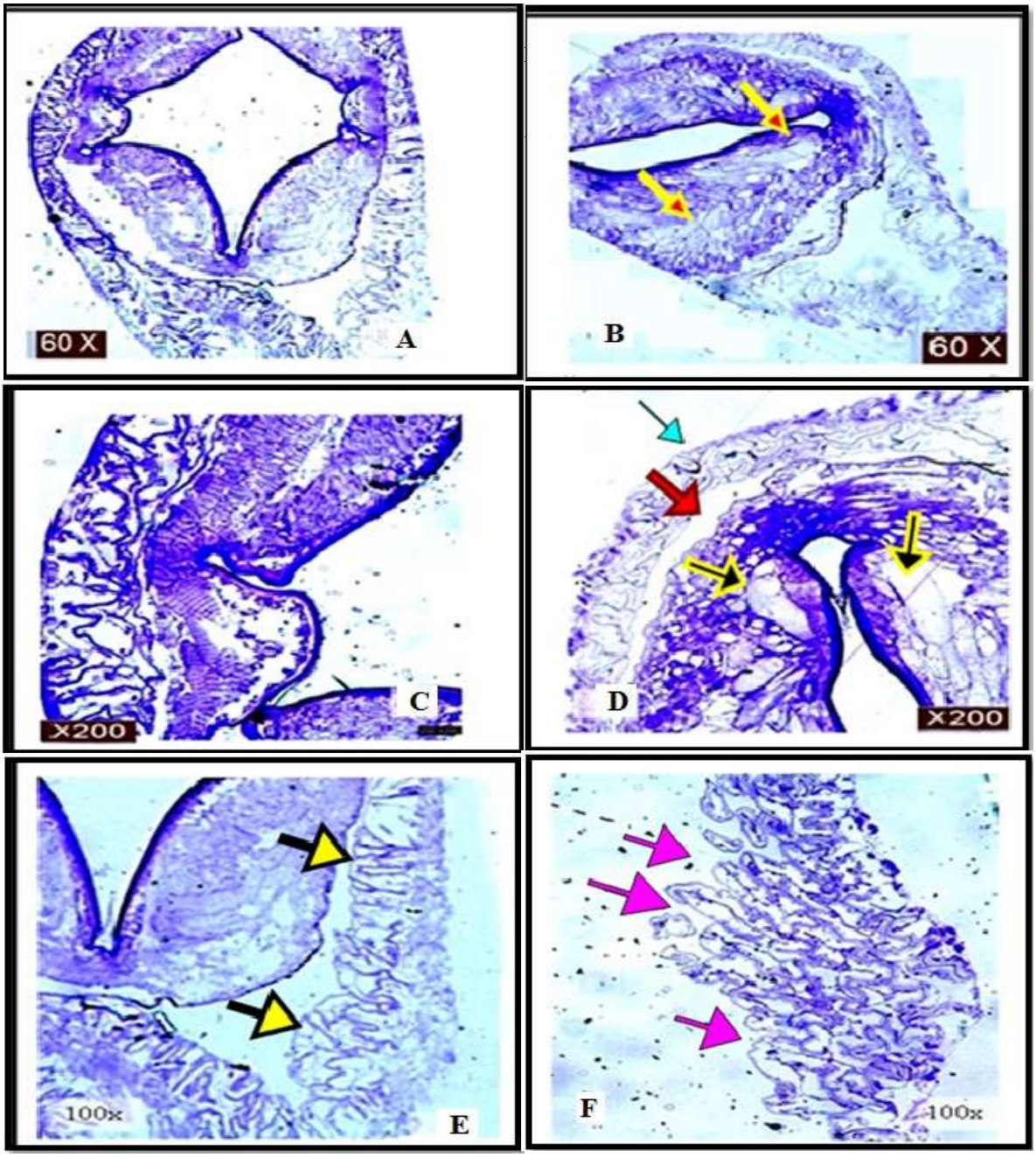
4.4.1.1 At Four hours post *P. larvae* infection

Significant effects were observed on the proventriculus of *A. m. jemenitica* 3rd instar larvae followed by the gut tissue. Proventriculus of the larvae exhibited many vacuoles as shown in (Figures 28 B , D) which were absent in control non injected larvae (Figures 28 A , C).

Microbial invasion caused the separation of the epithelial layer as shown in (Figure 28 D). The circular muscle layer was also affected as indicated by blue arrow in (Figure 28 D).

4.4.1.2 At Twenty four hours post *P. larvae* infection

A. m. jementica 3rd instar larvae infected with *P. larvae* showed separation and elongation of the epithelial cells of the stomach and the



Figures (28): A,C,E) Light microscopy stained sections of Stomach of : non infected (control) *A. m. jemenitica* 3rd instar larvae. B,D) Stomach 4 h post infection with *P. larvae* . F) Stomach 24 h post infection with *P. larvae*.

4.4.2 Midgut of *A. m. jemenitica* 3rd instar

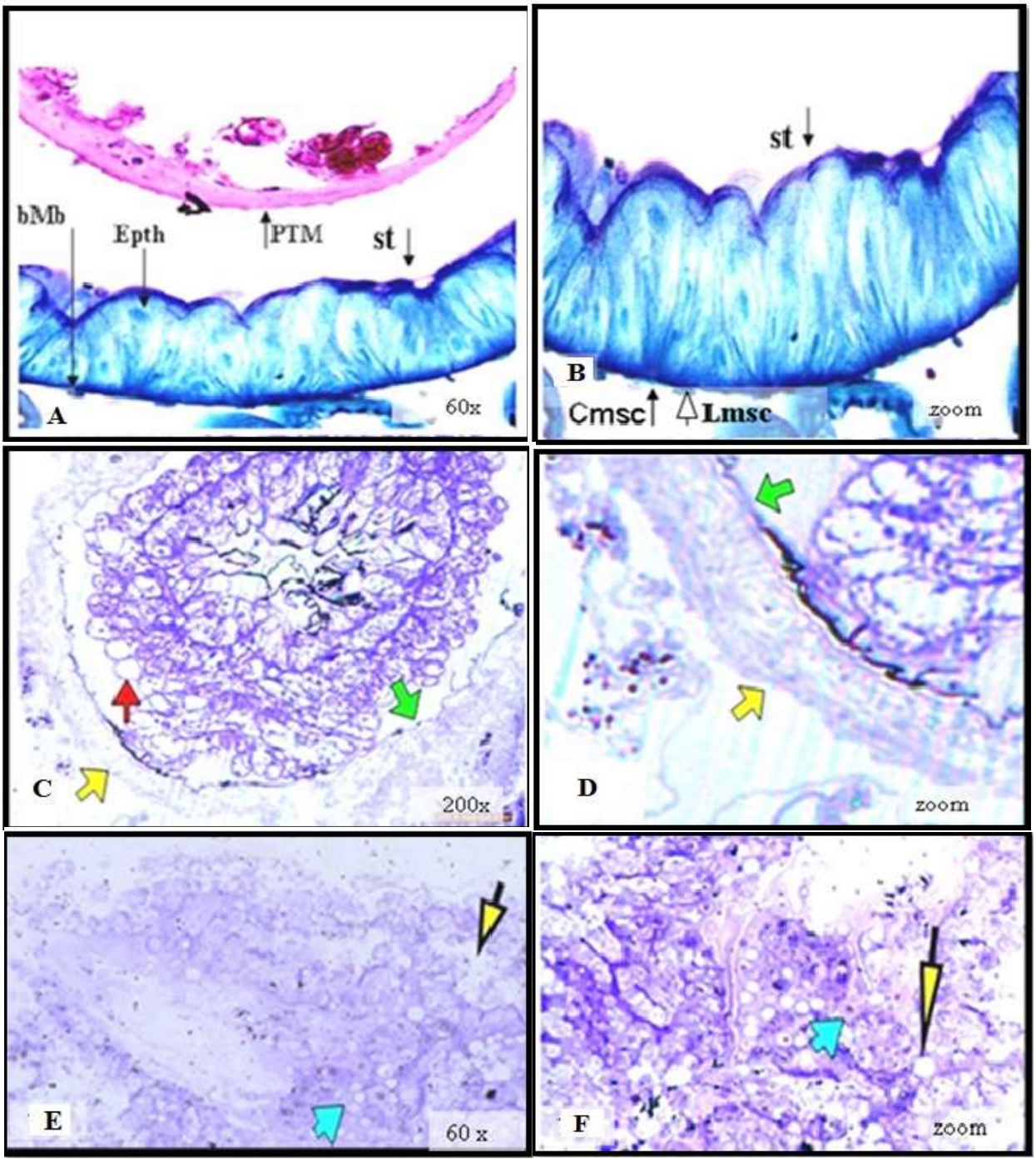
Results of *P. larvae* artificially infected *A. m. jemenitica* 3rd instar *larvae* showed gradual effects on the midgut tissue that increased as the time of microbial infection increased.

4.4.2.1 At Four hours post *P. larvae* infection

Laceration was observed in the basement membrane surrounding the stomach in the infected larvae (Figures 29 C, D) when compared to control ones (Figures 29 A , B). The infected larvae also showed loss of muscle layer and striated border (Figures 29 C , D) when compared to control larvae (Figures 29 A , B). Large vacuoles were also observed in the infected *A. m. jemenitica* 3rd instar larvae which were absent in the control group.

4.4.2.2 At Twenty four hours post *P. larvae* infection

Serious damage of the epithelial cells was also observed in the midgut tissues due to *P. larvae* infection. There was a severe laceration of the stomach layer as shown in the (Figures 29 E , F blue arrows). Generally, there were damages to all the components of muscles and epithelial cells layers. Increased number of vacuolations were observed in infected larvae



Figures (29): Light microscopy stained sections of : A,B) Mid gut of non infected (control) *A. m. jemenitica* 3rd instar larvae. C,D) Mid gut 4 h post infection with *P. larvae* . E,F) Mid gut of 24 h post infection with *P. larvae*. bMb: Basement membrane , pth: Epithelial cells ,PTM: Peritrophic membrane, St: Striated border , Lmsc: Longiudinal muscles,Cmsc :Circular muscles.

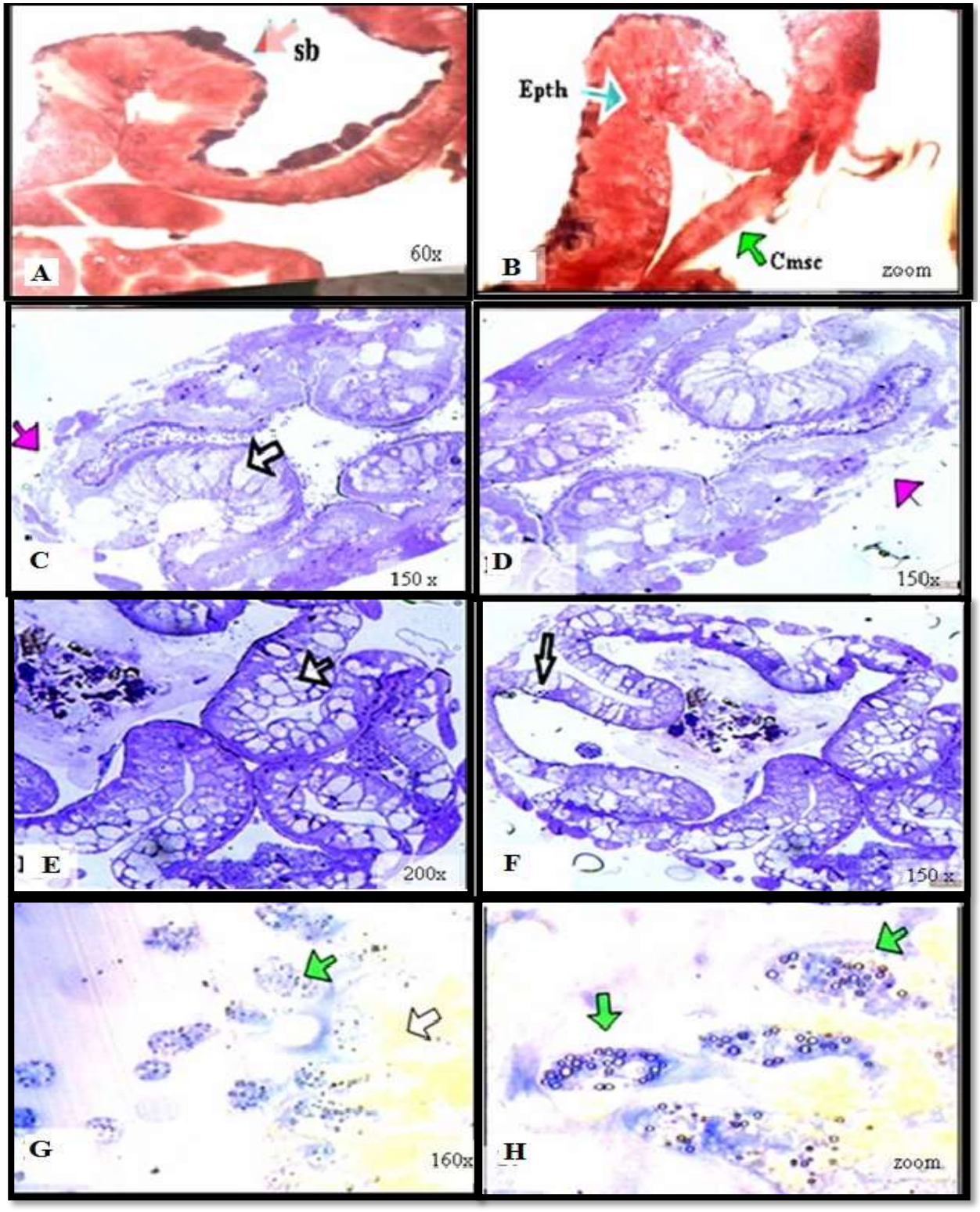
4.4.3 Hindgut of *A. m. jemenitica* 3rd instar

4.4.3.1 At Four hours post *P. larvae* infection

After four hours of infection of *A. m. jemenitica* 3rd instar larvae with *P. larvae*, vacuoles of different sizes were observed in the hindgut as shown in (Figure 30 B) when compared to control larvae (Figures 30 A , B). The hind gut of infected larvae showed loss of longitudinal muscular layer (Figures 30 C , D).

4.4.3.2 At Twenty four hours post *P. larvae* infection

Increased vacuoles were observed in the epithelial cells of the hindgut of *A. m. jemenitica* 3rd instar larvae infected with *P. larvae* (Figures 30 E , F). The infected larvae also showed the presence of microbial communities (Figures 30 G , H). Severe laceration was also observed in the epithelial cells (Figure 30 G).



Figures (30): Light microscopy stained sections of : A,B) Hindgut of non injected *A. m. jemenitica* 3rd instar larva. Arrows indicate Sb: Striated border, epth : epithelial cells, CMSC: circular muscles. C,D) Effect of *P. larvae* injection on epithelial cells (arrow) indicates different vacuoles. E,F) Heavy deployment and increased vacuoles of epithelial cells. G,H) Aggregation of *P. larvae* in epithelial cells.

4.6 Anti-bacterial activity (MIC)of purified peptide fractions from plasma of injected *A. m. jemenitica* 3rd instar larvae against different standard bacterial strains

The induction of the humoral immune response in *A. m. jemenitica* 3rd instar larvae 72h old was performed by injection with either *P. larvae* or *S. enteritidis*. The induced peptides were isolated using RP-HPLC resulting in fractions (A, B and C) from *P. larvae* /or (D, E and F)from *S. enteritidis* injected *A. m. jemenitica* 3rd instar larvae plasma. These fractions were subjected to Minimal Inhibitory Concentration (MIC) and tested against different standard strains viz., *S. enteritidis*, *E. coli* , *M. luteus*, *St. aureus*, *B. subtilus*, *P. aeruginosa* and *P. larvae* using CIP antibiotic as reference. Table (9) shows that, the purified peptide fraction B showed the best antibacterial potency compared with the reference CIP antibiotic against *S. enteritidis* followed by fractions (A, C and F) that have the same antibacterial activity. Fractions A, B, E and F showed similar activity, while C and D fractions produced same antibacterial activity equal to that of CIP against *Staphylococcus aureus*. Fractions B and F showed the highest antibacterial activity than the other fractions while they were less effective than CIP towards *P. larvae*. On the other hand fractions A, B, C and D showed similar activity as that of CIP

against *E. coli* and *M. luteus*. Fraction D was the most potent when compared to other fractions towards *Ps. aeruginosa*. In case of *B. subtilis*, fraction A showed most potency followed by fractions C and D that exhibited the similar activity compared to other fractions and also than CIP activity.

Table (9): Anti-bacterial activity using minimum inhibitory concentration (MIC) of purified peptides after 24 h incubation at 37°C with selected standard bacterial strains.

Bacteria	A	B	C	D	E	F	CIP
	MIC of Peptide Fractions						**(MIC*)**
Salmonella enteritidis 13076	64	32	64	128	128	64	0.25
Staph aureus 6538	4	4	0.25	0.25	4	4	0.25
Panbacillus larvae 10801	128	64	128	128	128	64	4
Micrococcus luteus 2665	0.25	0.25	0.25	0.25	4	4	0.25
Pseudomonas aeruginosa 9027	64	64	64	32	128	128	0.25
*Escherichia coli*10536	0.25	0.25	0.25	0.25	4	4	0.25
Bacillus subtilus 6051	4	32	8	8	64	64	0.5

*Minimum inhibitory concentration ($\mu g ml^{-1}$)
A,B,C,D,E,F: isolated purified peptide fractions
CIP: Ciprofloxacin antibiotic as reference

4.7 Susceptibility of *S. enteritidis* towards different *A. m. jemenitica* 3rd instar larvae purified peptide fractions

Antibacterial susceptibility of *S. enteritidis* for the six purified peptides (A,B,C) or (D,E and F) were done according to NCCLS, (2003) by disk diffusion method. Zones of inhibition for each peptide fraction (A,B,C) as well as (D , E and F) were 19.5±0.71, 21±0, 18.5±0.71, 19±1.41, 18±1.41and 19±0 mm, respectively (Figure 31 and Table 10) .

It was deduced that the peptide fraction B showed the highest antibacterial activity as it produced the largest inhibition zone diameter about 21±0 mm compared with ciprofloxacin. Based on this result the

ability of the purified peptide fraction B was evaluated for the prevention of the attachment of *S. enteritidis* to intestinal epithelial cells *in vitro*.

(Figure48) shows the inhibition efficacy of the isolated purified peptide fractions on adhesion capability of *Salmonella* bacterial strain to human epithelial cells. The diffusion assay for inhibition of bacterial growth was used to determine the most efficient peptide fraction, which would be applied in the adherence study for *S. enteritidis* (the causative agent for food poisoning) to intestinal epithelial cells *in vitro*.

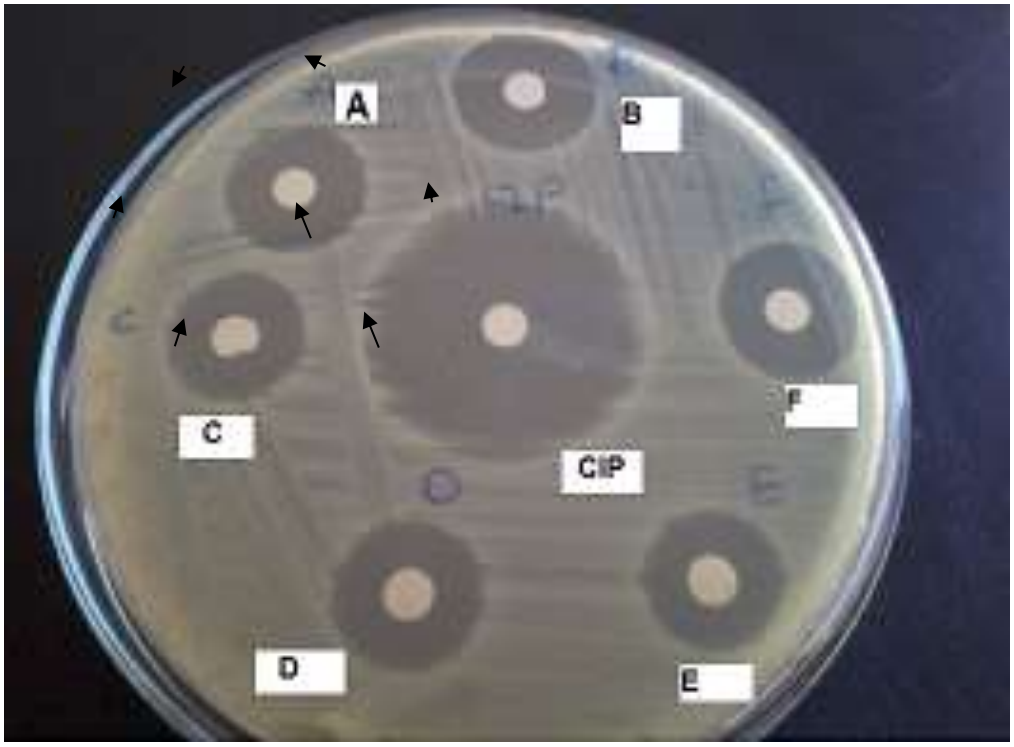


Figure (31): **Mueller Hinton agar plate showing antibacterial activity of *A. m. jemenitica* 3rd instar larvae purified peptide fractions disk (A,B,C,D,E,F) against *S. enteritidis* using ciprofloxacin (CIP) antibiotic as reference.**

Table (10): **Susceptibility test using disk diffusion method for peptide fractions against *S. enteritidis* (inhibition zone in mm (mean±SD) using Ciprofloxacin (CIP) as reference.**

Disk content (25µg) of peptide	A	B	C	D	E	F	CIP
Salmonella enteritidis *ATCC13076* (25µg)	19.5±0.71mm	21±0mm	18.5±0.71mm	19±1.41mm	18±1.41mm	19±0mm	37±1.41 mm

4.8 Anti-adhesion of *S. enteritidis* to human epithelial cells

By count method, peptide fractions A, B, C and D significantly decreased the adhesion ($P<0.001$) while fractions E and F showed less significant reduction of adhesion ($P=0.009$). Peptide fraction B showed the highest efficiency in adhesion reduction followed by fraction A ($P=0.02$) (Figures 32 and 33).

Crystal violet method using light microscopy by oil immersion lens (x1000) of peptide fraction (B) showed significant reduction in *S. enteritidis* adhesion to epithelial cells (Figure 33). Results revealed a quantitative aspect that was evaluated by image software analysis as well as the distribution of adherent bacteria onto the surface.

Epithelial cells were co-cultured with *S. enteritidis* in absence (control) and presence of purified peptide fraction B in concentrations of ½ MIC and ¼ MIC. Cells were incubated at 37◦C overnight, fixed and stained

fraction B on *S. enteritidis in vitro*. All the peptides exhibited a reduction of *S. enteritidis* adhesion to human epithelial cells as shown in (Figure 34).

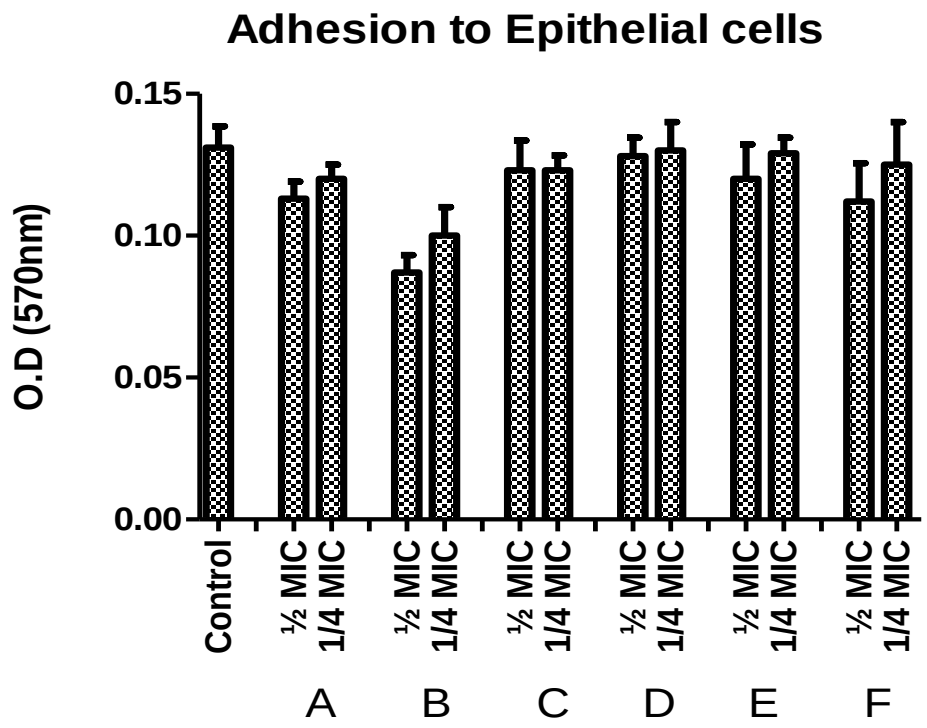


Figure (32): Adhesion of *S. enteritidis* to epithelial cells in (control) and in the presence of purified peptide fractions (A, B, C, D, E and F) in concentrations ½ MIC and ¼ MIC.

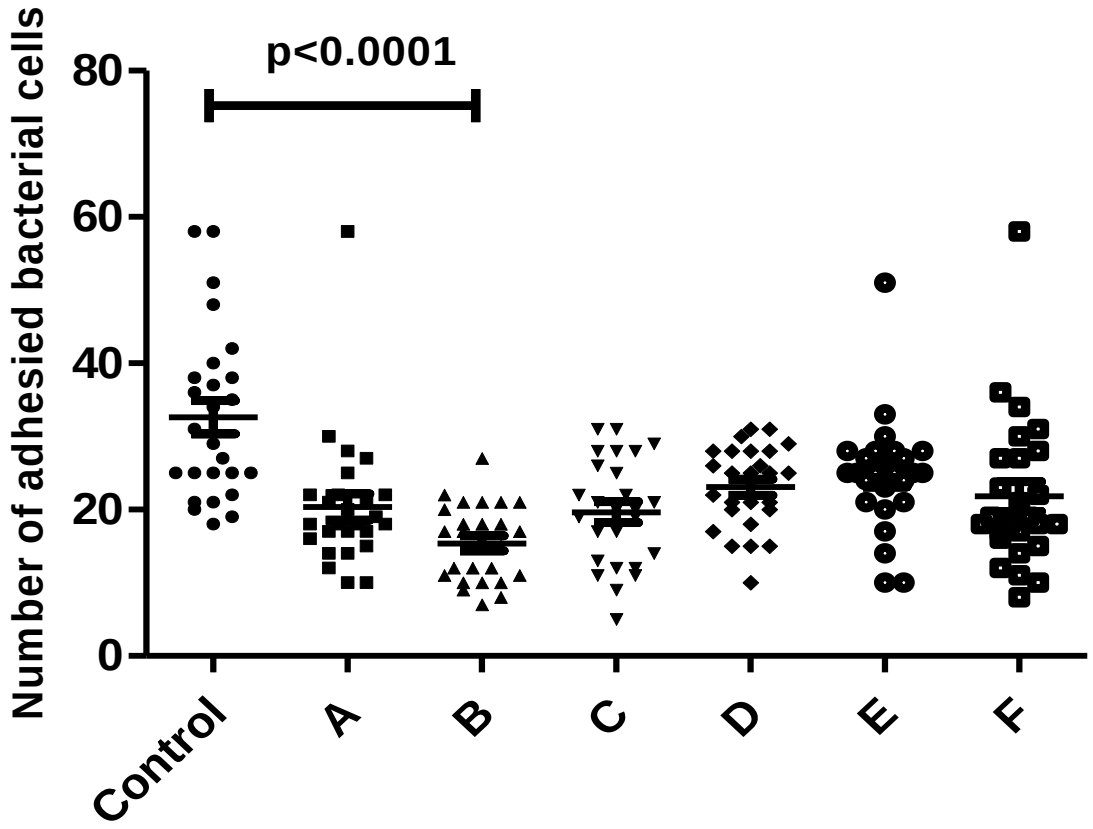
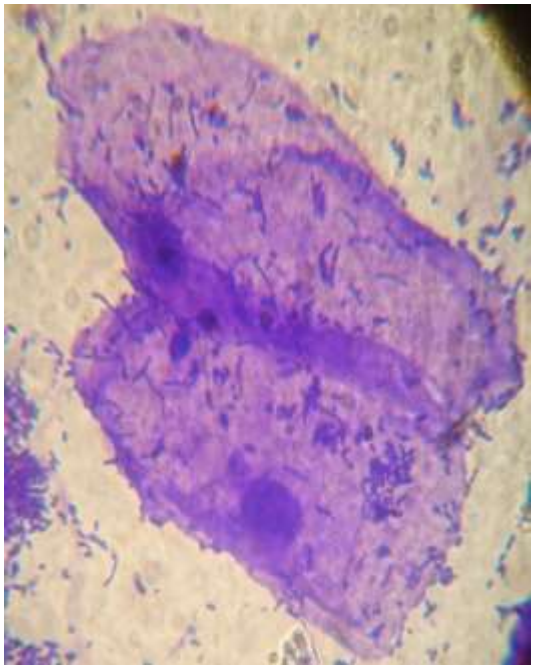
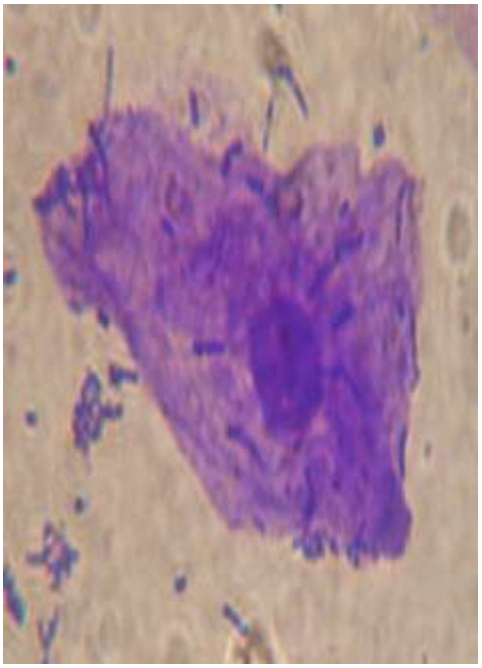


Figure (33): Scatter plot of number of adhered *S. enteritidis* to epithelial cells using purified peptide fractions (A, B, C, D, E, F). Adhered stained bacterial cells with crystal violet were counted under microscope (x 1000 magnification).





(A) **(B)**

Figure (34): Light microscopy showing the adherence of *S. enteritidis* to human epithelial cells in absence and presence of purified peptide fraction. (A) Untreated *S. enteritidis* and (B) *S. enteritidis* after treatment with ½ MIC peptide fraction B.

DISCUSSION

AND

CONCLUSION

5 DISCUSSION

American foulbrood (AFB), caused by the spore- forming *Paenibacillus larvae ssp.* (formerly classified as *Bacillus larvae*), is the most widespread and destructive of the bee brood diseases. In the past decade, considerable progress has been made in the understanding and taxonomic reclassification of the causative agent as well as the diagnosis of AFB. Traditional methods such as the recognition of typical clinical symptoms of infection, culture of *P. larvae* from diseased brood, and microscopy provide effective and inexpensive means of diagnosing the disease. In addition, improved detection of *P. larvae* from brood and bee products using molecular techniques, immunotechniques and superior culture techniques have emerged in recent years providing a broader range of methodologies for efficient diagnosis (de Graaf *et al.*,2006).In the present study, the immune systems of healthy, *P. larvae/S. enteritidis* infected *A. m. jemenitica* 3rd instar larvae were compared.

The innate immune response in honey bees is manifested in three major ways: a constitutively active cellular system that includes phagocytosis and nodulation response, an induced humoral immune response that ultimately generates small antimicrobial peptides (AMPs) and the activation of Prophenoloxidase (Propo) by a serine protease cascade resulting in the transient synthesis of quinones and melanin

(Gätschenberger *et al.,* 2013). In the present study the total protein content of plasma of *A. m. jemenitica* 3rd instar larvae injected with *S. enteritidis* and *P. larvae* bacteria was the highest followed by that of LPS and *M. luteus* compared with that of control values. SDS-PAGE electrophoresis of plasma of *A . m. jemenitica* 3rd instar larvae injected with *P. larvae* and/or *S. enteritidis* confirmed that the bands with protein over expression were similar to PO exhibiting a molecular mass of 70 kDa and lysozyme with a molecular mass ~ 15 kDa. This is in accordance with the earlier studies of Osawa et al., 2001. Same authors have demonstrated that the activity of this enzyme during development of larvae appears to correlate very tightly with susceptibility of the larvae to infection. It has been postulated that the host larva responds to infection by producing proteins that can fight the infection which explains the up-regulation of PO in larvae infected with *E. coli* or *S. enteritidis* which are not natural pathogens of bees (Osawa *et al.,* 2001). Another protein involved in immune response is lysozyme, The primary known function of lysozyme is to degrade the peptidoglycan shell of Gram-positive bacteria (Kunieda *et al.,* 2006) and is therefore expected to have a significant role in inhibiting *P. larvae*. The constitutive lysozyme concentration in plasma is considered as a background level of activity that participates in the initial immune responses against the invading

bacteria. It is believed to degrade the bacterial debris released during the initial cellular immune response (Klara Randolt *et al*.,2008).The increased total protein content in *P. larvae* infected *A. m. jemenitica* 3rd instar larvae plasma may therefore be due to upregulation of these enzymes.

Phenoloxidase is a vital enzyme essential for a number of processes such as cuticular hardening (sclerotization), pigmentation, wound healing and nodulation. It is present as an inactive precursor, called proPO zymogen, and is activated by a cascade of serine proteases (Gätschenberger *et al.*, 2001; Cerenius *et al*., 2008; Zufelatoa *et al*., 2004). Phenoloxidase (PO) activity is widely used as an indicator of insects' immunocompetence (In the present study the initial PO activity was low in the larvae. However with the progression of time there was a significant increase in the plasma PO activity of *P. larvae* infected *A. m. jemenitica* 3rd instar larvae. This may be due to activation of PO from its zymogen Propo by immune receptor protein present on the surface of blood cell and hemolymph.

Lysozymes and other lytic proteins can be active against both Gram-positive and Gram-negative bacteria, potentially activating the Toll-related signaling pathway by releasing bacterial cell wall material. Lysozyme activity is usually more related to bacterial infection and PO activity to fungal and multicellular hemolymph-invading organisms

(Freitak *et al*., 2007). This was observed in the present study where the hemolymph of *P. larvae* infected *A. m. jemenitica* 3rd instar larvae showed an increased lysozyme activity with the progression of time. The results of the present study suggests that lysozyme can be important in host defense, which is also supported by the observation that its homolog has antimicrobial activity in the medicinal leech *Hirudo medicinalis* (Weidang *et al*., 2014).

The melanization of the nodules in *A. m. jemenitica* 3rd instar larvae in response to infection has also been reported in other insects (Lackie, 1988) similar to deposition of melanin in nodules on the surface of encapsulated parasites and in hemolymph clots at the vicinity of the wound. In the present study also melanization was observed in *A. m. jementica* larvae injected with *P. larvae.*

Nodulation is a predominant cellular immune reaction in many insects, and is activated by bacteria and fungi and involves the interaction between circulating hemocytes and microorganisms (Horohov and Dunn, 1983). Nodulation is a predominant cellular defense reaction to microbial invasion in insects (Horohov and Dunn, 1983;Koella and Sørensen .,2002). Nodulation is a complex process; it involves prominently hemocyte migration, aggregation, and adhesion .It is initiated by the release of adhesion proteins from granulocytes, trapping the

microorganisms and forming microaggregates with other granulocytes. These protonodules are ultimately ensheathed by a wall of plasmatocytes (Ratcliffe and Rowley, 1979 ; Boman , 1991; Gasic *et al*., 2007). In the present work, intra hemocoelic injection of *A. m. jemenitica* 3rd instar larvae with the *P. larvae* pathogenic bacteria evoked strong cellular immune responses, as measured by the number of the formed nodules.

The time course of nodulation in bee larvae showed that maximum number of nodules/h were produced 48 h PI with *P. larvae* bacterial injection. The highest rate of nodulation varies considerably among insects; i.e. 8h in *Chrysomya megacephala* (Zhao *et al.,* 2009), 4h in *Chilo suppressalis* (Mirhaghparast *et al.,* 2015) , 6h in *Manduca sexta* (Lord *et al.,* 2002), 1-2h in *Pimpla turionellae* (Kayis *et al*., 2012). The pattern generally shows increased number of nodules with increased incubation time PI, up to about 8h PI. The difference between the different insect species may be due to variation in the elucidation potential of the PAMPs of the different pathogens and to the efficiency of the subsequent response. The variation in population density of the circulating hemocytes and the rate at which they are recruited may also effect nodule formation (Ratcliffe and Rowley, 1979; Lackie, 1988; Kotthoff *et al*., 2011).

Antimicrobial peptides (AMPs) are basic molecules of small size. According to their biochemical characteristics, they are classified in three main groups: (1) linear peptides without cysteine (e.g., cecropins); (2) linear peptides that are enriched in one amino acid, e.g., glycine or proline; and (3) peptides with an even number of cysteine residues resulting in several intramolecular disulfide bridges(Boman and Hultmark 1987; Hoffmann, 1995; Evans *et. al*., 2006 ; Chen *et al*., 2009). In the present study AMP production was induced in the plasma of *P. larvae* infected *A. m. jemenitica* 3rd instar larvae. They were purified by RP-HPLC and designated as A, B and C. They were shown to possess antibacterial activity against *E. coli, M. luteis, B. subtilis* and *P. aeruginosa* using CIF as the standard by disc diffusion method. One of the fractions was found to show cecropin like antibacterial activity. This may lead us to speculate that it is a linear peptide without cysteine residue.

The anti-adhesive effect of the isolated purified peptide fraction B in the present work towards eukaryotic epithelial cells supports its role as a bactericidal agent. There are several possible explanations for prevention of bacterial adherence demonstrated by purified peptides isolated from honeybee *Apis mellifera* i) non-specific mechanical inhibition through coating the bacteria by the peptides; ii) peptide fractions may alter

bacterial electrostatic charge or hydrophobicity which have been reported to be important factors in the interaction of bacteria with host cells (Zasloff, 1992; Huang, 2000;Ratcliffe *et al.*, 2011) or iii) killing the bacteria due to the antibacterial activity of peptides.

Various products compounds have shown various biological effects including anti-adhesive properties (Daniel Amsterdam., 1996;Osawa *et al*., 2001; Xiao *et al*., 2007). It has been shown that microbial resistance is generally less likely to occur with insect antibacterial peptides than with conventional antibiotics (Steinberg *et al*., 1997; Zhang *et al*., 2005). This is because insects produce different antibacterial peptides simultaneously, which work together synergistically against the different invading bacteria (Tzou *et al*., 2002). The characterization of these peptides would provide suitable alternatives to classical antibiotics because they are characterized by selectivity, fast killing, broad antimicrobial spectra, and little resistance development against them (Zasloff, 1992; Huang, 2000). Expression of these peptides in bacteria is, by far, the simplest and most inexpensive means to produce large amounts of these products (Valore and Ganz, 1997; Rao *et al*., 2004 ; Niu *et al*., 2008).

The digestive tract of insects is divided into foregut, midgut and hindgut. In bees, the foregut consists of the pharynx, esophagus, crop and

proventriculus, the midgut corresponds to the ventriculus and the hindgut is divided into ileum and rectum (Santos and Serrao, 2006). *A. mellifera* larvae are susceptible to American foulbrood (AFB) caused by the endospore-forming, Gram-positive bacteria *Paenibacillus larvae* (*P. larvae*), (Shimanuki, 1997). The response of the gut of honey bee to *P. larvae* infection was that of inflammation and degeneration. The histological picture of the honey bee ventriculus undergoes changes as a result of action of factors as such as toxic substances and bacterial infections (Szymas and Je druszuk,2003). There was generalized separation and detachment of the epithelial cells and increased laceration and vacuole formation. In control guts, the peritrophic membrane and epithelial cells were homogenous while in the gut of parasitized honey bees they showed signs of degeneration. Similar results were reported by Zakaria, 2011. The degeneration of the epithelial cells is be due to secretion of metalloproteases by *P. larvae* that are involved in the larval degradation that occurs after infection (Antu´nez *et al.,* 2009).

The larval stages of *A. m. jemenitica* 3rd instar represents a unique system for applying proteomics to probe host-pathogen interactions. Unlike most other systems, proteins in larvae not only play major roles in immune defense but also constitute one of their primary stores of energy. Studying such a response in most other systems with more conventional energy

reserves (e.g., lipids) would necessitate a wide variety of tools in order to monitor energy usage, immune factor production and metabolic flux all at the same time. By monitoring all these aspects simultaneously, our data demonstrates that host defense against bacterial challenge is a concerted response involving proteins that kill the microbes directly, as well as metabolic and cell/protein repair enzymes that indirectly support this defensive effort. By using proteomic techniques on this unique model organism where immunity and protein energy flux are tightly coupled, we have been able to build a more comprehensive picture of the eucsocial and economic important insect innate immune response.

In conclusion, the current work suggests that natural peptides isolated from injected *A. m. jemenitica* 3rd instar larvae have potential effective antimicrobial agents. Moreover it was observed it has potent efficiency against the *A. m. jemenitica* 3rd instar larvae pathogenic bacterium, *P. larvae*. The disease which is considered as the most serious widespread bacterial disease of honeybee larvae AFB (Brodsgaard *et al* ., 1998).

Additionally the future work that is still underway to be investigated by homology from well-studied species are often very helpful for understanding how various genes and proteins attribute to the anatomy and physiology of this less studied subspecies bees that is well

prevalent in Arabian Pensuila. Algorithms such as BLAST or similar utilities are commonly under investigation .

Further usage of sequence data in a laboratory context however, frequently involves high-throughput methodologies such as microarrays for gene expression studies, and MS-based proteomics for protein expression studies are recommended.

RECOMMENDATIONS

Based on the results recorded in the present study it became necessary to further characterize the structure and function of AMPs purified from plasma of *A. m. jemenitica* 3^{rd} instar larvae which is still under exploration and comparison with the protein components separated from other homologous species to clarify their role in the immune resistance to insect diseases .

The development of future research in relation to the chemical synthesizing of these characterized peptides, where they are less expensive and more effective against the over resistant microbes towards conventional antibiotics.

Emphasizing the importance of correlating the results that have been obtained for the first time of the existing work on Yemeni honeybee type *A. m. jemenitica* 3^{rd} instar larvae, that are native in Saudi Arabia to studies that have been done by other scientists on this type of bees all over the world for the establishment of a database that would categorize them in promising research of insects of economic importance .

The identification and characterization of these antibacterial peptides may help to identify a potential natural candidate antibiotic against bacterial diseases that may further provide a boast to bee keeping industry in KSA.

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ARABIC SUMMARY

الملخص العربي

لقد أجريت هذه الدراسة لتقييم وتتبع كفاءة الببتيدات المناعية التي تم تنقيتها وعزلها من الطور اليرقي الثالث (عمر ثلاث أيام) لنحل العسل المحلي في المملكة العربية السعودية، *Apis mellifera jemenitica* (*A. m. jemenitica* 3rd instar) بعد استحثاثه بالحقن بأنواع من البكتيريا سالبة وموجبة صبغة جرام .

إذ أن تلك الببتيدات التي درست اثبتت تأثير فعال مضاد لبكتيريا *Paenibacillus larvae* (*P. larvae*) (ATCC 10801) ، المسببة لمرض عفن الحضنة الأمريكي (AFB) الذي يؤثر على يرقات النحل .

وبكتيريا *Salmonella enteritidis* (*S. enteritidis*) American Type Culture Collection ATCC 13076، المسببة لمرض السالمونيلا، والمنقول عن طريق الغذاء للإنسان. إذ تمت معايرة نشاط تلك الببتيدات المضاد لذلك النوعان من للبكتيريا مع مسببات الأمراض البكتيرية اللاهوائية والهوائية القياسية المختلفة.

تم تتبع الاستجابة المناعية فى اليرقات بعد حقنها بجرعة معايرة من (LPS) Lipopolysaccharide الببتيد عديد التسكر ،المكون لجدار الخلية البكتيرية سالبة صبغة جرام (20 نانوغرام / يرقة) وكذلك بجرعة معايرة(1×10^6 cfu / يرقة) من أنواع مختلفة من البكتيريا سالبة صبغة جرام ممثلة بـ(ATCC10536) *Escherichia coli* (*E. coli*) ، *S. entritidis*،والبكتيريا موجبة صبغة جرام ممثلة بـ *P. larva* و(*M. luteus*) *Micrococcus luteus* (NCTC 2665) كل على حدة. وقد أظهرت النتائج سلسلة من الاستجابات المناعية الخلطية والخلوية مع أنشطة مضادة للبكتيريا

ولقد تم قياس الاستجابات المناعية باستخدام طرق فحص مناعية قياسية : منها فحص منطقة تثبيط الانتشار البكتيري في أطباق الآجار. وتكوين العقيدات الخلوية حول البكتيريا المصاحبة لظاهرة الملننة (melanization) و الابتلاع الخلوي ضد جسيمات Latex beadsو تقدير نشاط مثيل الليسوزيم (Lysozyme-like activity)، ونشاط مثيل cecropin (Cecropin-like activity) وكذلك قياس نشاط إنزيم phenoloxidase .

وعند فحص ظاهرة تكون العقيدات الخلوية في اليرقات بعد الحقن بكل من بكتيريا *P. larvae*و *S. entritidis*. أظهرت النتائج تكون العقيدات وسجلت أعلى معدل لها بعد 48 ساعة من الحقن

ومن ثم سجلت اليرقات أعلى معدل اسوداد بكامل الجسم ثم الموت والتي تم رصدها بالتصوير باستخدام المجهر الضوئي والإلكتروني الماسح.

وعند حقن اليرقات بالجرعة المعايرة 0.9µl من مادة carboxylate-modified polystyrene latex beads، أظهرت خلايا يرقات العمر الثالث نشاطا ملحوظا ممثلا لظاهرة الالتهام الخلوي وذلك بتكوين عدد من الفجوات الهاضمة بمراحل مختلفة تحتوي على حبيبات لاتكس (latex beads) خلال 24 ساعة بعد الحقن والتي تم رصدها وتصويرها باستخدام المجهر الإلكتروني النافذ.

وقد تم قياس نشاط مماثل الليسوزيم (lysozyme-like) في بلازما دم اليرقات والذي تم حقنها ببكتيريا *P. larvae* وذلك باستخدام طريقة فحص العكارة (disk diffusion method Kirby- Bauer technique) حيث أظهرت النتائج المتحصل عليها أن مستوى التركيز الرئيسي لليسوزيم مماثلة لتلك التي لوحظت في المجموعة الضابطة (غيرالمحفزة) (5.8±0.016) ثم زاد مستوى النشاط بشكل ملحوظ ومعنوي ($P < 0.05$) في بلازما دم اليرقات المحقونة (6.1 ± 0.013) كانت الزيادة في نشاط الليسوزيم متناسبة تناسبا طرديا مع الزمن حيث أن هناك زيادة في نشاط الإنزيم في الفترات الزمنية المختلفة بعد الحقن ($P > 0.05$).

وعند قياس نشاط مماثل السيكروبين Cecropin-like ضد بكتيريا *P. larvae*، قد سجل إزدياد للنشاط المضاد للبكتيريا (قطر منطقة التثبيط 11.3 ± 0.5 ملم) في بلازما دم اليرقات المحقونة بـ *P. larvae*. مقارنة مع معيار مماثل من Cecropin B والتي لها نفس التركيز من البروتين (1.725 ± 0.214). كانت نفس النتائج قد لوحظت مع تدرج تنازلى لتركيزالبروتين الكلى في كل من التركيز 5 و2.5 ميكروجرام / مل ، في حين لم يتم التحقق من تواجد النشاط المماثل للسيكروبين في بلازما دم يرقات المجموعة الضابطة (غير المحقونة).

وقد أوضحت النتائج المتحصل عليها باستخدام كل من المجهر الضوئي والمجهر الإلكتروني النافذ أن إصابة يرقات نحل العسل *A. m. jemenitica* 3rd instar عن طريق التغذية بجراثيم بكتيريا *P. larvae* بجرعة (1×10^6 cfu / يرقة) قد نتج عنه أعراض الاصابة بمرض عفن الحضنة الأمريكي AFB. حيث تأثرت عدة مناطق من الجهاز الهضمي لليرقات المصابة تأثراً بالغاً وكان الضرر واضحاً في أنسجة أجزاء مختلفة ممثلة بالمعدة والمعي الأوسط والمعي الخلفي. كانت استجابة امعاء يرقات النحل للإصابة ببكتيريا *P. larvae* حدوث الالتهاب والتحلل. الصورة النسيجية من المعي الاوسط ليرقات نحل العسل تعرضت لتغيرات كنتيجة

لتأثير عوامل مثل المواد السامة والالتهابات البكتيرية، وبصفة عامة كان واضح حدوث انفصال وزيادة تمزق وتهتك الخلايا الطلائية وتشكل الفجوات. وكذلك تحلل الخلايا الطلائية قد يكون بسبب إفراز إنزيمات metalloproteinases بواسطة الاصابة بتلك البكتيريا الممرضة *P. larvae* التي تشارك في تدهور اليرقات.

وكذلك تم عزل الببتيدات المناعية وتنقيتها من بلازما دم يرقات النحل (عمر 3 أيام) التي تم حقنها وذلك باستخدام جهاز الفصل الكروماتوغرافي ذو الكفاءة العالية (RP-HPLC) و عند فحص مقدرتها التثبيطية تجاه نمو البكتيريا باستخدام تجربة (MIC) Minimum Inhibitory Concentration الذى يعبر عن أقل تركيز يُحدث أعلى تثبيط لنمو البكتيريا . إذ أستخدم كل من البكتيريا القياسية التالية *S. enteritidis* (ATCC 13076; Basingstoke, UK), *E. coli* (ATCC 10536), *M. luteus* (NCTC 2665; Sigma, UK), *Staphylococcus aureus* (ATCC 6538) , *Bacillus subtilis* (ATCC 6051), *Pseudomonas aeruginosa* (ATCC 9027) and *P. larvae* (ATCC 10801).

حيث أظهرت النتائج أن محتوى البروتين الكلي في بلازما دم الطور اليرقي الثالث المحقونة ببكتيريا *S. entritidis* و*P. larvae* كان أعلى (1.799 ± 0.152, 1.725 ± 0.214) من تلك المحقونة بأي من مادة LPS أو بكتيريا *M. luteus* (1.54 ± 0.40, 1.54 ± 0.41) مقارنة مع تلك الموجودة في بلازما دم المجموعة التي لم يتم حقنها (1.24 ± 0.32) (المجموعة الضابطة).

وقد تم تنقية عوامل ببتيدية أطلق عليها تسمية كودية ممثلة بالعوامل A, B, C, D, E, and F وتلك الببتيدات قد تم تجفيفها باستخدام جهاز التجفيف تحت التبريد للحفاظ على المكون البروتيني والكفاءة الفعالة، هذا وقد أظهرت النتائج أن كل من عوامل الببتيد المنقى (A، B، C و D) لها نفس الفعالية المضادة للجراثيم مقارنة مع المضاد الحيوي سيبروفلوكساسين (Ciprofloxacin) ضد البكتيريا سالبة صبغة جرام *E. coli* والبكتيريا موجبة صبغة جرام *M. luteus* وكانت أكثر البكتيريا المقاومة هي *P. larvae* متبوعة بـ *S. enteritidis* ثم *P. aeruginosa* ضد كل من الببتيدات النقية التي تم اختبارها على حدة.

وقد أظهر عامل الببتيد (B) أدنى تركيز مثبط (MIC) (32µg/ml) تجاه بكتيريا *S. enteritidis*.

وقد أجريت تجربة الفصل الكهربي باستخدام هلام البولي أكريلاميد (SDS-PAGE) وقد أوضحت النتائج المتحصل عليها أن بلازما دم اليرقات قد أظهر عن التعبير عن جزيئات بروتينية ذات أوزان جزيئية تتراوح بين 70 kDa وقد تشير لتحرير انزيم phenoloxidase، من صورته الخاملة وكذلك المكون الجزيئي المعبر عنه بـ 15 kDa والذي قد يشير إلى lysozyme ، كذلك التعبير البروتينى للجزيئات الأقل من 11 kDa والتي تتوافق مع تكون المضادات البكتيرية عديدة الببتيدات والتي في طريقها للإثبات من حيث التوصيف البروتيني باستخدام بعض تقنيات فصل البروتين المختلفة.

بالإضافة إلى ذلك، تم تقييم فعالية هذه العوامل الببتيدية المنقاة والمعزولة A و B و C أو E و FوD من بلازما اليرقات المحقونة بـ *S. enteritidis* ، وذلك بإجراء اختبار تثبيط الالتصاق ببكتيريا سالبة صبغة جرام *S. enteritidis* المعروفة بالسالمونيلا المسببة لمرض التسمم الغذائي للإنسان من خلال منع التصاقها بالخلايا الطلائية للإنسان .

وقد أجريت التجربة في الفراغ على الخلايا الطلائية للإنسان معمليا. وقد أظهرت النتائج أن كل من العوامل المنقاة المكودة بـ A,B,C,D على التوالي قد أدت إلى انخفاض معنوي ($P < 0.05$) (19.5±0.71, 21±0, 18.5±0.71,19±1.41) من الالتصاق البكتيري بالخلايا الطلائية قيد التجارب .

وقد اختير كل من عاملي A وB المسجلين لأعلى كفاءة معنوية وكان أكثرهم فاعلية لتثبيط الالتصاق هو عامل B ($P < 0.05$) لترشيحه كمضاد حيوي طبيعي مستخلص من يرقات نحل العسل يمكن استخدامه على نطاق أوسع في مقاومة الميكروبات المسببة للأمراض في الإنسان .

خلاصة

أظهرت نتائج الدراسة أن الببتيدات الطبيعية غير المستحثة من نحل العسل *A. m. jemenitica* 3rd instar الطور اليرقي الثالث المحقونة بأنواع مختلفة من البكتيريا تمتلك النشاط المضاد للبكتيريا ضد فريق من مسببات الأمراض البكتيرية وهي *E. coli* ، *M. luteus* ، *B. subtilis* و *P. aeruginosa*

وعلاوة على ذلك فإن تنقية عوامل المكون الببتيدى من بلازما دم اليرقات المستحث باستخدام تقنية RP-HPLC من نحل العسل *A. m. jemenitica* الطور اليرقي الثالث أوضحت أن لديه كفاءة قوية ضد البكتيريا *P. larvae* المسببة لمرض عفن الحضنة الأمريكي (AFB).

هذا بالإضافة للتأثير المضاد لالتصاق بعض عوامل الببتيد المنقي نحو الخلايا الطلائية للكائنات حقيقية النواة (الإنسان) وبكتيريا *S. enteritidis* الكائن المسبب لمرض السالمونيلا في الانسان وهذا يقوي ويدعم دورها كعامل مبيد قوي للجراثيم .

هذه الدراسة تُعد خطوة واعدة إلى الأمام نحو تحديد عوامل مناعية لترشيحها كمضادات حياتية مستمدة من يرقات نحل العسل *A. m. jemenitica* 3rd instar لاستخدامها ضد الأمراض المنقولة عن طريق الأغذية سواء للنحل أو الإنسان وكذلك تشجيع استخدامها لتقوية حضنة النحل ضد بعض الأمراض البكتيرية التي قد تسبب القضاء على كامل مملكة النحل مما يؤثر بالسلب على صناعة النحل التي تمثل أحد أهم مصادر الدخل لصغار النحالين وكذلك يمكننا من استخدامه على نطاق آخر في مراحل الحفاظ على الصحة العامة للإنسان في المملكة العربية السعودية.