In vitro effects of natural purified peptides from the native *Apis mellifera jemenitica* larvae on *Salmonella* adherence to epithelial cells

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Abstract

Food borne non-typhoidal Salmonellosis outbreaks are an increasing public health problem in Saudi Araba. Resistance of Salmonella to therapeutic agents currently used for treatment of Salmonella infections is emerging as a global problem Adhesion of Salmonella to intestinal epithelial cells, an important step in the development of disease, is currently the focus of novel anti-bacterial strategies. Six natural antimicrobial peptide fractions from plasma of local honeybee (*Apis mellifera jemenitica*) larvae (3rd instars, 72h) were isolated by reversed-phase high-performance liquid chromatography (RP-HPLC) that recovered as 3 peaks for each treatment, separately. Either of post- injection with *Paenibacillus larvae*, the primary pathogen of American foulbrood disease (A, B, C) or *Salmonella enteritidis*, the causative agent for food poisoning (D, E and). All the isolated peptide fractions showed antibacterial activity with moderate variable minimum inhibitory concentration (MICs) against standard bacterial strains using Broth Micro dilution Assay. Antimicrobial susceptibility of *S. enteritidis* or *P.larvae* was determined ,

separately by Kirby-Bauer Disk Diffusion Method. The obtained results showed that all peptide fractions had an inhibitory effect against *S. enteritidis* as well as against the honeybee pathogen *P. larvae*. The peptide fraction B showed the highest antibacterial activity with inhibition zones of 21±0 mm and 20.8±0.71mm against *S. enteritidis* and *P. larvae,* respectively compared with Ciprofloxacin antibiotic. Purified peptide fractions A, B, C and D significantly decreased the adhesion ($p<0.001$) of *S. enteritidis* to epithelial cells. Peptide fractions A and B showed the highest efficiency in this concern. Moreover, peptide fraction B significantly reduced the adhesion of *S. enteritidis* more than peptide A ($p=0.02$). *In vitro* mimic experiment of the partial isolated peptide fractions A, B, C and D significantly decreased the adhesion ($P < 0.001$) of *S. enteritidis* to epithelial cells. Peptide fractions A and B showed the highest efficiency in this concern. Moreover, Peptide fraction B significantly showed the highest inhibitory activity against the adhesion of *S. enteritidis* than the peptide fraction A ($P=0.02$).

Keywords: Adhesion; epithelial cells; *Apis mellifera jemenitica,* partial purified peptides, *Salmonella*, *Paenibacillus larvae***,** American foulbrood disease.

Introduction

Food poisoning is becoming a globally health problem and recognized as a major health concern in the Kingdom of Saudi Arabia (KSA). Resistance of *Salmonella* to therapeutic agents currently used for treatment of *Salmonella* infections is emerging as a global problem. 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). Expansion of fast food businesses established by poorly educated personnel from some developing countries who do not possess adequate training in food safety as improper handling of food stuffs and unhygienic cooking conditions has further contributed to this situation (Cetinkaya et al., 2008). Contaminated food contributes to 1.5 billion cases of diarrhea in children each year, resulting in more than three million premature deaths, according to the World Health Organization (WHO, 2003). As early as 1998, Al-Ahamadi et al. studied an outbreak of food poisoning associated with restaurant-made mayonnaise in Abha, Saudi Arabia. They have isolated *Salmonella enterica* from 124 (78%) of 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 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 led 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 derived from natural products which are less likely to cause pathogen resistance. The discovery of antimicrobial peptides (AMPs) in insects and animals, has gained the attentions from theresearchers and pharmaceutical companies towards these newly, promising alternative sources of today's antibiotic (Nguyen et al., 2011). This finding indicates the importance of nature to be the rich source for developing drugs against seriousbacterial infection diseases.

Insects are capable of producing different multiple antibacterial peptides depending on the type of invading bacteria (Boman & Hultmark 1987; Boman, 1991

and 1995). They are synthesized mainly in insect fat bodies and in certain hemocytes as a response to microbial infection (Irving et al., 2005; Amaral et al., 2010). Upon microbial infection, these peptides are rapidly released into hemolymph where they act against the invading microorganisms (Hetru et al., 1998; Kavanagh & Reeves, 2004; Yang et al., 2013). Recently the immune peptides from the honeybees, *Apis florae* and *Apiscarnica* have been isolated, and showed antibacterial activities against the human pathogenic bacterium, *Klebsiella pneumoniae* strain American Type Culture Collection (ATCC 11678) that causes urinary tract infections (Ayaad et al., 2012).

Paenibacillus larvae are the etiological agent of American foulbrood disease (AFB), a serious disease of the honey bee (*Apis mellifera*) larvae (Genersch et al., 2006). AFB is the most contagious and destructive bacterial disease affecting honey bees and able to kill entire colonies. As a result, AFB leads to considerable economic loss in a bee culture. In addition, colony losses due to AFB also compromise agricultural and natural environment because of the essential role of honey bees as fertilizer of many fruit, crops, and wild flowers (Garcia-Gonzalez et al., 2014).

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 that radiate from the bacterial surface for initial adherence as pili (fimbriae) and flagella (Allen-Vercoe& 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).

The aim of this work is to explore and highlight the diverse natural purified peptide fractions (A, B, C, D, E and F) isolated from honeybee (*A, mellifera jemenitica*) larvae that have a potential antimicrobial effect. Furthermore, the inhibitory effect of these isolated purified peptides on adhesion of *S. enteritidis* to

human epithelial cells *in vitro* was assessed compared to the specific efficiency of these peptides against the honeybee pathogenic bacterium, *P. larvae.*

Materials and Methods

Bacterial strains and growth conditions

To characterize antibacterial activity of isolated peptide fractions quality control strains were purchased for the experimental purposes of this study. The set contains three Gram-negative strains: *Salmonella enteritidis* American Type Culture Collection (ATCC 13076; Basingstoke, UK), *Escherichia coli* (ATCC 10536) . *Pseudomonas aeruginosa*(ATCC9027**),** as well as four Gram positive strains: *Micrococcus luteus* National Collection of Type Cultures (NCTC 2665; Sigma, UK), *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilus* (ATCC 6051)and *Paenibacillus larva ksuPL5* which was isolated and identified molecularly for the first time from local Saudi honey bee apiaries(Ansary et al. (2017).

All strains were maintained at – 20°C in brain heart infusion broth with 20%v/v glycerol. Before experimental use, *P. larvae* cultures were propagated at 35°C in PCBY medium (0.6% bactopeptone, 0.4% casein hydrolysate, 0.15% beef extract, 0.3%yeast extract, 0.1% glucose, pH 7.9) according to Bilikova et al.,(2001).

Induction and isolation of antibacterial peptides

Immune induction of A. mellifera jemenitica larvae

Hemocoelic bacterial injections were carried out using Hamilton micro syringe with a fine needle as described by Laughton et al., (2011). Larvae (3^{rd} instars,72 h) either, control (none injected) or injected with *P. larvae or S.entritidis* (2×10^5cfc/larva) as described by Ahmad & Hoopingarner, (2002). 24 hours post injections, larvae (10 each) were maintained in suitable containers for recovery in standard suitable rearing conditions for the following procedures. Before hemolymph collection 24 post-injection, active larvae were surface-sterilized with 70% (v/v) ethanol. Clear hemolymph samples were obtained by puncturing the abdomen with a sterile needle as described by Zufelatoa et al., (2004).

Hemolymph extraction of A. mellifera jemenitica larvae

Larvae (3rd instars,72 hours) either control (none injected) or injected (n=10) were washed in 10 ml of 70% (v/v) ethanol to reduce contamination from worker royal jelly. A small incision was then made two-thirds of the way down one side of the larva 24hours post larval injection for isolation of hemolymph plasma from hemocytes as described by Laughton et al., (2011). Milky white hemolymph was decanted into microfuge tubes containing equal volume of anticoagulant (AC) (98 mMNaOH, 186 mMNaCl, 17 mM EDTA and 41 mM citric acid, pH 4.5) with EDTA-free Protease Inhibitor Cocktail. The microfuge tubes were spun at 1500 rpm in a micro centrifuge (Biofugefresco, Heraeus, and D- 3752) for 10 minutes at 4°C to pellet hemocytes which were discarded. Clear plasma were decanted into 1.5 ml Eppendorf tubes and kept at -80° C for further experiments.

Protein estimation

Total protein concentrations of clarified plasma of 3rd larval instar (hemocyte - free) were estimated by the method of Bradford, (1976) using Coomassie Brilliant Blue (CBB) (ICI Americas, Inc.) according to the manufacturer's instructions. For the calibration curve, bovine serum albumin was used.

Partial peptides isolation by reversed-phase high-performance liquid chromatography (RP-HPLC)

The crude plasma obtained from each *A. mellifera jemenitica* larval injection, separately were dissolved in 10% acetic acid and purified by a reversed-phase high-performance liquid chromatography (RP-HPLC) on a C18 Bondapak column (1μm, 10 × 250 mm) using acetonitrile gradient in water [30-70%/ 15 minutes] containing 0.05% trifluoroacetic acid (TFA). The peptides were further separated by a RP-HPLC on an analytical (12 μm, 4.6 × 120 mm) C18 Pepmap column using acetonitrile gradient in water [30 - 70% / 30 min] and 0.05% trifluoroacetic acid (TFA) as described by Venkatasami &JohnSowa, (2010) with some modifications. The isolated peptide fractions obtained from each injection were, separately hydrolyzed with 6 N HCl at 100°C for 24 hours, and then dried in the vacuum and stored till used.

Inhibition of bacterial growth

Minimum inhibitory concentration of partially purified peptide fractions against different standard bacterial strains using broth micro dilution assay

The antimicrobial activity of the purified peptide fractions isolated from plasma either of post – injection of *A.m. jemenitica* larvae (3^{rd} instars, 72hours) with *P. larvae* (A, B, C) or *S. enteritidis* (D, E and F) bacteria were determined by the microtiter dilution plate method. Minimal Inhibitory Concentrations (MICs) of six purified peptide fractions were determined against different standard strains; *S. enteritidis* (ATCC 13076),*E. coli* (ATCC 10536), *M. luteus* (NCTC 2665), *St.aureus* (ATCC 6538), *B. subtilus* (ATCC 6051),*P. aeruginosa* (ATCC 9027) and *P. larvae(9545)* using Ciprofloxacin(CIP) antibiotic as a reference. Bacterial suspensions 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 according to the method used by CLSI, (2010) which is based on the protocol of Amsterdam (1996) with some modifications.

Microtiter plates 96-well (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 dilution of peptide solutions was made by withdrawing 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. Fifty microliter aliquots of the bacterial suspensions in Mueller-Hinton broth were inoculated into wells of the microtiter 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-microtiterplates were sealed using a perforated plate seal (TREK Diagnostic Systems Inc., Cleveland, OH) and incubated at 37^oC for 24 hours. The MICs were recorded as the lowest concentration where no viability was observed in 96-microtiterplates after incubation for 24 hours. Experiment was conducted in triplicate.

Susceptibility of P. larvae and S. enteritidis to partially purified peptide fractions of injected A. mellifera jemenitica larvae by disk diffusion method.

The susceptibility of either *S. enteritidis* or *P. larvae* bacteria to isolated peptide fractions (A, B, C, E, D, and F) were determined using disk diffusion method (Kirby-Bauer technique) according to Clinical and Laboratory Standards Institute (CLSI, 2013)

For preparation of *P. larvae* spore suspension, 2% agar in the PCBY medium was used. 20µl of *P. larvae* bacterial overnight culture (1 ×10 ^{6} CFU) in the liquid PCBY medium was spread over the surface of 2% agar PCBY plates. The plates were incubated at 30 °C for 20 hours and the colonies of *P. larvae* were subsequently replaced into the sterile glass tube and suspended in physiological saline (PBS). The bacterial suspension (vegetative form of *P. larvae*) was stored at 15°C. After one month, the bacterial suspension was incubated at 80°C for 20 min in order to kill non-spore-forming bacteria according to Bilikova et al. (2001).

Antibacterial diffusion test on agar plates was performed by spreading 8 ml of 0.5% agar PCBY medium mixed with spore suspension of *P. larvae* (1 ×10^{5} spores per ml) in Petri dishes. On the other hand, Müeller-Hinton agar plate (Oxoid, U.K.)was inoculated by streaking the suspensions of *S. enteritidis* ATCC 13076 (1x10^{6} CFU/ml) using swab stick.The inoculums were 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. Five micro-liters (25 µg) of each peptide was applied on to each sterile disk and dried in sterile laminar flow cabinet before used. The disks impregnated in peptide fractions were placed on the agar plates with sterile forceps.Inoculated plates were incubated within 15 minutes of disk application inverted at 35^{0}C for 24 hours.Plates were read and photographed (DC120 Zoom Digital Camera, Kodak, USA)from the back against a black background to visualize the inhibition zones.The inhibition zone (mm) was measured and interpreted according to Clinical and Laboratory Standards Institute (CLSI, 2013), CIP disk was used as a control.

In vitro adherence assays

Bacteria preparation for adherence

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

Preparation of epithelial cells

Human epithelial cells were used in this assay. The urine samples bwere obtained
in the morning from healthy pregnant woman (prefer the concentrated first morning specimen, collected mid-stream) and harvested by centrifugation at 1000xg for 10 min. The cells sediments were washed twice with PBS at pH 7.2 and re-suspend in 2ml of PBS, and then standardized to a concentration of approximately 10^5 cells/ ml using haemocytometer.

Measurement of the adhesive effect of isolated peptide fractions from A. m. jemenitica larvae on S. enteritidis to epithelial cells by crystal violet method in vitro.

The anti-adhesive effect of natural isolated *A. m. jemenitica* larvae peptides on *S. enteritidis* bacteria adhesion to epithelial cells was determined by microscopic analysis using crystal violet method (Vesterlund et al.,2005).Bacterial suspension was prepared and standardized with turbidity test 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. Approximately 150µl of *S. enteritidis* ATCC 13076was added into microtiter plate wells coated with 150 µl of epithelial cells in absence and presence of peptides in concentrations of ½ MIC or ¼ MIC, separately .Then incubated at 37 °C for 1 hour. The non-adherent bacteria were removed by washing the wells three times with 250 µl of PBS (pH 7.2) . The adherent bacteria were fixed at 60 °C for 20 minutes and stained with crystal violet (100 µl/ well, 0.1% solution) for 45 minutes. 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 cells for 45 minutes at 37°C and optical density was measured at 570nm.

Count method

Epithelial cells were counted by methylene blue method, distributed evenly in 6 well plates and co-cultured with different experimental bacterial strains in total volume in absence and presence of peptides in concentration of ½ MIC. Cells were incubated at 37°C for 1 hour, washed 3 times with PBS, fixed at 60°C for 30 minutes, stained with methylene blue for 45 minutes and washed 5 times with PBS. Adhered bacterial cells to each individual epithelial cell of selected 25 epithelial cells were counted under microscope (x1000 magnification) and images were taken. All experiments were repeated three times.

Statistical analysis

Experiments were conducted in triplicate, and statistical analysis was done by GraphPad prism 5. Mean, standard deviation and significance were calculated.

Results

RP- HPLC profile of induced peptides of *A.mellifera jemenitica* larvae

Natural peptide fractions isolated from *A. mellifera jemenitica* larvae either induced with *P. larvae* or *S. enteritidis* were initially fractionated by using 30-70% v/v acetonitrile containing 0.05%v/v trifluoroacetic acid. They were recovered as 3 peaks for each treatment, separately in the final RP-HPLC step; peaks A, B, C as shown in(Fig.1a) and peaks D, E, F as shown in (Fig.1b).

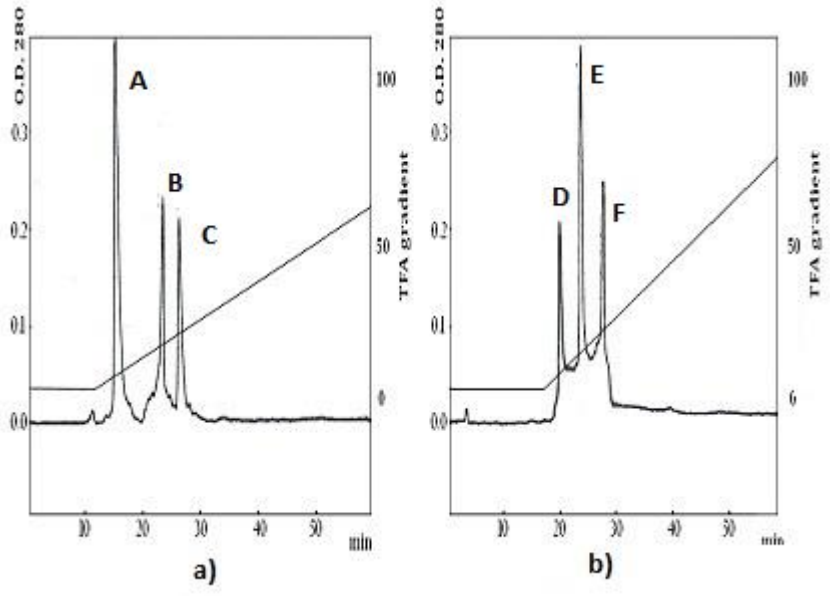


Fig. (1) RP-HPLC of isolated peptide fractions from *A. m. jemenitica* larvae induced with *P. larvae* or *S. enteritidis* bacteria, panels a) and b), respectively. Heat treated plasma was fractionated, separately, on a C18 Bondapak and finally on C18 Pepmap columns. Fractions A, B, C, D, E and F developed using a water-acetonitrile gradient [30 - 70% / 30 min] containing 0.05% trifluoroacetic acid (TFA).

Anti-microbial activity

Minimal inhibitory concentration of partially isolated peptide fractions against different standard bacterial strains using broth micro dilution assay

The isolated peptide fractions (A, B, C, D, E and F) showed a moderate inhibitory effect on bacterial growth after 24 hours incubation at 37°C. MIC values revealed that peptide fractions (A, B, C and D) are with the same antibacterial potency if compared with a the reference Ciprofloxacin antibiotic against both of the

gram negative *E. coli* and gram positive *M. luteus* (table 1).However the gram positive *P. larvae* was the most resistant organism followed by *S. enteritidis* and *Ps. aeruginosa*. Peptide fraction B showed the lowest MIC among the isolated peptide fraction against*S. enteritidis* and *P.larvae*. It has MIC 32µg/ml and 64µg/ml, respectively.

Anti-microbial activity of partially isolated peptides against P. larvae and S. enteritidis

Antimicrobial susceptibility for the six purified peptides (A, B, C, D, E and F) was done according to (CLSI, 2013) by disk diffusion method against *P. larvae* ATCC 9545 and *S. enteritidis* ATCC 13076. After incubation, each plate was examined. The diameters of the zones of complete inhibition were measured, including the diameter of the disk, using a ruler, as shown in table (2) and Figs. (2&3).
Peptide fraction B showed the highest antibacterial activity as it produced the largest inhibition zone diameter about20.8±0.71mm and 21±0 mm respectively, compared with ciprofloxacin antibiotic.

Bacterial adhesion

Antiadhesive effect of purified peptides on S. enteritidis by crystal violet method in vitro

Epithelial cells were co-cultured with *S. enteritidis* strain in absence and presence of purified peptide fractions in concentration(sub MIC) ½ MIC and ¼ MIC. All the peptides exhibited a reduction in *S. enteritidis* adhesion to epithelial cells as shown in Fig. (4).

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 fractions A and B showed the highest efficiency in adhesion reduction. Moreover, peptide fraction B reduced the adhesion of *S. enteritidis* significantly more than peptide fraction A ($P = 0.02$) as shown in Fig. (5). In addition, adhered bacterial cells to each individual epithelial cell were counted after treatment with peptide fraction (B) under microscope and images were taken.

By crystal violet method, peptide fraction B showed significant reduction in *S. enteritidis* adhesion to epithelial cells under microscope using oil immersion lens (x1000 magnification) as shown in Fig. (6). This microscopypicture reveals not only a quantitative aspect by using image tool software but also shows the distribution of adherent bacteria onto the surface.

Table (1): Minimum inhibitory concentration (MIC) of purified peptides after 24hours incubation at 37°C with selected standard bacterial strains

Peptide fractions / **Microrganisms**	A (MIC*)	B (MIC*)	C (MIC*)	D (MIC*)	E (MIC*)	F (MIC*)	CIP (MIC*)
Salmonella enteritidis ATCC (13076)	64	32	64	128	128	64	0.25
Staphylococus aureus ATCC(6538)	4	4	0.25	0.25	4	4	0.25
Paenibacillus larvae ATCC(9545)	128	64	128	64	128	128	4
Micrococcus luteus NCTC (2665)	0.25	0.25	0.25	0.25	4	4	0.25
Pseudomonas aeruginos ATCC(9027)	64	64	64	32	128	128	0.25
Escherichia coli ATCC (10536)	0.25	0.25	0.25	0.25	4	4	0.25
Bacillus subtilus ATCC *(*6051)	4	32	8	8	64	64	0.5

*Minimum inhibitory concentration (μgml^{-1})
A, B, C, D, E, F: purified peptide fractions isolated from *A. m. jemenitica* larvae induced with *P. larvae* (A, B, C) and /*S. enteritidis* bacteria (D, E, F)
CIP: Ciprofloxacin antibiotic as a reference

Table (2): Susceptibility test using disk diffusion method for peptide fractions against *S. enteritidis* and *P. larvae* ATCC 9545 (inhibition zone in mm (mean ± SD)) using Ciprofloxacin (CIP) as a reference.

Disk content (25µg) of peptide	A	B	C	D	E	F	CIP
Salmonella enteritidis *ATCC* 13076	19.5±0.71	21±0.0	18.5±0.71	19±1.41	18±1.41	19±0.0	37±1.41
Paenbacillus larvae ATCC 9545	17.5±0.71	20.8±0.71	19.5±0.71	20±1.41	18±0.71	19±0.71	26.5±0.71

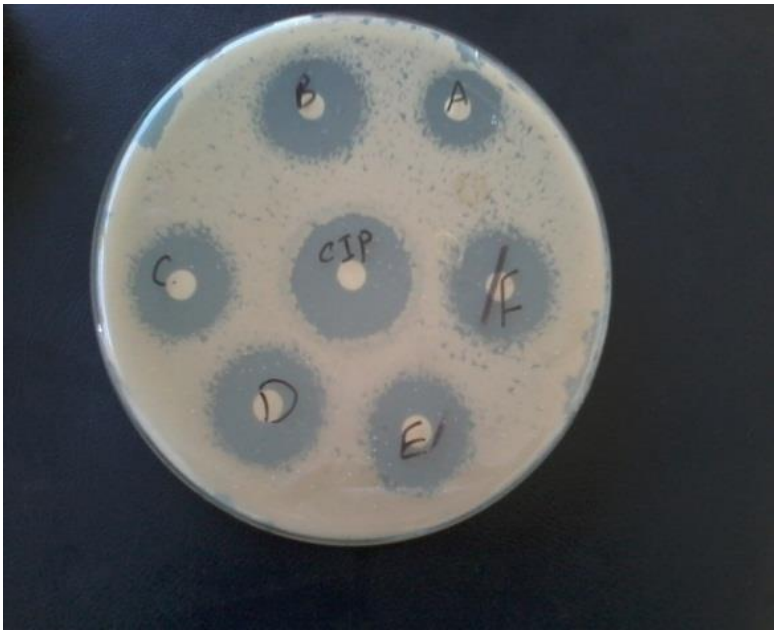


Fig. (2) PCBY agar plate showing antibacterial activity of purified peptide fractions disks (A,B,C,D,E,F) against *P. larvae* ATCC 9545 using ciprofloxacin (CIP) antibiotic as a reference

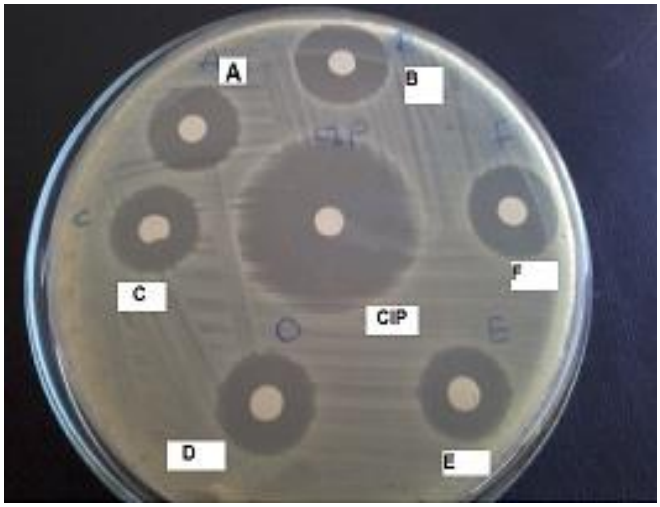


Fig.(3):Mueller Hinton agar plate showing antibacterial activity of purified peptide fractions disks (A,B,C,D,E,F) against *S. enteritidis* using ciprofloxacin (CIP) antibiotic as a reference.

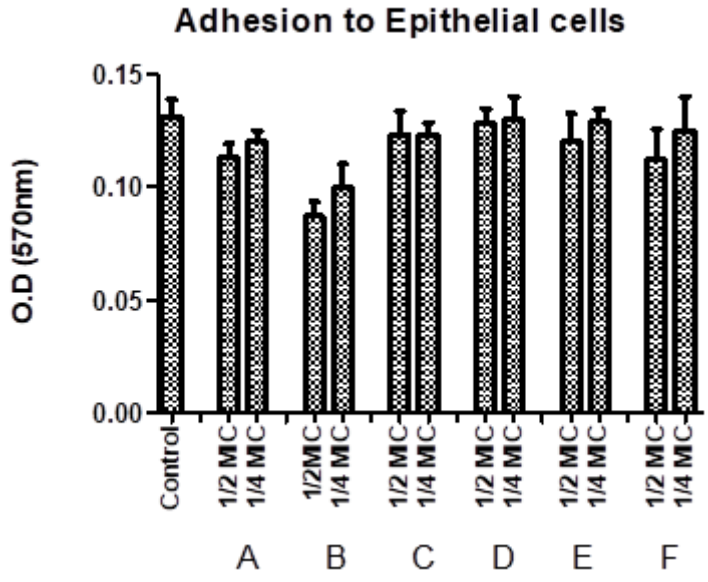


Fig. (4): Adhesion of *S. enteritidis* to epithelial cells in (control) and in the presence of purified peptide fractions (A, B, C, D, E and F) in concentrations of ½ MIC and ¼ MIC.

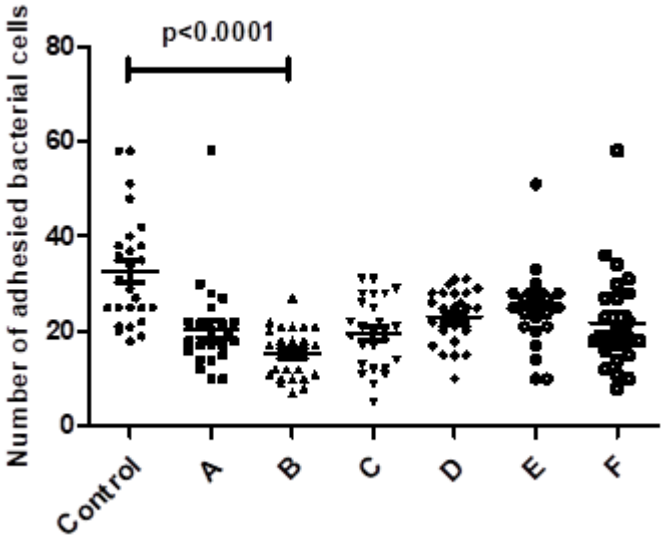
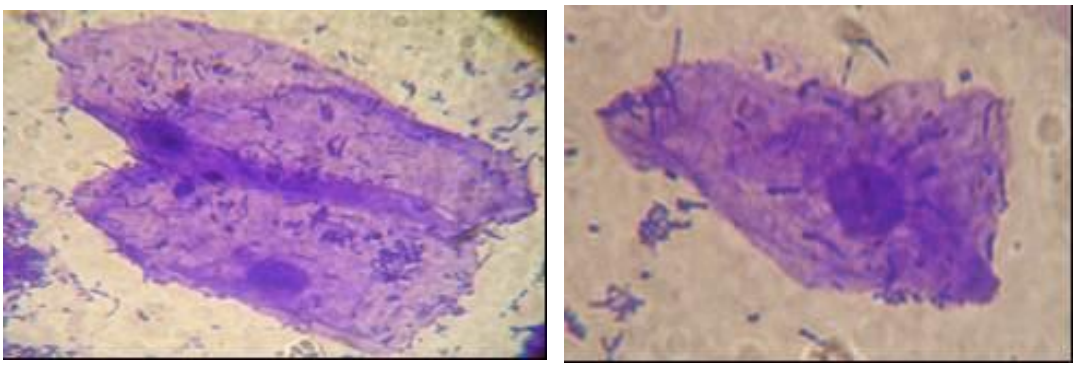


Fig. (5) Scatter plot of number of adhered *S. enteritidis*to epithelial cells using purified peptide fractions (A, B, C, D, E, F). Adhered stained bacterial cells with crystal violet were counted under microscope (x 1000 magnification).



(a) **(b)**

Fig. (6): Crystal violet method using light microscopy by oil immersion lens (x1000). (a) Untreated *S. enteritidis* (control), (b) Showed significant reduction in *S. enteritidis* adhesion to epithelial cells after treatment with ½ MIC peptides fraction B.

4. Discussion

The alarming increase of antibiotic-resistant pathogenic bacteria all over the world make the search for new ways, to fight bacterial infections, very essential and the need to antimicrobial agents derived from natural products, that are less likely to cause resistance to pathogens, more ultimate. Natural products compounds have already shown various biological effects including anti-adhesive properties (Osawa et al., 2001; Daglia et al., 2002; 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). Therefore, these peptides, as further characterization, would investigate that they are suitable alternatives for the classical antibiotics because they are characterized by selectivity, fast killing, broad antimicrobial spectra, and little resistance development against them (Huang, 2000; Zasloff, 2002).

Three peptide fractions (A, B and C fractions) were isolated from plasma of post- injection of honeybee (*Apismellifera jemenitica*) larvae (3^{rd} instars, 72hours) with *P. larvae*, the primary pathogen of American foulbrood disease, while D, E and F peptide fractions were isolated after injection by *S.enteritidis*, the causative agent for food poisoning,and purified by reversed-phase high performance liquid chromatography (RP-HPLC).

Minimal Inhibitory Concentration (MIC) of the isolated purified peptide fractions from immune honeybee lymph were determined using brothmicro dilution assay **(**CLSI, 2010). According to table (1), it wasrevealed that the purified peptide fractions (A, B, C and D) have the same antibacterial potency compared with a reference ciprofloxacin antibiotic against gram negative bacteria *E.coli* and gram positive bacteria *M.luteus*. Peptide fraction B showed the lowest MIC among the isolated purified peptides; it has MIC values of 32µg/ml and 64µg/ml with *S. enteritidis* and *P.larvae* respectively.

Susceptibility of *P. larvae* to purified isolated peptide fractions by disk diffusion method (25 µg/ disk) was represented in table (2) and fig. (2) using stock peptide solution containing (5 mg/ml for each purified peptide fractions) according to CLSI, (2013). Diffusion tests on agar plates showed that the all peptide fractions had an inhibitory effect with comparable values against the honeybee pathogen *P. larvae***.** Moreover**,** peptide fraction B showed the highest antibacterial activity as it produced the largest inhibition zone diameter (about 20.5±0.71mm) compared with ciprofloxacin antibiotic. Brødsgaard et al., (1998) found that 24–28 hours old larvae were susceptible to infection with *P.larvae*. Older larvae became more resistant to the infection and no significant dose-mortality relationship existed when the larvae were older than 48 hours.

A pathogen's ability to colonize and invade host tissues strictly depends on the adhesion. Thus, interference with adhesion is an efficient way to prevent bacterial infections. Salmonella virulence behavior is directly based on its invasiveness capability, and adhesion is prior step to invasion.

In this study, we tried the inhibition efficacy of the isolated purified peptide fractions on adhesion capability of *Salmonella* bacterial strain (ATCC 13076) to human epithelial cells. Initially, it was intended to use the diffusion assay for inhibition of bacterial growth as a screening method to determine the most efficient type of peptide fractions which would then be applied in the adherence study for *S. enteritidis* to intestinal epithelial cells *in vitro*. Antimicrobial susceptibilityof *S. enteritidis* for the purified peptide fractions (A, B, C, D, E and F) was done according to CLSI, (2013) by disk diffusion method. Each peptide fraction inhibited the growth of *S. enteritidis* in the immediate area surrounding the well. Peptide fraction B showed the highest antibacterial activity as it produced the largestinhibition zone diameter (about 21±0 mm) compared with ciprofloxacin antibiotic as reference as shown in table (2) and Fig. (3). According to these results, we evaluate the ability of the isolated purified peptide fraction B to prevent the attachment of*S. enteritidis* to intestinal epithelial cells *in vitro*. Epithelial cells were co-cultured with *S.enteritidis* strain in absence and presence of purified peptide fraction B with concentration ½ MIC and ¼ MIC. All the isolated peptide fractions

exhibited reduction in S.*enteritidis* adhesion to human epithelial cells as shown in Fig. (4) with variable degree.

Count method used foradhesion assay *in vitro*as shown in Fig. (5) indicated that the purified 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 fractions A and B showed the highest efficiency in adhesion reduction. Moreover, Peptide fraction B significantly reduced the adhesion of *S.enteritidis* more than peptide fraction A ($P=0.02$).

Our results are consistent with previous studies which reported that antibacterial peptides had antiadhesive property. Ochoa et al., (2003) proved that Lactoferrin with a sub-MIC of 10 mg/ml (0.125 mM) can inhibit the adhesion of enteropathogenic *Escherichia coli* to HEp2 cells. In addition, Guerra et al., (2005) observed that nisin, an antibacterial peptide produced by lactobacillus, reduced the attachment of *Listeria monocytogenes* strains.

The bacterial adhesion is a complex biological process involving many physicochemical forces, which primarily include hydrophobic interactions, cation bridging and receptor-ligand binding. The surface components responsible for the hydrophobic interactions include hydrocarbons, aromatic amino acids, fatty acids and mycolic acids. The adhesion and hydrophobicity of interface has the positive correlation when other physicochemical forces are the same (Mack & Sherman, 1999; Doyle, 2000). The mutual repulsion between the surfaces of bacteria and host cells with net negative charge can be counteracted by divalent metal ions, which thereby act as a cation bridge between the two surfaces. The receptor on the surface of host cell can recognize the ligand on the bacteria surface with a complementary structure and form a strong (but non-covalent) bond. The change of these physicochemical forces will influence the adherence between bacteria and host cells (Wilson et al., 2002).

There are several possible explanations for prevention of bacterial adherence demonstrated by purified peptides isolated from honeybee *A. m. jementica*. It may be

due to non-specific mechanical inhibition through the coating of the bacteria by the peptides; or due to change in bacterial electrostatic charge or hydrophobicity which have been reported to be important factors in the interaction of bacteria with host cells by peptide fractions (Edebo et al., 1980; Sakai, 1987). The last explanation is killing of the bacteria due to the antibacterial activity of peptides.

The antiadhesive effect of the antibacterial peptide fraction B isolated from honeybee *Apismellifera jementica* towards eukaryotic epithelial cells promises possible applications as a compound in prevention of bacterial Salmonella food poisoning and infections.

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